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The relaxin receptor as a therapeutic target – perspectives from evolution and drug targeting

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ABSTRACT

The peptide relaxin was first identified as an important circulating hormone during pregnancy over 90 years ago. Research over many years defined the numerous biological roles that relaxin plays throughout pregnancy in many mammalian species. These important biological actions have led to the testing of relaxin as a therapeutic agent for a number of indications. The discovery of the relaxin receptor, RXFP1, in 2002 facilitated the better understanding of the cellular targets of relaxin, its mechanism of action and enabled the development of relaxin mimetics and screening for small molecule agonists. Additionally, the rapid expansion of the genome databases and bioinformatics tools has significantly advanced our understanding of the evolution of the relaxin/RXFP1 signaling system. It is now clear that the relaxin-RXFP1 signaling axis is far more ancient than previously appreciated with important roles for invertebrate relaxin-like peptides in reproductive and non-reproductive functions. This review summarizes these advances as well as developments in drug targeting of RXFP1. Hence the complex mode of activation of RXFP1 is discussed as is the discovery and development of a peptide mimetic and small molecule agonist. Detailed signaling studies are summarized which highlight the cell specific signaling of a peptide mimetic and biased signaling of a small molecule agonist. These studies highlight the complexities of targeting peptide GPCRs such as RXFP1.

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Abbreviations: 2R, two rounds; AT2, angiotensin type-2; Anc, ancestral; Dilp, Drosophila insulin-like peptide; EL, Exoloop; INSL, insulin-like; LDLa, Low Density Lipoprotein type A; LGR, leucine-rich repeat containing G protein-coupled receptor; LRR, Leucine rich repeat; RLN, relaxin; RGP, relaxin-like gonad simulating peptide; RXFP, Relaxin family peptide; TMD, transmembrane domain.

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1. Introduction

The peptide hormone relaxin was first identified in 1926 as a substance in the serum of pregnant guinea pigs or rabbits that when injected into virgin guinea pigs promoted the relaxation of the pubic ligament (Hisaw, 1926). Research in the 1940s to the 1960s determined many of the biological actions of relaxin on the female reproductive tract providing the first insights into the important roles that relaxin played during pregnancy and parturition in many species (reviewed in Bathgate, Hsueh, & Sherwood, 2006). It was not until the 1970s that an understanding of the primary structure of the peptide was obtained and detailed information as to its tissue expression and plasma profile. These studies highlighted that relaxin is produced in the ovary of pregnant mammals and circulates in the plasma during pregnancy where it mediates numerous actions on the reproductive tract (reviewed in Bathgate, Hsueh, et al., 2006; Sherwood, 2004). The cloning of the rat (Hudson, Haley, Cronk, Shine, & Niall, 1981) and porcine relaxin cDNAs (Haley et al., 1982) and the human relaxin genes (Hudson et al., 1983, 1984) in the 1980s demonstrated that relaxin was produced as a prohormone with a B-, C- and A-chain structure like insulin. Like insulin, the C-peptide is cleaved *in vivo* such that the majority of circulating relaxin is in the heterodimeric A/B chain form with two inter-chain and one intra-chain disulphide bonds (Fig. 1). The cloning of human relaxin also demonstrated that there were two homologous relaxin genes in humans *RLN1* and *RLN2*. The product of the *RLN1* and *RLN2* genes are called H1 and H2 relaxin, respectively. Importantly, H2 relaxin was shown to be the "relaxin" produced in the ovary and circulating during pregnancy in humans (Hudson et al., 1984). The function of the *RLN1* gene in humans and higher primates is unknown. Hence H2 relaxin is considered to be the ortholog of the

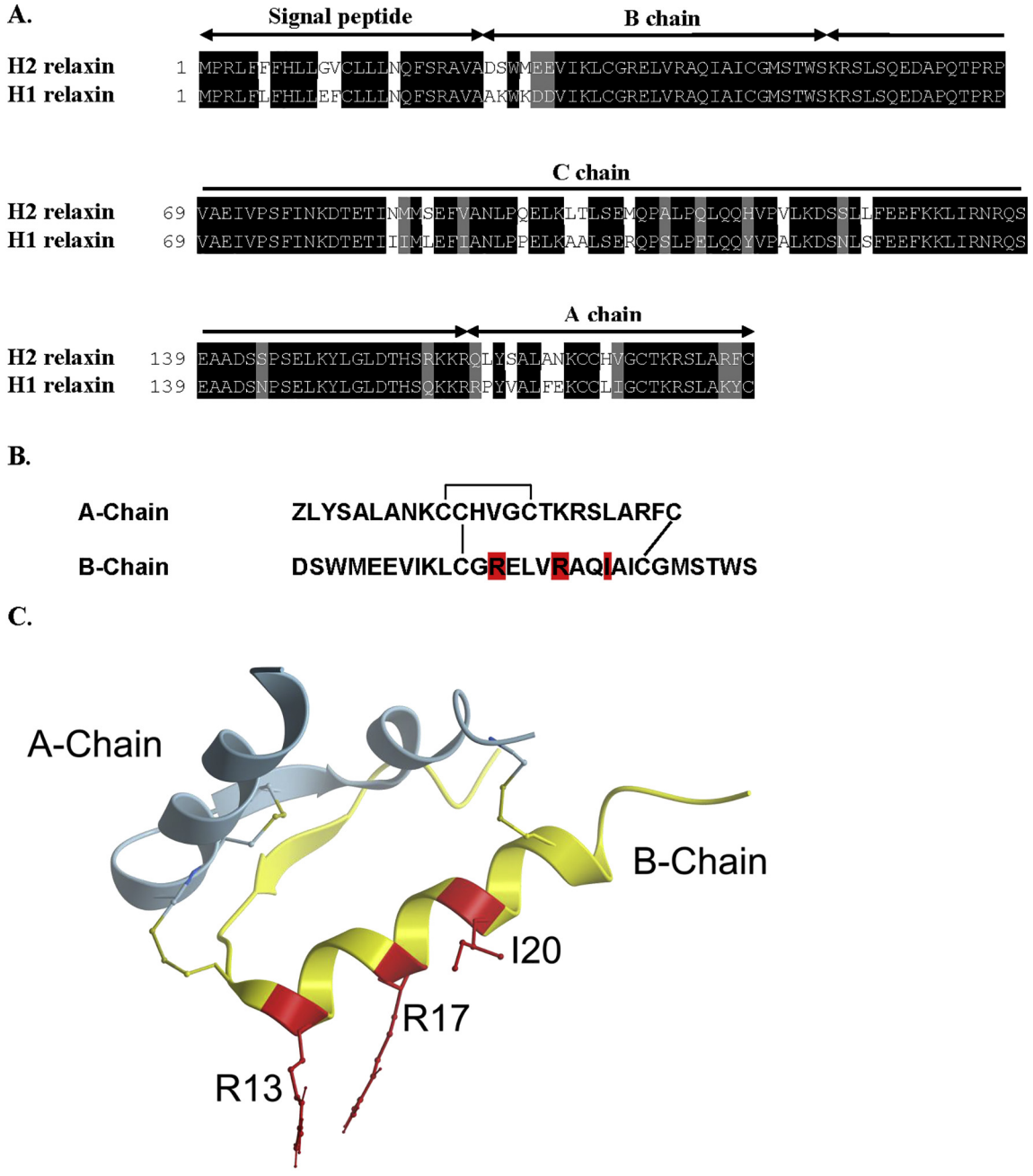


Fig. 1. A. Alignment of pre-prohormone sequences of human relaxin-1 (H1) and relaxin-2 (H2). The signal peptide, B-, C- and A-chains are indicated. Identical residues are highlighted in black and similar residues in grey. B. Mature H2 relaxin peptide sequence with key residues for receptor binding highlighted. C. Crystal structure of H2 relaxin

single *RLN* gene product in non-primate mammals which is referred to as "relaxin".

Research on relaxin progressed for 76 years without knowledge of the identity of the relaxin receptor. It was not until 2002 that the then orphan leucine-rich repeat containing G protein-coupled receptor 7 (LGR7) was shown to be the relaxin receptor (Hsu et al., 2002). In the same article the authors indicated that the related receptor LGR8 also bound relaxin. However, LGR8 was subsequently shown to be the receptor for the related peptide insulin-like peptide 3 (INSL3; see below) (Kumagai et al., 2002). Importantly, LGR8 is still often reported in the literature as a "relaxin" receptor which is incorrect, especially as relaxin peptides from some species do not bind to their corresponding LGR8 receptor (Scott et al., 2005). LGR7 and LGR8 belong to subgroup δ of the rhodopsin class (Type I or Class A) of GPCRs and are further classified as subtype C LGRs (Fredriksson, Lagerström, Lundin, & Schiöth, 2003) (Fig. 2a). The type A LGRs are the three glycoprotein hormone receptors for thyroid-stimulating hormone (TSHR), luteinizing hormone/chorionic gonadotropin (LHCGR) and follicle-stimulating hormone (FSHR) (Hsu & Hsueh, 2000). LGR7 and LGR8 show lower sequence similarity to the type B LGRs, LGR4, LGR5 and LGR6 which have recently been shown to be receptors for the R-spondin peptides (Carmon, Gong, Lin, Thomas, & Liu, 2011; de Lau et al., 2011; Glinka et al., 2011). LGR7 and LGR8 are distinct from class A and B LGRs in having an N-terminal Low Density Lipoprotein A (LDLa) module at their N-terminus and are indeed the only mammalian GPCRs to have such a domain (Fig. 2b). Due to the increasing understanding of the broader relaxin family of peptides and their GPCR partners, LGR7 was classified by IUPHAR as the relaxin family peptide 1 (RXFP1) receptor (Bathgate, Ivell, Sanborn, Sherwood, & Summers, 2006).

There have been considerable advances in the field since the identification of the relaxin receptor, including the development of both relaxin (Zhao et al., 1999) and RXFP1 (Kamat et al., 2004; Krajnc-Franken et al., 2004) KO mice. Importantly the phenotypes of these KO models correlate closely, further highlighting that RXFP1 is the native receptor for relaxin. These model systems have backed up much of the original biology of the relaxin system and its essential roles during pregnancy, but also highlighted some novel aspects of relaxin biology. Both relaxin (Zhao, Samuel, Tregear, Beck, & Wintour, 2000) and RXFP1 (Kamat et al., 2004) KO mice show age-dependent increases in tissue fibrosis, most notably in the lung which is accompanied by functional deficits (Samuel et al., 2017). It has been long known that relaxin is involved in collagen metabolism in reproductive tissues (reviewed in (Bathgate, Hsueh, et al., 2006)) but it was not expected that relaxin would be associated with collagen turnover in males and non-pregnant females. Notably, relaxin shows anti-fibrotic actions in numerous animal models of disease (reviewed in (Samuel et al., 2017)) and therefore there is still considerable interest in the clinical use of relaxin as an anti-fibrotic agent. Another area of relaxin biology that has expanded considerably in recent years is the important role of relaxin in regulating cardiovascular and renal function during pregnancy. These roles have been the topic of many excellent reviews in the past years (Conrad, 2011; Conrad & Davison, 2014; Conrad & Shroff, 2011; Leo et al., 2017; Leo, Jelinic, Ng, Tare, & Parry, 2016). These studies have also directly led to the use of H2 relaxin for the treatment of heart disease which is outlined in this review in the context of "RXFP1 as a therapeutic target".

The discovery of RXFP1 has led to an improved understanding of the interaction of relaxin peptides with the receptor and the design of peptide mimetics. It has also led to the identification of small molecule agonists of RXFP1 which are proving to be valuable research tools to understand the cellular actions of relaxin. These important studies are a major focus of the current review and are covered in detail in the sections "The complex mode of RXFP1 activation", "Development of peptide and small molecule H2 relaxin mimetics" and "RXFP1 signaling".

There have been other recent interesting developments in the field, including the discovery of novel ligands for RXFP1 (Shemesh et al., 2008)

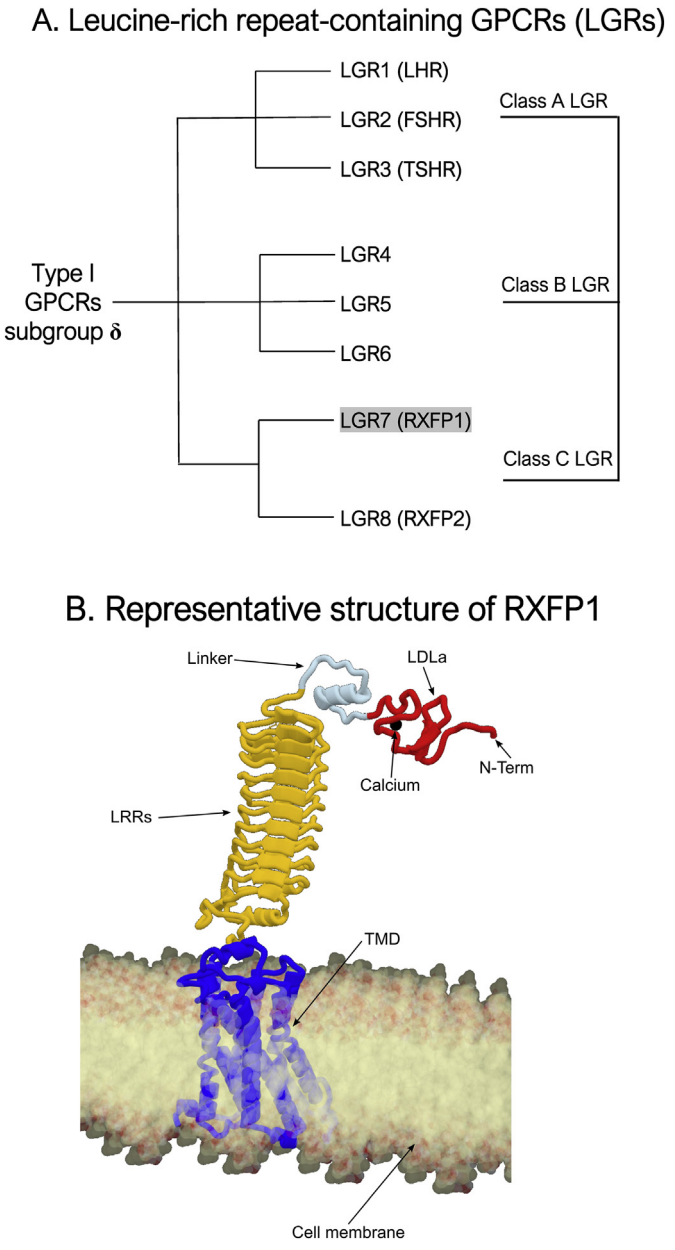


Fig. 2. A. Makeup of the leucine-rich repeat-containing GPCR (LGR) family. B. Cartoon of the putative structure of the RXFP1 receptor highlighting the key structural features. LDLa: Low Density Lipoprotein Type A module, LRRs: Leucine rich-repeats, Linker: linker region between the LRRs and LDLa, TMD: Transmembrane domain.

and the potential interaction of relaxin with the glucocorticoid receptor (Dschietzig, Bartsch, Stangl, Baumann, & Stangl, 2004). Novel ligands of RXFP1 were initially discovered through a screening project of putative bioactive peptides encoded within larger proteins. Hence short linear peptides (named P59 and P74 or CGEN25009) derived from the precursor for the complement C1q tumor necrosis factor-related protein 8 (CTRP8) were shown to activate RXFP1 and RXFP2 expressed in CHO cells (Shemesh et al., 2008). Further studies on CGEN25009 demonstrated that the peptide was able to activate fibroblasts and THP1 cells expressing RXFP1 *in vitro* and reduce lung inflammation and injury as well as to ameliorate adverse airway remodeling and peribronchial fibrosis *in vivo* in mice, in a similar manner to H2 relaxin (Pini et al., 2010). A more recent study demonstrated that the precursor protein CTRP8 induces tumor cell migration and invasion in an RXFP1-dependent manner in glioblastomas isolated from human patients (Glogowska et al., 2013). The authors proposed a molecular model of CTRP8 interacting with LRR7 and LRR8 of

the RXFP1 extracellular domain, however this has not been experimentally validated. The significance of CTRP8 as a novel ligand of RXFP1 is currently unknown.

Due to the rapid expansion of the genome databases, our understanding of the evolution of the relaxin/RXFP1 signaling system has also rapidly advanced in recent years. It is now clear that the relaxin/RXFP1 signaling axis is far more ancient than previously appreciated. This review will therefore also highlight these recent developments starting with an overview of the evolution of the relaxin peptide and its receptor.

2. Evolution of the relaxin/RXFP1 signaling axis

An understanding of the evolution of the relaxin/RXFP1 system requires an introduction to the larger relaxin peptide family in vertebrates. Relaxin family peptides are part of the insulin superfamily and include both relaxin and insulin-like (INSL) peptides. They have gene and protein structures similar to insulin; the gene contains two exons coding for a pre-prohormone, translation of which results in the synthesis of a prohormone, containing three protein domains (B-C-A), which is proven or proposed to be post-translationally modified in the Golgi and secreted as a mature peptide consisting of two peptide chains (B and A domains) joined together by both inter- and intra-chain disulphide bonds at six highly conserved cysteine residues. The relaxin peptide family in humans consists of human relaxin-1 (H1 relaxin) (Hudson et al., 1983), human relaxin 2 (H2 relaxin) (Hudson et al., 1984), human relaxin 3 (H3 relaxin) (Bathgate et al., 2002), insulin-like peptide 3 (INSL3 also known as relaxin-like factor or Leydig insulin-like peptide) (Adham, Burkhardt, Benahmed, & Engel, 1993), insulin-like peptide 4 (INSL4 or placentin) (Chassin, Laurent, Janneau, Berger, & Bellet, 1995), insulin-like peptide 5 (INSL5) (Conklin et al., 1999; Hsu, 1999) and insulin-like peptide 6 (INSL6) (Hsu, 1999; Kasik, Muglia, Stephan, & Menon, 2000; Lok et al., 2000) (Fig. 3). Relaxin and INSL peptides mediate a broad range of physiological functions (reviewed in (Bathgate, Hsueh, et al., 2006)). Importantly, unlike insulin and insulin-like growth factors (IGFs), which signal via receptor tyrosine kinases (RTKs), relaxin and INSL peptides signal via two distinct classes of type I G protein-coupled receptors (GPCR) known as the relaxin family peptide (RXFP) receptors (Bathgate, Ivell, et al., 2006).

Four RXFPs, RXFP1-RXFP4, are found in human and serve as the cognate receptors for four peptides, relaxin, INSL3, RLN3 and INSL5, that arose early during vertebrate evolution (Fig. 3). Notably, the receptors for the most recently evolved peptides in this family, INSL4 and INSL6 are currently unknown. RXFP2 is closely related to the relaxin receptor RXFP1 and was initially reported as a relaxin receptor as it can bind and be activated by relaxin peptides (Hsu et al., 2002). Subsequent studies demonstrated that RXFP2 is actually the receptor for INSL3 (Kumagai et al., 2002) and importantly relaxin peptides from some species including rodents, do not activate RXFP2 (Scott et al., 2005). The important roles of INSL3 in testicular descent and male and female gonadal function have been reviewed elsewhere (Bathgate, Hsueh, et al., 2006; Ferlin et al., 2006; Ivell, Agoulnik, & Anand-Ivell, 2017).

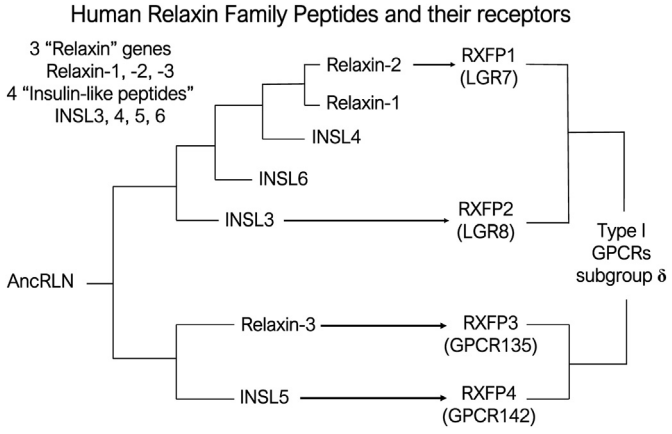


Fig. 3. Phylogenetic tree of the human relaxin family of peptides and their G protein-coupled receptors.

The RXFP3 and RXFP4 receptors are structurally and phylogenetically more closely related to small peptide receptors such as the somatostatin, angiotensin and bradykinin receptors than to the LGR-type RXFP1/2 (Fredriksson et al., 2003). Relaxin-3 and its receptor RXFP3 are highly expressed in the brain with this pairing being considered an important neuropeptide receptor system. Relaxin-3 expression in the brain is highest in the *nucleus incertus* in the brainstem, with smaller populations of neurons expressing relaxin-3 in the pontine raphé nucleus medial and ventrolateral periaqueductal grey, and in an area dorsal to the substantia nigra (Bathgate et al., 2002; Liu et al., 2003; Matsumoto et al., 2000; Tanaka et al., 2005). The distribution of relaxin-3-containing nerve fibres closely matches the distribution of RXFP3 in numerous brain regions (Ma et al., 2007; Sutton et al., 2004). Relaxin-3 has been reported to play a role in the modulation of many different behaviours including arousal, motivation, emotion and related cognition, appetite regulation, stress responses, anxiety, memory, sleep and circadian rhythm (Calvez, de Avila, & Timofeeva, 2017; Kumar et al., 2017; Ma, Smith, Blasiak, & Gundlach, 2017). INSL5 and its receptor RXFP4 are expressed in the colon (Conklin et al., 1999; Grosse et al., 2014; Liu et al., 2005). Recent research indicates that as a gut hormone, INSL5 is highly expressed in enteroendocrine cells (L-cells) while RXFP4 is expressed in the gut, specifically the myenteric and submucosal ganglia that innervate it (Grosse et al., 2014). The precise function of INSL5 is unknown, but it has been hypothesized to play roles in glucose metabolism, satiety, colon motility and microbial sensing (Ang et al., 2017; Burnicka-Turek et al., 2012; Grosse et al., 2014; Lee et al., 2016; Luo et al., 2015; Wagner et al., 2016).

2.1. Evolution of the genes coding for relaxin family peptides and their receptors in vertebrates

All vertebrate relaxin family peptides and their receptors appear to have arisen from three genes in the ancestral vertebrate genome, denoted as *ancrln*, *ancrxfp1/2* and *ancrxfp3/4* (the prefix *anc* denoting ancestral) which were subsequently duplicated during the 2 rounds (2R) of whole genome duplication that occurred ~550 million years ago (Fig. 4). Reconstructions of the post-2R vertebrate gene repertoire indicate that these three ancestral genes (one ligand and two receptor genes) diversified into three *rxfp1/2* (*rxfp1*, *rxfp2* and *rxfp2-like*), four *rxfp3/4* (*rxfp3*, *rxfp3-2*, *rxfp3-3* and *rxfp4*) and four *rln/insl* genes (*rln*, *insl3*, *rln3* and *insl5*), indicating that there were up to 11 *rln/insl/rxfp* genes present in the gnathostome ancestor of tetrapods and teleosts (Fig. 4) (Yegorov, Bogerd, & Good, 2014 ; Yegorov & Good, 2012).

2.2. Changes in rxfp and rln/insl genes and ligand-receptor binding relationships across vertebrate groups

Throughout vertebrate evolution, *rln/insl/rxfp* peptide and receptor genes have changed dramatically in different vertebrate lineages both in terms of gene numbers as well as deduced amino acid sequences. For example, in humans, the peptide family is composed of seven genes: the *RLN* locus (containing *RLN1*, *RLN2*, *INSL4* and *INSL6* genes), *RLN3*, *INSL3* and *INSL5* loci (Fig. 3), while teleosts carry paralogous copies of *rln3* and *insl5* (owing to the fish specific third round (3R) whole genome duplication event which happened ~300 million years ago) and have six genes (*rln*, *insl3*, *rln3a* and *rln3b*, *insl5a* and *insl5b*), whereas birds show signs of extreme gene loss with only *rln* and *insl5* present in chicken (Good, Yegorov, Martijn, Franck, & Bogerd, 2012; Yegorov et al., 2014) (Fig. 4).

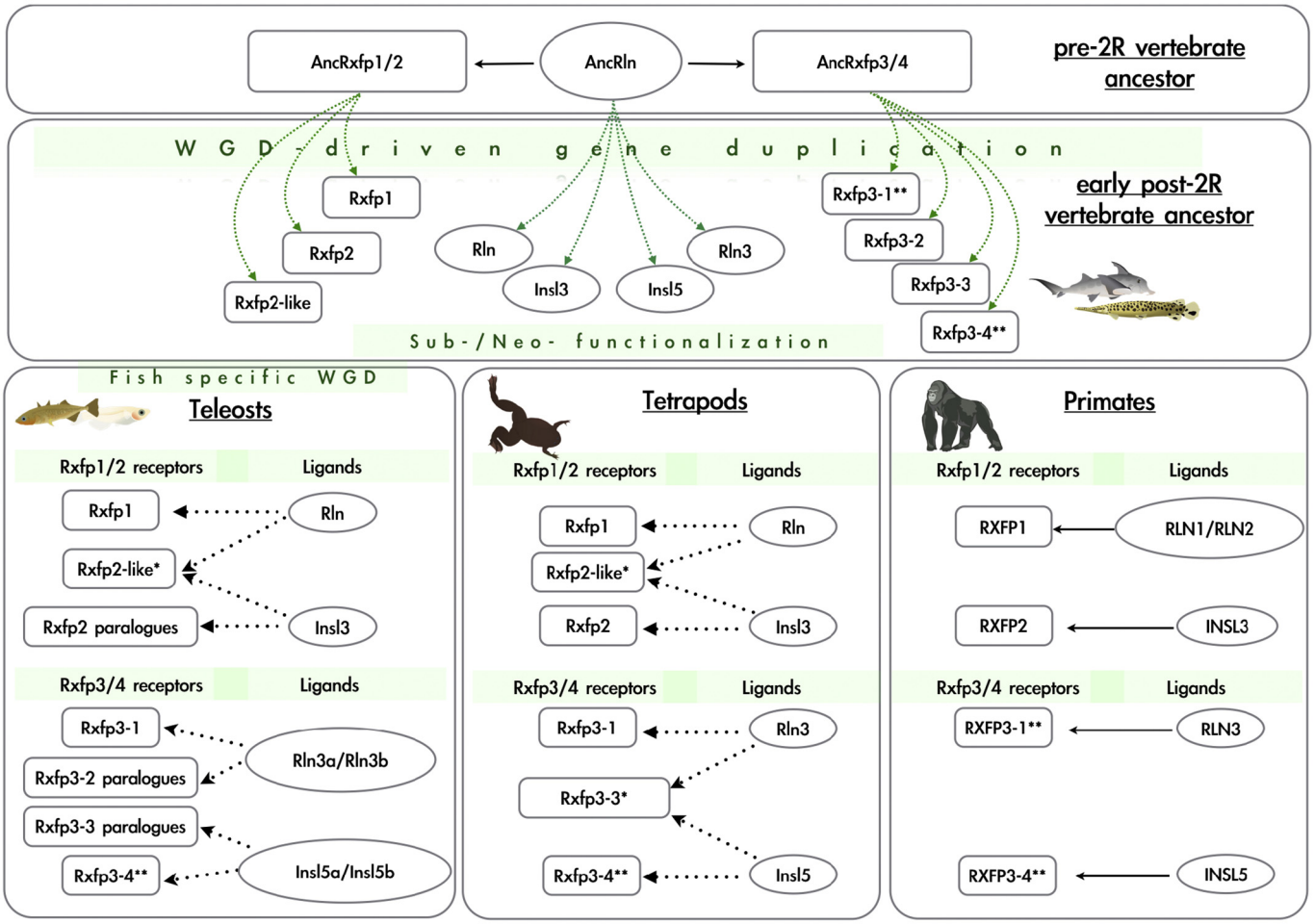


Fig. 4. Evolution of the relaxin family of peptides and receptors in vertebrates. The pre-2R vertebrate ancestor is hypothesized to have had a single relaxin peptide, AncRln (oval), and single copies of both the ancestral Rxfp1/2-like (AncRxfp1/2) and ancestral Rxfp3/4-like (AncRxfp3/4) receptors (rectangular boxes). This tri-partite system underwent duplication (green dotted arrows) during the two rounds (2R) of whole genome duplication in early vertebrate evolution, followed by subsequent sub and neo-functionalization and differential gain and loss of genes in distinct vertebrate lineages, including: duplication of the genes coding for two of the ligands (Rln3 and Insl5) as well as all of the Rxfp3/4 type receptors (lower left panel) in teleosts during the fish specific whole genome duplication; loss of *rxfp3-2* in all tetrapods, loss of *rxfp3-3* in most, but not all, tetrapods, and expansion of the relaxin locus in mammals giving rise to tandemly duplicated copies of *RLN1* and *RLN2* in primates (lower right) as well as *INSL4* and *INSL6* (not shown) that remain orphan ligands. While the primary ligand-receptor binding relationships for primates are known (solid black arrows, lower right panel), the ligand-receptor binding relationships in other vertebrates have been hypothesized but not confirmed experimentally (dotted black arrows). **Rxfp2-like* and *Rxfp3-3* are novel receptors recently identified in several vertebrate genomes, *Rxfp2-like* is the only *rxfp2* gene in chicken, and was also detected as a pseudogene on human chromosome X. ** *Rxfp3-1* and *Rxfp3-4* pertain to the genes traditionally known in mammals as *Rxfp3* and *Rxfp4* respectively.

Another characteristic of the relaxin family, is the dynamic changes in receptor-ligand binding, including binding promiscuity and the evolution of new ligand-receptor pairs. In humans and mice, the receptor-ligand ratio is close to 1, although several of the ligands can bind to multiple RXFP receptors, and the cognate relationships are relatively well understood (reviewed in Bathgate et al., 2013) (Fig. 3), while the co-evolution and molecular biology of *rln/insl/rxfp* peptide and receptor genes in teleosts is more diverse with a receptor-ligand ratio in zebrafish of almost 2:1. In other vertebrates (for example, frog and chicken), the receptor-ligand ratios and binding partners appear to be different from those in mammals and are yet to be explored (Good et al., 2012; Yegorov et al., 2014; Yegorov & Good, 2012). In keeping with these dynamic receptor-ligand binding affinities, the relaxin family of peptides has recruited new receptors at least once throughout evolution. It is thought that one such major event occurred during early chordate evolution when the Rxfp3/4 type receptors were recruited to supplement the more ancient Rxfp1/2 LGR subtype C receptors (Fig. 2). Additionally, the receptors for novel mammalian peptides Insl4 and Insl6, remain unidentified. Notably, there is evidence that both RXFP1 and relaxin have additional binding partners in mammals as relaxin has been shown to bind to the glucocorticoid receptor (Dschietzig et al., 2004), and the human C1q-tumour necrosis factor-related protein 8 (CTRP8) (Glogowska et al., 2013) and fragments of the protein (Shemesh et al., 2008) have been shown to bind to RXFP1. The physiological significance of these interactions is not yet understood.

2.3. Evolution of rln and rxfp1 genes in vertebrates

Of all of the relaxin family receptor-ligand pairs, the evolution of the RXFP1-relaxin system is perhaps the most enigmatic. For one, in teleosts, the *rln* gene has higher sequence similarity with teleost (and mammalian) *rln3* than with mammalian *rln*. The ratio of non-synonymous to synonymous mutations (d_N/d_S), an indicator of the level of constraint on amino acid changes, in teleost *rln* is $d_N/d_S = 0.09$ which is similar to teleost *rln3* ($d_N/d_S = 0.07$), but very different from mammalian *rln* ($d_N/d_S = 0.5$) (a d_N/d_S of 0 indicates very high conservation, a $d_N/d_S > 1$ suggests evidence of positive selection) (Good-Avila et al., 2009; Wilkinson, Speed, Tregear, & Bathgate, 2005). Furthermore, teleost relaxin differs from mammalian relaxin at 61.5% of the amino acids, while it differs from mammalian relaxin-3 at only 36.5% of the amino acids. The similarity and conserved nature of teleost

rln3 (which duplicated during 3R giving rise to paralogous genes *rln3a* and *rln3b*) with teleost *rln* lead Wilkinson et al. (2005) to name teleost *rln*, *rln3c* in the first bioinformatic study of the family in vertebrates (Wilkinson et al., 2005), something that was later shown to be incorrect based on syntenic data analyses, but persists erroneously in some databases (Good-Avila et al., 2009; Park et al., 2008).

2.4. Accelerated evolution of rln-rxfp1 in mammals

Despite its highly conserved nature in non-mammals, relaxin has changed extensively during recent mammalian evolution. Beginning with marsupials and monotremes, the rate of evolution of *rln* accelerates rapidly: this increased rate of evolution is evident in both, 1) the rate of amino acid change, 2) duplication of the relaxin locus giving rise to *insl4* and *insl6* as well as other duplications in lineages such as rabbit and primates (*rln1*/*rln2*) (Arroyo, Hoffmann, Good, & Opazo, 2012; Arroyo, Hoffmann, & Opazo, 2014; Hoffmann & Opazo, 2011) and, 3) loss of relaxin genes in ruminants (Malone, Opazo, Ryan, & Hoffmann, 2017). The coding sequence of *rln2* is highly variable among mammalian species. The d_N/d_S ratio of the *RLN2* in human vs chimpanzees is 1.6 indicating evidence of positive selection which is heavily concentrated on the B-chain region with a d_N/d_S ratio of 5.0 (Wilkinson et al., 2005). Furthermore, while mammal-wide estimates of d_N/d_S were found to be 0.64 for *rln2*, indicating more selection in some species than others, but only 0.08 for *rln3* (Good-Avila et al., 2009).

Despite the high divergence in relaxin peptide sequences across mammalian species, the amino acid sequences and their complete loss in some lineages (see below), the Rxfp1 proteins across vertebrates are quite conserved (Good et al., 2012; Yegorov et al., 2014). This suggests that only those residues of relaxin that are required for binding to Rxfp1 may be conserved, while other residues have responded to different selective pressures. Notably, the RXXXRXXI/L sequence in the B-chain responsible for Rxfp1 binding is highly conserved in mammalian and vertebrate *rln* sequences. The rapid evolution of other residues in the *rln2* gene may be driven by diversifying selection to prevent binding to non-cognate receptors of other genes at the *rln* locus (*insl4*, *insl6* and/or *rln1*), and/or by the presence of multiple interacting partners for the relaxin peptide. Interestingly, ruminants show a lack of functional *rln* genes despite their responsiveness to the relaxin peptides derived from other mammals (Bagna, Schwabe, & Anderson, 1991; Malone et al., 2017). Since at the same time, ruminants harbour all four *rxfp* receptor genes orthologous to human RXFPs (i.e. RXFP1-4) and an additional *RXFP3-like* gene (Yegorov et al., 2014), this implies that the ruminant lineages experienced a recent reorganization of relaxin peptide-receptor pairing.

All in all, we believe that a better understanding of the mechanisms driving the rapid evolution of mammalian relaxin-Rxfp systems will facilitate the development of clinical therapies targeting them.

2.5. Relaxin family peptides and receptors in protostomes

Protostomes harbour various numbers of insulin-like peptides (Ilps) (Fig. 1B), but until recently it was not clear whether any of these signaling molecules were relaxin-like. More than two decades ago, the *Drosophila* insulin receptor homolog gene (*inr*) was identified (Fernandez, Tabarini, Azpiazu, Frasch, & Schlessinger, 1995), which led the discovery of seven *Drosophila insulin-like peptide* genes (*dilp1–7*), of which the gene products of four (*dilp2*, *-3*, *-5* and *-6*), appear to have functions similar to vertebrate Ins/Igf peptides. In 2012, another *dilp*, *dilp8*, was identified in *D. melanogaster* (Garelli, Gontijo, Miguela, Caparros, & Dominguez, 2012), when it was demonstrated that Dilp8 delays the onset of adulthood by slowing growth of the imaginal discs, to ensure developmental stability, symmetry and robustness. These imaginal discs are part of juvenile insects (larva) and will form wings, legs, antenna and other external structures in the adult during pupal transformation. In a following paper Dilp8 was demonstrated to exert these effects via a signaling pathway involving the *Drosophila* relaxin-like receptor LGR3, an LGR type C receptor homologous to RXFP1 and RXFP2 (Garelli et al., 2015). The authors localized LGR3 activity to a new subset of neurons in the central nervous system (CNS) in the *pars intercerebralis* (PIL) region. Stimulation of LGR3 positive PIL neurons with ectopic Dilp8 peptide increased intracellular cAMP, suggesting that Dilp8 was a relaxin-like peptide that signaled through the orphan LGR3. A second group independently replicated these findings and demonstrated that LGR3 is the cognate receptor for Dilp8 (Vallejo, Juarez-Carreno, Bolivar, Morante, & Dominguez, 2015), confirming that a relaxin-like signaling system exists in protostomes. Garelli et al. (2015) argue that the neurosecretory cell-rich PIL region that expresses LGR3 in *Drosophila* connects the CNS to the endocrine ring gland in an analogous way that the hypothalamus links the CNS to the endocrine system via the pituitary gland in vertebrates. As such, they propose that the relaxin signaling system in both *Drosophila* and vertebrates, influence similar physiological responses related to stress, energy metabolism, growth, water retention and reproduction, although no relaxin signaling system, like Dilp8, has been found to play a role in protostome juvenile development *per se*.

These findings in *Drosophila* imply that relaxin-like peptides are probably widespread in protostomes. The genome of *D. melanogaster*, encodes two putative type C LGRs, i.e. CG31096 (dLGR3) and CG34411 (dLGR4), initially known as CG5042 and CG4187, respectively. Recent phylogenetic analyses across metazoa revealed that type C LGRs are present in sea anemones, coral, molluscs, annelid, arthropods as well as in Cephalochordates and Urochordates (Roch & Sherwood, 2014; Van Loy et al., 2008). Since Ilps are also widespread in protostomes (Leevers, 2001), this suggests that other relaxin signaling systems are likely present in protostomes. Given the presence of multiple orphan LGRs, including LGR4 in *Drosophila*, and orphan Ilps in diverse taxa, this opens the door for research into the presence, diversity and function of relaxin-like Ilps that signal via LGR type C receptors in protostome biology.

2.6. Relaxin family peptides in deuterostomes

A relaxin-like peptide was identified and biochemically characterized as a gonad-stimulating substance (Gss) in *Asterina pectinifera* (Mita et al., 2009; Mita, Yamamoto, & Nagahama, 2011) and subsequently in other starfish species (Mita, Daiya, Haraguchi, Tsutsui, & Nagahama, 2015; Mita & Katayama, 2016) and named relaxin-like gonad stimulating peptide (Rgp). Rgp, secreted by radial nerves induces follicular cells to produce 1-methyladenine, the echinoderm counterpart of vertebrate progestin, thereby promoting oocyte maturation. While the receptor of Rgp remains unknown, its signal transduction is mediated by a GPCR that results in a dose-dependent increase in intracellular cAMP concentration (Mita et al., 2012; Mita, Haraguchi, Uzawa, & Tsutsui, 2013), as would be expected for a relaxin LGR type C receptor homologous to RXFP1/2. The signaling role of Rgp in starfish is reminiscent of vertebrate LH, which is produced in the pituitary and signals via the hr (an LGR type A receptor) located on follicular cells (Mita et al., 2013). This function suggests a reproductive role for Rgp compared to Dilp8, although Lin et al. (2017) propose that the cells secreting Rgp in the radial nerve of starfish are functionally equivalent to neurons secreting Rln3 in mammals, supporting a similar role of the CNS in relaxin-like signaling systems in both *Drosophila* and starfish.

Deuterostome genomes also possess multiple LGR-type C receptors. For example, the sea urchin *Strongylocentrotus purpuratus* (echinoderms) genome has up to 35 *rxfp1/2* genes, of which only two were found to be similar to *Drosophila lgr3* and *lgr4* and vertebrates *rxfp1/2*. On the other hand, *Amphioxus* and *Ciona* both harbour *rxfp1/2*-like genes: all five of *rxfp1/2* type genes in *Amphioxus* appear closely related to vertebrate *rxfp1/2* genes, and one of seven *rxfp1/2*-like genes in *C. intestinalis* was found to be closely related to vertebrate *rxfp1/2s* (Yegorov & Good,

2012). Notably, three out of the five *rxfp1/2*-like genes in *Amphioxus* were found to be in the same genomic linkage group (CLG8 in Putnam et al., 2008) as human *RXFP1* and *RXFP2* (Yegorov & Good, 2012), providing strong support that the orthologue of vertebrate *rxpf1/2* gene was already present in the chordate ancestor (Yegorov & Good, 2012). Despite the extensive representation of *rxfp1/2* genes in protostomes and early deuterostomes, *rxfp3/4* type receptors have only been found in the *Ciona* genome, but not in earlier diverging taxa and it is not known if any of the peptides coded by the *Ciona rxfp3/4* genes are functionally related to relaxin peptides (Fig. 5).

Olinski, Lundin, and Hallbook (2006) identified three paralogons containing insulin-like genes in *Ciona intestinalis* (*ins-L1*, *ins-L2* and *ins-L3*), all of which displayed conserved synteny to *INS/IGF/RLN-INSL* gene clusters in humans. They proposed that the *Ciona* genome contained *rln*-like (*ins-L1*), *ins*-like (*ins-L2*) and *igf*-like (*ins-L3*) genes; experimental work has supported the possible orthology of *Ciona ins-L2* and *ins-L3* to vertebrate *ins* and *igf* genes respectively (Thompson & Di Gregorio, 2015). Additionally, a fourth Ilp has been identified in *Ciona*, which exhibits greater similarity to vertebrate relaxin-like peptides (Yegorov & Good, 2012), suggesting there may be one or two *rln*-like genes in *Ciona*. On the other hand, bioinformatic data mining in the *Amphioxus* genome (Holland et al., 2008; Olinski et al., 2006), originally led to the identification of three *ilps*, but more recently analyses indicate that there are eight or more *ilps* in *Amphioxus*, several of which appear to code for relaxin-like peptides (Lecroisey, Le Petillon, Escriva, Lammert, & Laudet, 2015; Good et al., unpublished) (Fig. 5).

Overall, the vertebrate phylogenomic data combined with the fragmented data from invertebrates point at an evolutionary scenario whereby *rln/insl* and *rxfp1/2*-like receptors had a common ancestor in protostomes and deuterostomes and co-evolved to produce expanded gene families in some pre-vertebrate groups (e.g. sea urchins and lancelets). At some point in the pre-vertebrate genome, Rxfp3/4 was recruited to function as a second receptor for a relaxin-like peptide forming a tri-partite signaling system (a relaxin-like peptide and two receptors, possibly sub-functionalized by expression in different tissues). It further appears that only a single *rxfp1/2*, *rxfp3/4* and *rln*-like gene were retained in the early vertebrate genome and then duplicated during the 2R of whole genome duplications that occurred in early vertebrate evolution to give rise to expanded families of vertebrate genes (Yegorov & Good, 2012). A plausible scenario is that the recruitment of genes coding for *rxfp3/4* type receptors into the relaxin–like peptide signaling repertoire facilitated sub- and neo-functionalization of the neuroendocrine and reproductive roles of these hormones during the rapidly changing and newly compartmentalized vertebrate body plan that included the development of the hypothalamus-pituitary-gonadal and hypothalamus-pituitary-adrenal axes (Good et al., 2012) in which the relaxin family peptides play diverse roles.

2.7. Evolutionary hints at broader physiological roles of RLN-RXFP systems

A broader view of the evolution and function of the relaxin gene in vertebrates may help explain the finding that the *Drosophila* relaxin-like peptide, Dilp8, and its receptor, LGR3, are co-localized to the CNS and play roles in mediating developmental processes. While in mammals, relaxin/RXFP1 signaling is predominantly associated with reproduction, relaxin/RXFP1 are also widely expressed in the mammalian brain and play significant roles in other tissues (such as the heart and renal system), and, in lower vertebrates and teleosts, the *rln* gene is

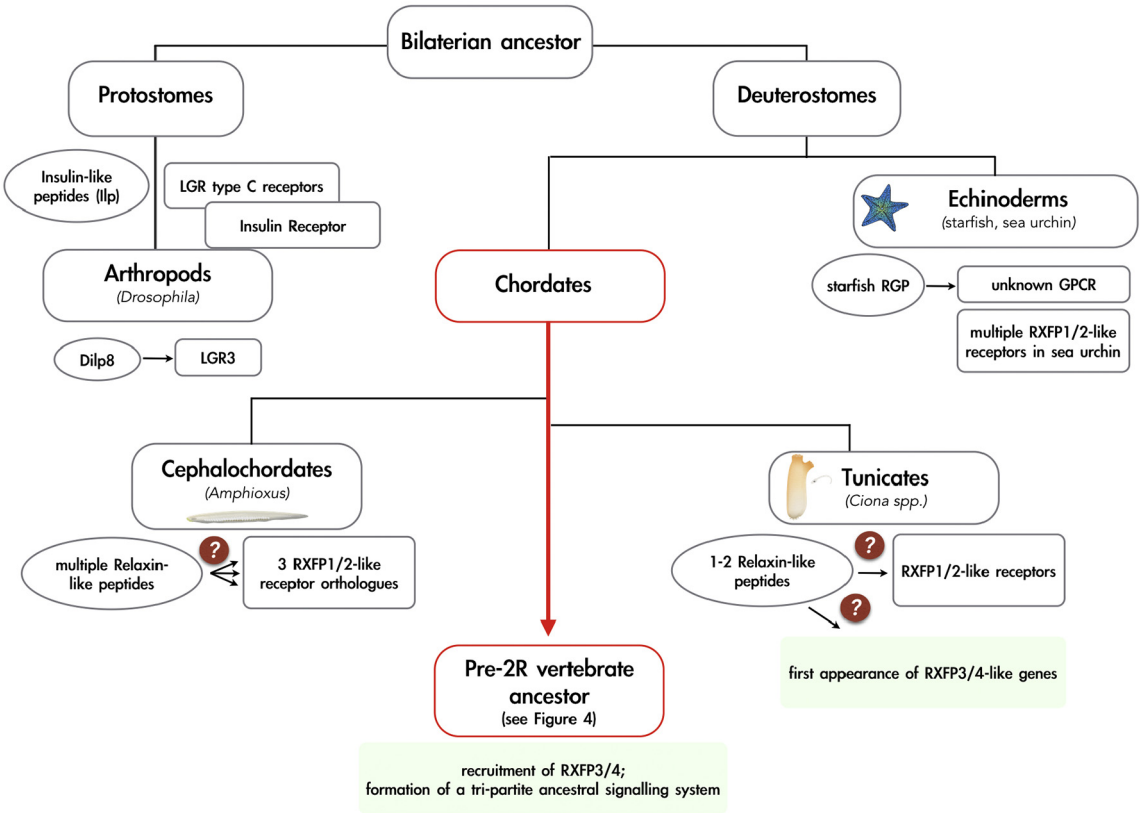


Fig. 5. Evolution of the relaxin peptides and candidate receptors in pre-vertebrates. Data mining and phylogenetic analyses of early chordate genomes has led to the identification of multiple relaxin-like peptides in both tunicates (*Ciona*) and cephalochordates (*Amphioxus*); both lineages harbour multiple *rxfp1/2* genes but candidate ancestral *rxfp3/4* genes have only been identified in *Ciona*. Experimental work in the early deuterostome starfish identified a relaxin-like peptide called relaxin-like gonad-stimulating peptide (Rgp) that is involved in oocyte maturation and signals via a yet-to be described GPCR. Although the receptor for Rgp remains unknown, multiple *rxfp1/2* genes have been identified in other echinoderms. In protostomes, multiple insulin-like peptides (Ilps, of which there are eight in *Drosophila*) have been characterized as well as multiple LGR type C receptors (the class of receptors to which *rxfp1/2* genes belong), and, in most species, a singular insulin receptor. Recent evidence indicates that *Drosophila* Ilp8 (Dilp8) is the endogenous ligand for Lgr3, suggesting that Dilp8-Lgr3 represents an ancestral relaxin signaling system in protostomes.

less associated with reproduction. As described above, teleost *rln* is more similar to *rln3* than to mammalian *rln*, and expression of teleost relaxin and RXFP1 are higher in brain than reproductive tissues (Fiengo, Donizetti, del Gaudio, Minucci, & Aniello, 2012; Hu, Kusakabe, & Takei, 2011). Moreover, a novel member of the relaxin gene family of receptors, *rxfp2*-like that was recently discovered through data-mining and phylogenetic reconstruction of vertebrate *rxfp* sequences (Yegorov & Good, 2012) is also present in many lower vertebrates (including birds, reptiles, coelacanth, spotted gar and zebrafish), and was found to be predominantly expressed in zebrafish brain based on RNA sequencing data from multiple tissues (Al N'Afea et al., in preparation). These evolutionary insights suggest that at least some of the ancestral functions of relaxin and RXFP1 may have less to do with reproduction, but with some of the broader physiological effects of relaxin/RXFP1.

In support of this, Jaszczak, Wolpe, Bhandari, Jaszczak, and Halme (2016) recently showed LGR3 functions in the prothoracic gland (PG) of *Drosophila melanogaster* where it signals upstream of the nitric oxide synthase (NOS) pathway to coordinate developmental timing. Using RNAi, they demonstrated that l*gr3* $^{-/-}$ flies continued development of their imaginal discs even when damaged, and demonstrated that LGR3 activity in both the CNS and PG is necessary for NOS activation in the PG following damage (Jaszczak et al., 2016). This dependence of NOS activation on Dilp8/LGR3 signaling is reminiscent of many of the key signaling actions of relaxin through RXFP1 in mammals (outlined in RXFP1 signaling section). Nitric oxide signaling is a key component of the vascular (Leo et al., 2017) and tissues remodeling actions of relaxin acting through RXFP1 (Samuel et al., 2017). Although there is no evidence that relaxin/RXFP1 signaling plays a role in the developmental process *per se*, the parallel upregulation of NOS by both LGR3 and RXFP1 suggests that this may be one of the oldest pathways by which these receptors exert their physiological effects.

In summary, the recent detailed evolutionary analysis made possible by the availability of multiple vertebrate and chordate genome sequences, highlights that the relaxin/RXFP1 axis constitutes an ancient signaling system with roles extending beyond the regulation of reproductive functions.

3. RXFP1 as a therapeutic target

The clinical history of using relaxin as a therapeutic goes back over 60 years with early studies using partially purified extracts of pig ovary containing large amounts of porcine relaxin, named releasin, to treat various patients including those with scleroderma (Casten & Boucek, 1958) or peripheral artery disease (Casten, Gilmore, Houghton, & Samuels, 1960). The cloning of the human relaxin-2 gene enabled the development of recombinant technology to produce recombinant H2 relaxin, known as serelaxin. The availability of the drug serelaxin, able to be produced in large amounts, has enabled numerous randomized, placebo-controlled clinical trials. Before the recently completed acute heart failure (AHF) Phase IIIb clinical trials, serelaxin had been administered in clinical trials to more than 1500 human subjects (Unemori, 2017). Upon completion of the Phase IIIb trials which involved 3274 serelaxin-treated patients, this number now stands at nearly 5000 human subjects. The Phase IIIb trial once again reinforced that there were no adverse effects of serelaxin treatment mirroring previous trials where the drug was well tolerated with limited side effects (Teichman et al., 2009).

Initial clinical efforts were targeted at cervical ripening as there was, and still is, a need for clinical agents to assist in inducing labour when the cervix has not ripened (Weiss et al., 2016). Relaxin had been demonstrated to induce cervical ripening in various mammals (reviewed in (Bathgate, Hsueh, et al., 2006)). There was evidence for relaxin receptors in the human cervix (Kohsaka et al., 1998) and human cervical cells had been shown to respond to relaxin (Hwang, Macinga, & Rorke, 1996). Clinical trials utilized serelaxin administered as an intravaginal gel which was shown to be safe (Bell et al., 1993); however, serelaxin had no effect as a cervical ripening agent before induction of labour at term (Brennand et al., 1997). It was suggested that the trial failed because serelaxin did not reach the target cervical cells; indeed studies have shown that serelaxin is not adsorbed by this route of administration (Chen et al., 1993). A subsequent Phase II randomised, double blind, placebo controlled trial utilized intravenous infusion of serelaxin (Weiss et al., 2016). Unfortunately, although serelaxin infusion was well tolerated, it did not advance cervical ripening in the patients. It should be noted that in those species of mammals where relaxin acts as a cervical ripening agent, there is a large antepartum surge in plasma relaxin levels (Bathgate, Hsueh, et al., 2006), whereas in humans this does not occur (Quagliarello, Lustig, Steinetz, & Weiss, 1980). It should be noted however that relaxin concentrations in late pregnant women are still quite high and it is possible that the maximum effect of relaxin may be already achieved physiologically and adding more does not have additional effect.

It has long been known that relaxin modulates connective tissue remodelling in numerous reproductive organs during pregnancy (Bathgate, Hsueh, et al., 2006). It was therefore envisaged that relaxin would be an effective anti-fibrotic agent. Subsequently, relaxin has been successfully used to reverse fibrosis in numerous animal models of organ fibrosis (reviewed in Samuel et al., 2017). Early studies using partially purified porcine relaxin showed some promise for treating scleroderma or systemic sclerosis (Casten & Boucek, 1958), a disease characterized by fibrosis of the skin, vasculature, and internal organs. Clinical trials with serelaxin were therefore performed using continuous subcutaneous infusion over six months. An initial small Phase II study demonstrated encouraging results with reduced skin thickening, improved mobility, and improved function in patients with moderate to severe diffuse scleroderma (Seibold et al., 2000). However, a larger follow up Phase II/III trial did not meet its primary endpoint, which was a measure of skin thickness (Khanna et al., 2009). It has been suggested that this failure was due to the larger patient cohort which potentially included more patients with severe disease. An interesting result of the study was the significant increases in creatinine clearance observed in serelaxin-treated patients (Erikson & Unemori, 2001). These changes are consistent with increases in renal perfusion which are also observed in rats treated with relaxin (Danielson, Sherwood, & Conrad, 1999). Moderate decreases in diastolic blood pressure were also seen during serelaxin treatment. The renal perfusion and blood pressure results highlighted that serelaxin infusion was having biological actions and demonstrated that relaxin has cardiovascular and renal effects in humans.

These renal effects of relaxin together with its vasodilatory actions (Leo et al., 2017) and known ability to increase global arterial compliance (Conrad, Debrah, Novak, Danielson, & Shroff, 2004) suggested it may be a unique potential therapeutic for the treatment of heart failure. The first clinical study was a Phase I/II safety and dose finding study in patients with stable heart failure with reduced ejection fraction (HFrEF) (Dschietzig et al., 2009). Serelaxin infused over 24 hours at multiple doses was well tolerated by all the patients. Systemic vascular resistance (SVR) was reduced at all doses with trends in improvement of a number of hemodynamic parameters seen mostly at the lower doses (Dschietzig et al., 2009). Notably, bell-shaped concentration responses were seen for some parameters similar to signaling responses seen with relaxin in some cell systems (see RXFP1 signaling section).

The encouraging renovascular results from this trial prompted a larger clinical trial in AHF, where renal dysfunction is a common comorbidity and major predictor of poor patient outcomes (Teichman et al., 2009). Originally a multicenter, international pilot Phase IIb study (Pre-RELAX AHF) was initiated to determine the optimal dose of serelaxin to test in a larger Phase III trial (RELAX-AHF). AHF patients with normal or increased blood pressure were infused with different doses of serelaxin or placebo over 48 hours (Teerlink et al., 2009). Numerous clinical endpoints were observed during and up to 180 days

after infusion. Serelaxin at all doses once again showed a good safety profile and was associated with favourable relief of dyspnoea and other clinical outcomes. Notably, there were improvements in cardiovascular death or readmission at 60 days, and 180-day cardiovascular mortality especially in the group receiving serelaxin at 30 μg/kg per day (Teerlink et al., 2009).

The Phase III trial, RELAXIN AHF was therefore initiated with 1161 AHF patients randomly assigned to serelaxin 30 μg/kg per day or placebo 48 hour infusion. Serelaxin treatment significantly improved one of the primary endpoints of dyspnoea relief, but had no significant effect on the other dyspnoea measurement (Teerlink et al., 2013). There were also no significant effects on secondary endpoints of cardiovascular death or readmission to hospital for heart failure or renal failure or days alive out of the hospital up to day 60. However, serelaxin treatment was associated with a significant reduction in mortality at day 180 (Teerlink et al., 2013). Measurement of plasma biomarkers demonstrated that markers of cardiac (high-sensitivity cardiac troponin T), renal (creatinine and cystatin-C), and hepatic (aspartate transaminase and alanine transaminase) damage and of decongestion (N-terminal pro–brain natriuretic peptide) were all significantly improved in serelaxin treated patients (Metra et al., 2013). These results are consistent with serelaxin infusion preventing organ damage and this being a potential mechanism by which the 24 hour infusion could improve mortality 180 days later (Metra et al., 2013).

A Phase IIIb trial was then initiated (RELAX AHF-2) which was designed to demonstrate an improvement in cardiovascular and all-cause mortality, consistent with the Phase III RELAX-AHF (Teerlink et al., 2013) and Phase II Pre-RELAX-AHF trials (Teerlink et al., 2009). An AHF program was also initiated in Asia (Sato et al., 2015, 2017) as well as a Phase II trial assessing the safety and pharmacokinetics of serelaxin in paediatric heart failure patients was also started (Unemori, 2017). Unfortunately, it was recently announced that RELAX-AHF-2 did not meet its primary endpoints of reduction in cardiovascular death through Day 180 or reduced worsening heart failure through Day 5 when added to standard therapy in patients with AHF. (https://www.novartis.com/news/mediareleases/novartis-provides-update-phase-iii-study-rlx030-serelaxinpatients-acute-heart). The results were presented at the European Society of Cardiology meeting in April 2017, but have not been published as yet; hence, there is no account so far for this striking discrepancy between the two Phase III trials in AHF. It is therefore not known if the AHF programme will continue or if other trials that were ongoing in patients with hepatic impairment (Kobalava et al., 2015), renal impairment (Dahlke et al., 2016), and patients with compensated cirrhosis (Snowdon et al., 2017) will also continue.

Interestingly, an additional clinical study was completed which examined the safety and antibody responses to repeated serelaxin infusions in CHF patients (RELAX-REPEAT). The results indicated that three sequential 48 hour infusions of serelaxin four weeks apart were safe and well tolerated with only one patient out of 200 developing non-neutralizing antibodies (Unemori, 2017). This study highlights that chronic serelaxin treatment may be possible and may be more efficacious for heart failure treatment. Chronic treatment would certainly be necessary for the treatment of fibrosis.

The future of serelaxin as a therapeutic is unclear. However, what is clear is that nearly 5000 human patients have been treated with serelaxin with no serious adverse effects or incidence differences between treatment and placebo groups. Serelaxin infusion is well tolerated even up to very high doses of 250 μg/kg per day used in CHF trials (Teerlink et al., 2009). Efficacy of serelaxin action was demonstrated by various biological parameters in all the trials highlighting the clear biological actions of serelaxin. However, the AHF trials also highlighted that much is not known about the mechanism of relaxin action. Future clinical development will be aided by advances in the understanding of the relaxin/RXFP1 interaction, the development of cell specific peptide analogs and small molecule RXFP1 agonists and a detailed understanding of the cellular actions of relaxin which are outlined in the sections below.

4. The complex mode of RXFP1 activation

Before the identification of RXFP1, studies primarily focused on understanding which residues in the relaxin peptide were responsible for its biological activity (reviewed in (Bathgate, Hsueh, et al., 2006)). A comparison of relaxin sequences from different species highlighted the low sequence similarity in the primary structures, except at the conserved cysteine residues and the two arginine residues (Arg/R) in the middle of the B-chain. Subsequent studies on synthetic H2 relaxin, involving chemical modification or mutation of these arginine residues at position 13 and 17 in the B-chain, demonstrated that they are essential for relaxin activity (Bullesbach & Schwabe, 1991; Bullesbach, Yang, & Schwabe, 1992). Subsequently a third amino acid, isoleucine (Ile/I) at position 20 which is valine in some species, was shown to also be important for activity (Bullesbach & Schwabe, 2000). The primary H2 relaxin receptor-binding site is therefore considered to comprise of these three residues forming a so-called “relaxin binding cassette” RXXXRXXI/L (Bullesbach & Schwabe, 2000).

Upon the discovery of RXFP1, much effort was then focussed on the understanding of the mode of H2 relaxin-mediated binding and activation of the receptor. As discussed above, the domain structure of RXFP1 comprises of a large central Leucine Rich Repeat (LRR) domain, joined by two linker regions to an N-terminal Low Density Lipoprotein A (LDLa) module, and a C-terminal transmembrane domain (TMD) of seven transmembrane helices, typical of the Rhodopsin class of GPCRs. Historically, through *in silico* modelling of the LRR domain and site-directed mutagenesis of RXFP1, the LRR domain was identified as the high-affinity binding site for the “relaxin binding cassette” (Bullesbach & Schwabe, 2005; Scott, Rosengren, & Bathgate, 2012). The experiments suggested Arg13 and Arg17 to interact with acidic residues (Asp231, Glu233, Glu277 and Asp279) within LRR6 and LRR8 of RXFP1, whereas Ile20 of relaxin interacts with hydrophobic residues (Trp180, Ile182 and Leu204) of LRR4-5.

Importantly, relaxin binding to the LRR alone is not sufficient for receptor activation. Studies on a native splice variant of RXFP2 and an engineered equivalent variant of RXFP1 both lacking the N-terminal LDLa module showed that while the LDLa module did not influence peptide hormone binding, it is essential for RXFP1 and RXFP2 activation (Scott et al., 2006). Subsequent studies tested whether the RXFP1 variant lacking the LDLa module could signal through any known GPCR signaling pathway using a series of reporter gene assays (Kong et al., 2013). These studies clearly demonstrated that RXFP1 absolutely requires the LDLa module for signaling. Similar studies demonstrated the same for RXFP2 in response to H2 relaxin or INSL3. These experiments suggest that the LDLa module is an example of a tethered agonist, similar to the Protease Activated and Adhesion GPCRs (Schoneberg, Kleinau, & Bruser, 2016). In this section of this review we focus on recent experiments that support the role of the LDLa module as a tethered agonist. We further summarize new information that shows the peptide linker between the LDLa module and the LRR domain is not a simple spacer, but plays essential roles in both ligand binding and activation.

The presence of an LDLa module distinguishes RXFP1 and RXFP2 from other LGRs, and indeed mammalian RXFP1 and RXFP2 and their invertebrate orthologs are the only known GPCRs to contain this module. The LDLa module was initially characterized as a repeating module in the LDL receptor and related proteins involved in lipid metabolism (Sudhof, Goldstein, Brown, & Russell, 1985; Yamamoto et al., 1984). Since then, LDLa modules have been characterized in protein-protein interactions in a diverse number of systems including viral entry, cancer metastasis and invasion and cell differentiation (Bates, Young, & Varmus, 1993; Demczuk et al., 1995; Takeuchi, Misaki, Chen, & Ohtsuki, 1999). The fold of the LDLa modules of both RXFP1 and RXFP2 has been solved by solution NMR (Hopkins, Bathgate, & Gooley, 2005; Hopkins, Layfield,

Ferraro, Bathgate, & Gooley, 2007; Kong et al., 2014). These LDLa modules are similar in size, 4 kDa, comprise three strictly conserved disulphides and a conserved Ca^{2+} ligation motif (D/NxxxDxxD/NxxDE), whereby calcium binding is required to stabilize the fold (Hopkins et al., 2005). These structures guided gain-of-function and loss-of-function experiments which highlighted that hydrophobic interactions of the RXFP1 LDLa residues Leu7, Tyr9 and Lys17, located in the N-terminal region of the module, were key for receptor activation (Hopkins et al., 2007; Kong et al., 2013). However, none of the mutants fully lost or gained activity indicating important interactions remained unknown.

The LDLa module and LRR domain are joined by a linker of variable length, 32 or 25 residues in human RXFP1 or RXFP2 respectively. This linker has been viewed as a spacer, until conflicting results suggested that these linkers may play roles in receptor activation. When the LDLa module of RXFP2, together with a portion of linker, was swapped onto RXFP1 signaling was lost, suggesting that the LDLa modules could not be swapped (Kern, Agoulnik, & Bryant-Greenwood, 2007). A more careful analysis where only the LDLa modules were swapped, thus preserving the LDLa, linker and LRR boundaries, showed activation was possible (Bruell et al., 2013). In this latter study, a series of chimeras of RXFP1 and RXFP2 were made, swapping the LDLa modules or both the LDLa module and TMD. For the chimera of the TMD, LRR domain and linker of RXFP1 with the LDLa of RXFP2 (referred to as RXFP211), binding of relaxin appeared normal, however, the receptor showed significant loss of both potency and efficacy. For the chimera of LRR domain and linker of RXFP1 combined with the LDLa module and TMD of RXFP2 (referred to as RXFP212) efficacy remained significantly poor, but potency was regained suggesting that in this combination, the LDLa was functioning as a full agonist. These results suggest that the lower efficacy in RXFP212 may reflect subtle differences within the LRR domain or linker that confer inefficiencies of how the binding of ligand coupled with activation by the LDLa module.

To further investigate the role of the linker, between the LDLa module and the LRR domain, residues of the linker of RXFP1 were mutated (Sethi et al., 2016). Although many residues in the linker show a modest level of conservation, the residues GDxxGW, immediately C-terminal to the LDLa module, are highly conserved in both RXFP1 and RXFP2. Unexpectedly, mutagenesis of these residues in RXFP1 showed both loss of receptor activation and relaxin binding. Specifically, mutation of Asp42, Gly45 and Trp46 in RXFP1 resulted in respectively >10,000-, 5000- and 10,000-fold losses of activation upon relaxin stimulation. These mutations also resulted in respectively 35-, 35- and 20-fold weakening of relaxin binding. These data encouraged a site-directed mutagenesis campaign involving assays of whole receptor ligand-binding and relaxin-induced cAMP activation combined with measurement by NMR spectroscopy of ligand-binding and structural change to recombinant RXFP1 LDLa-linker in the absence of the LRR domain. These experiments concluded that the linker can be divided into two regions: region 1, encompasses the GDxxGW motif and is involved in receptor activation; region 2, from Leu48 to Ser56 comprises a region that directly binds relaxin and on doing so forms a helical structure. It is potentially this formation of helix that is critical for orienting the LDLa module for TMD engagement and activation. This binding site for relaxin was determined by chemical shift changes monitored in titrations of ^{15}N-labelled LDLa-linker with relaxin. Although these changes were confined to region 2 (Leu48-Ser56), mutations of the GDxxGW motif, especially Gly45 or Trp46, effected the extent of the chemical shift changes observed in such titrations, suggesting that region 1 influences the conformation of region 2. This work, importantly, shows that the ectodomain of RXFP1 comprises two binding sites for relaxin. As a dissociation constant for relaxin of ~200 μM to the LDLa-linker was determined, this suggests that relaxin binds with ~1 μM to the LRR domain, although this remains to be proven. Testing a number of synthetic relaxin mutants further suggests that the binding site to the linker involves the A-chain of relaxin, especially His12-Val13, however, structural experiments to directly prove where relaxin binds to both the LDLa-linker and, for that matter the LRR domain also, remain to be conducted.

While the LDLa module may be considered as a tethered agonist and must make important interactions with the TMD that lead to stabilizing an active conformation of the receptor, it is generally thought that H2 relaxin also contacts the TMD (Halls et al., 2005; Sudo et al., 2003). A chimera of the ECD of RXFP1 and the TMD of RXFP2 showed poor binding with relaxin-3, a known activator of RXFP1, but not RXFP2 (Sudo et al., 2003). However, swapping the second extracellular loop (EL2) of the RXFP2 TMD with EL2 of RXFP1 restored activity suggesting that EL2 is a binding site for relaxin. EL1 and EL2 of RXFP1 were engineered onto a soluble scaffold that included formation of the disulphide bond between EL1 and EL2 which attempts to maintain the native structure of EL2 (Diepenhorst, Gooley, Stone, & Bathgate, 2013). Pull-down assays and titrations of ^{15}N-labelled scaffold monitored by NMR spectroscopy supported a specific interaction between relaxin and the EL2 of RXFP1 (Diepenhorst et al., 2014). Similar titrations with the LDLa module and also the reverse titration of ^{15}N-labelled LDLa module with unlabelled scaffold showed a very weak interaction between these molecules (Diepenhorst et al., 2014). However, titration of ^{15}N-labelled LDLa-linker by this scaffold, showed large and significant shifts of the resonances of the linker from residues Gly41 to Met60 (Sethi et al., 2016). Notably, mutations of the GDxxGW motif of the LDLa linker and loss of the disulphide between EL1 and EL2 in the scaffold abolished this interaction. Additional mutations of EL2 in this scaffold, equivalent to Phe564 and Pro565 of RXFP1, also attenuated this interaction, whereas mutating Trp479 of EL1 had no effect. These results were consistent with activity assays of the whole receptor (Diepenhorst et al., 2014) and collectively suggest that the C-terminal end of the LDLa module and linker residues interact with the TMD. Notably, these interactions between the linker and EL2 are observed in the absence of relaxin, implying for the whole receptor that these regions are precoupled. Consequently, the mechanism of RXFP1 may commence with a non-activating association of the linker with the exoloops of the TMD (Fig. 6). Binding of relaxin either simultaneously or sequentially with strong affinity to the LRR domain and weak affinity to the linker induces a conformational change within the linker. This rearrangement reorients the LDLa module and the GDxxGW motif of the linker that results in strong interaction of these regions, possibly along with the hormone, with the TMD stabilizing the active conformation of the receptor in common with other Rhodopsin class GPCRs.

Alignment of the amino acid sequence of the linker of RXFP2 with RXFP1 shows that the GDxxGW motif of region 1 is conserved, but seven residues of region 2, corresponding to Lys52 to Tyr58 of RXFP1, are deleted. H2 relaxin, however, is a weak agonist of RXFP2 and thus raises the question of how does the mechanism of H2 relaxin-stimulated activation of RXFP2 compare with RXFP1. Mutation of Asp43, Gly46 and Trp47 of the GDxxGW motif of RXFP2 abolished H2 relaxin binding and activation by H2 relaxin stimulation (Bruell et al., 2017). Importantly, INSL3 binding was not significantly affected indicating that the ectodomain is intact, but activation by INSL3 stimulation was also abolished. These results support a common role of the GDxxGW motif (region 1) in receptor activation of both RXFP1 and RXFP2, but propose different roles for the remainder of the linker (region 2), in that it contains a relaxin binding site for RXFP1, but no INSL3 binding site for RXFP2. Whether H2 relaxin interacted with region 2 of RXFP2 was explored by NMR-monitored titrations of ^{15}N-labelled LDLa-linker constructs of RXFP2 with H2 relaxin, and showed, in contrast to experiments for RXFP1, pronounced chemical shift changes in the C-terminal region of the LDLa module and smaller changes to resonances of residues in region 2 implying H2 relaxin interacts differently with the LDLa-linker of RXFP2. Overall, the interaction is 4-fold weaker between H2 relaxin and the LDLa-linker of RXFP2 compared to the LDLa-linker of RXFP1 (Bruell et al., 2017). Further, titrations of the RXFP2 LDLa-linker construct with soluble scaffolds containing the EL2 loops of RXFP1 or RXFP2 showed that the LDLa module, rather than

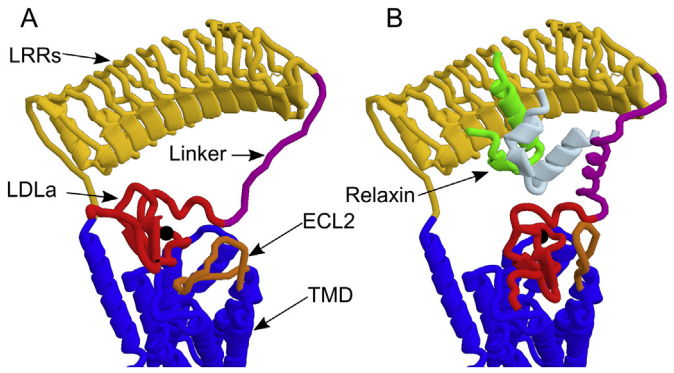


Fig. 6. Cartoon representation of the postulated mode of RXFP1 activation by relaxin. A. In the apo-state, the LDLa module (red) makes interactions with the transmembrane domains (TMD, blue) and potentially extracellular loop 2 (ECL2, orange). This places the leucine-rich repeats (LRR, yellow) in a closed conformation that disallows the binding of peptides such as INSL3. The linker (purple) exhibits little helical structure. B. Relaxin binds to the LRR via the B-chain (green) leaving the A-chain (pale blue) to potentially interact with the linker, promoting the formation of a helix. The resultant conformational change propagates back up the protein, influencing the binding mode of the LDLa module on the TMD and ECL2, promoting activation of the receptor.

the linker has the most significant interactions. For the scaffold containing the EL2 of RXFP2, significant shifts were noted for the C-terminal residues of the LDLa module consistent with the proposal that this region is important for receptor activation (Kong et al., 2014). Collectively these experiments suggest H2 relaxin may activate RXFP2 and RXFP1 by different mechanisms where the LDLa module, rather than the linker, of RXFP2 may have a more intimate coupling with the TMD of the receptor. This is not surprising considering that the binding mode of INSL3, and in fact relaxin, has been shown to be different on RXFP2 compared to RXFP1 (Scott et al., 2012).

Currently, no structural complexes of the full or truncated ectodomain of either RXFP1 or RXFP2 with relaxin or INSL3 are known and remain a challenge. Models through HADDOCK docking based on mutagenesis of both receptor and ligand, propose possible orientations of H2 relaxin with respect to the LRR domain, although this remains to be proven. NMR experiments have not succeeded in determining the precise orientation of H2 relaxin to the binding site on the LDLa-linker, although again, mutagenesis of H2 relaxin suggests that the A-chain of relaxin is likely to be the surface of interaction. Notably, in the work on the LDLa-linker construct of RXFP2 heterogeneity within the NMR spectra were observed, especially for the indole resonance of Trp47 of the GDxxGW motif (Bruell et al., 2017). The source of this heterogeneity was cis-trans isomerism of Pro4 in the N-terminus of the LDLa module suggesting that the linker folds back towards the N-terminus of the module. As the solution structures of the LDLa modules of RXFP1 and RXFP2 are similar (Hopkins et al., 2007; Kong et al., 2014) this overall fold and orientation may be present in both receptors. Consequently, an important role of the LDLa module may be to maintain this fold of the linker to correctly orient the GDxxGW motif for receptor activation.

Our proposed mechanism of relaxin-mediated activation of RXFP1 (Fig. 6) leaves open a number of questions, especially what is the precise structure of the relaxin/RXFP1 complex. Determination of the ectodomain structure free and in complex with relaxin will lead to an understanding of the possible specific residue interactions of relaxin with the LRR and LDLa-linker module, but this will not reveal how the ectodomain, or relaxin, interacts with the TMD. Curious results from truncates and chimeras of RXFP1 and RXFP2 highlight the complexity of this interaction. For example, replacement of the 7-transmembrane helical domain with a single helix retained relaxin binding, but also enabled high affinity binding to INSL3, which binds to full-length RXFP1 weakly (Scott et al., 2012). These data suggest steric hindrances or obscuring of the hormone binding sites by the coupling of the ectodomain and the TMD, thus to understand these details require a complete structure. Furthermore, as described below a relaxin B-chain mimetic is a weak full agonist of RXFP1 in some cells and a potent full agonist in others (Hossain et al., 2016), further adding additional complexities to the structure and function of this receptor.

5. Development of peptide and small molecule H2 relaxin mimetics

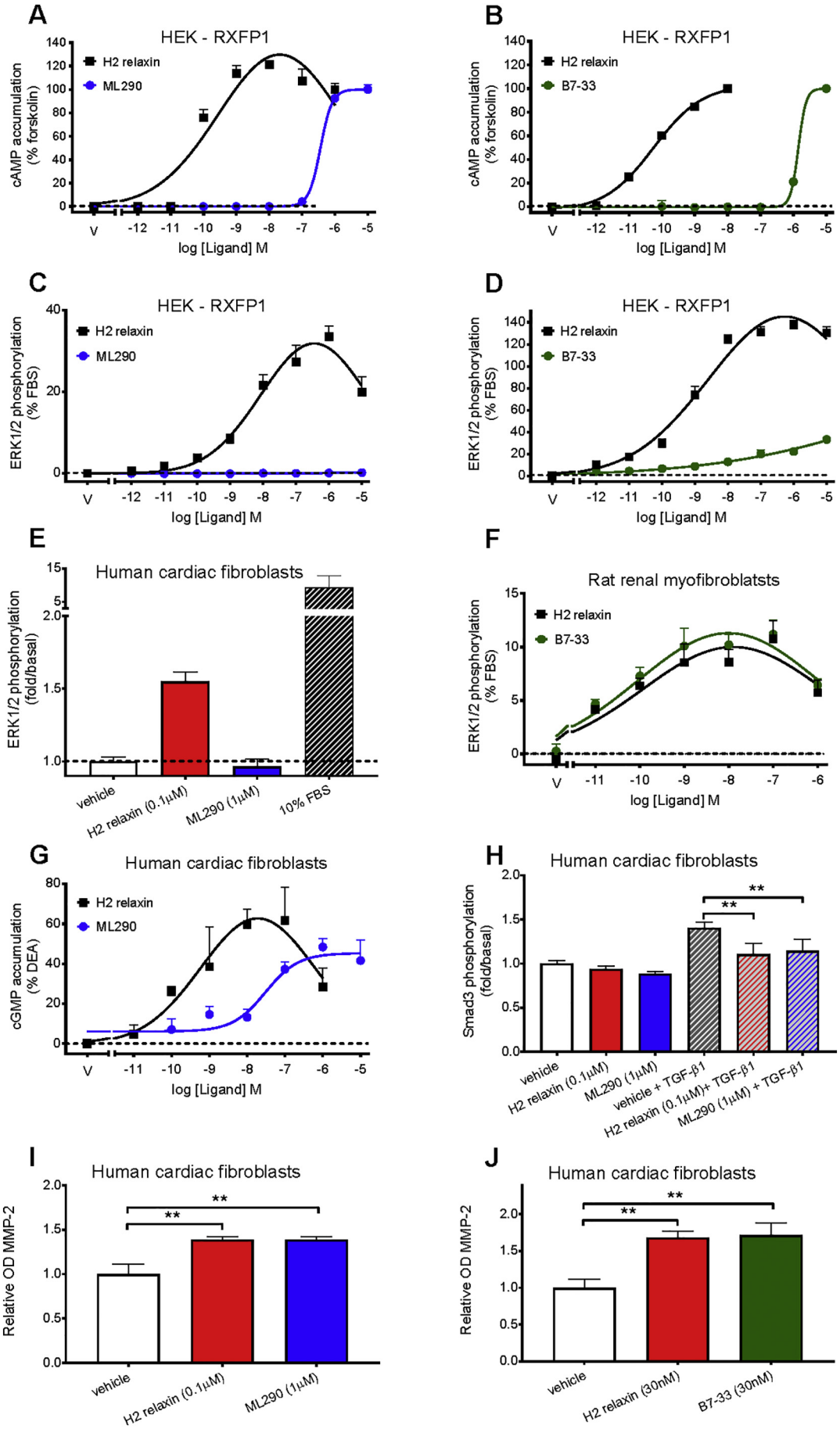
5.1. Peptide mimetics

Based on the extensive structure-function studies outlined above, both the A- and B-chain of relaxin are predicted to be necessary for activity and hence the design of smaller peptide mimetics of relaxin is likely to be difficult. Studies on truncated versions of H2 relaxin have indeed suggested that this is the case. While N-terminal truncation of the A-chain up to four residues has minimal effect on H2 relaxin activity, further truncation results in reduced binding and decreased potency. Interestingly, peptide activity can be rescued by non-native Alanine residue substitutions (Hossain et al., 2008) indicating the importance of the α-helical structure at the N-termini of the A-chain rather than residue specific function (Bullesbach & Schwabe, 1986; Bullesbach & Schwabe, 1987; (Hossain et al., 2008). Notably, truncation of the B-chain from either the N- or C-terminus results in progressive loss of binding affinity and potency which is also probably associated with loss of structural integrity rather than the loss of specific binding interactions (Hossain et al., 2011). These truncation studies led to the development of a minimally truncated H2 relaxin peptide A(4-24)(B7-24)H2 (also known as mini-H2 relaxin). While this peptide is smaller and easier to synthesize compared to H2 relaxin and is a full agonist at RXFP1, its potency is ~100-fold lower than H2 relaxin (Hossain et al., 2011).

As the relaxin binding cassette in the B-chain is responsible for high affinity binding to RXFP1, it was predicted that B-chain-only analogs of H2 relaxin could be developed. It was anticipated that such peptides may be RXFP1 antagonists in a similar manner to single-chain INSL3 analogs which are RXFP2 antagonists (Del Borgo et al., 2006). Unfortunately, early attempts to develop single-chain H2 relaxin peptides based on the 29-residue native B-chain sequence were not successful due to poor solubility (Del Borgo, Hughes, & Wade, 2005). More recently, a B-chain only H2 relaxin analog was produced by introducing positively charged native residues from the C-chain of pro-H2 relaxin onto the C-terminus of the B-chain and truncation at the N-terminus, hence improving the peptide solubility (Hossain et al., 2016). When this peptide, B7-33, was tested for its activity in HEK-293T cells stably expressing human RXFP1 (HEK-RXFP1), it demonstrated very poor affinity however, surprisingly, it was shown to be a weak full agonist of cAMP activation (Hossain et al., 2016) (Fig. 7B). More surprisingly, it was shown to have potent agonist actions in rat renal myofibroblasts, a cell system commonly used to demonstrate the anti-fibrotic actions of relaxin (Chow et al., 2012) (Fig. 7F). This greater potency was not due to species differences in RXFP1, as B7-33 was also a weak full agonist of cAMP signaling in HEK cells expressing mouse and rat RXFP1 (Hossain et al., 2016). Additionally, B7-33 demonstrated weak agonism of cAMP signaling in THP1 cells, another common cell line used to assess relaxin action (Parsell, Mak, Amento, & Unemori, 1996).

The full signaling profile of B7-33 is outlined in the signaling section below which illustrates that B7-33 has cell specific actions, whereby it has high potency at certain cell types and poor potency in others.

Fig. 7. Comparison of signaling pathways activated by H2 relaxin, ML290 and B7-33 in HEK-RXFP1 cells (A-D) human cardiac fibroblasts (E, G-J) and rat renal myofibroblasts (F). Effects of H2 relaxin, ML290 and B7-33 on short-term activation of cAMP accumulation (30-40 min; A,B); ERK1/2 phosphorylation (5-10 min; C-F) and cGMP accumulation (40 min; G) as well as long-term signaling: Smad3 phosphorylation (72 hours; H) and MMP-2 expression (72 hours; I, J). The results are discussed in the text and summarized in Table 1. Graphs are from Kocan et al. (2017) and Hossain et al. (2016).


A
HEK - RXFP1
H2 relaxin
ML290
cAMP accumulation (% forskolin)
log [Ligand] M
B
HEK - RXFP1
H2 relaxin
B7-33
cAMP accumulation (% forskolin)
log [Ligand] M
C
HEK - RXFP1
H2 relaxin
ML290
ERK1/2 phosphorylation (% FBS)
log [Ligand] M
D
HEK - RXFP1
H2 relaxin
B7-33
ERK1/2 phosphorylation (% FBS)
log [Ligand] M
E
Human cardiac fibroblasts
ERK1/2 phosphorylation (fold/basal)
vehicle
H2 relaxin (0.1μM)
ML290 (1μM)
10% FBS
F
Rat renal myofibroblatsts
H2 relaxin
B7-33
ERK1/2 phosphorylation (% FBS)
log [Ligand] M
G
Human cardiac fibroblasts
H2 relaxin
ML290
cGMP accumulation (% DEA)
log [Ligand] M
H
Human cardiac fibroblasts
Smad3 phosphorylation (fold/basal)
vehicle
H2 relaxin (0.1μM)
ML290 (1μM)
vehicle + TGF-β1
H2 relaxin (0.1μM)+ TGF-β1
ML290 (1μM) + TGF-β1
I
Human cardiac fibroblasts
Relative OD MMP-2
vehicle
H2 relaxin (0.1μM)
ML290 (1μM)
J
Human cardiac fibroblasts
Relative OD MMP-2
vehicle
H2 relaxin (30nM)
B7-33 (30nM)

Notably, it has recently been demonstrated that the effects of relaxin on rat renal myofibroblasts absolutely requires the angiotensin type-2 receptor (AT_2R), suggesting that relaxin action in these cells may be through an RXFP1-AT_2 complex (Chow et al., 2014). An AT_2R antagonist is able to block the actions of B7-33 in these cells, suggesting that B7-33 functions through the same mechanism hence providing a rationale for the high potency in these cells. However, it has recently been demonstrated that, similar to relaxin, B7-33 has potent actions in the rat mesenteric artery (Marshall et al., 2017), which cannot be explained by actions through a RXFP1-AT_2R complex. The exact mechanism by which B7-33 shows cell specific actions is currently unknown, however, it is clear that the mechanistic data on relaxin-mediated activation highlighted in the previous section may not be valid for all cell types and RXFP1 may act in complexes with other GPCRs which modifies its pharmacology.

5.2. ML290 - a novel small molecule RXFP1 agonist

Despite its enormous promise as a drug and its excellent safety profile in human patients the very short serum half-life of H2 relaxin requires continuous infusion for efficacy. Additionally, its complex, two-chain three disulphide-bonded structure means it is expensive to produce and store and it cannot be delivered orally. Hence the development of small molecular weight RXFP1 agonists would be highly desirable, especially for use as a long-term therapeutic. Importantly, a small molecule agonist would likely bind directly in the RXFP1 TMD bypassing the complex mechanism of activation and potentially avoiding the cell-type specific effects seen with the peptide mimetic B7-33, as described above.

Recently a high-throughput screening project was undertaken to identify RXFP1-specific small molecule agonists. HEK-RXFP1 and THP1 cells, which both exhibit robust activation of cAMP in response to relaxin treatment, were used for primary and secondary screens of a 365,677-compound NIH library (Chen et al., 2013; Xiao et al., 2013). Two agonist molecules were identified from these initial screens which also demonstrated more than 100-fold selectivity for RXFP1 over RXFP2. The cAMP activities of these initial hits were quite low (EC50 4–6 μM, 60–80% efficacy) but they demonstrated a similar structural scaffold and were hence subjected to further optimization by medicinal chemistry (Chen et al., 2013; Xiao et al., 2013). Ultimately, optimized hits were developed which demonstrated nanomolar potency in cAMP assays and other assays of relaxin activity with no activity in control cells. The best candidate compound, ML290, demonstrated excellent stability both *in vitro* and *in vivo* and low toxicity.

As anticipated ML290 binds directly to the TMD of RXFP1, bypassing the relaxin-mediated mechanism of activation described above. This was best demonstrated by showing that ML290 was able to activate an RXFP1 construct lacking the LDLa module (Xiao et al., 2013). Subsequent studies have confirmed this and demonstrated that ML290 exhibits unchanged potency or efficacy at mutant RXFP1 receptors for which H2 relaxin potency is severely perturbed (Diepenhorst et al., 2014; Sethi et al., 2016). Notably, ML290 was not able to activate cells transfected with mouse RXFP1, unfortunately precluding the testing of ML290 in rodent models. This information did provide the ability to analyse sequence differences in RXFP1 to identify potential interacting residues. Subsequently, residues in the third extracellular loop (EL3) of the TMD were shown to be involved in ML290, but not relaxin, activity (Xiao et al., 2013). A more recent study utilizing homology modelling of the RXFP1 TMD, docking, molecular dynamics simulations, binding free energy calculations and mutagenesis has resulted in a model of ML290 interaction with the RXFP1 TMD (Hu et al., 2016). This model highlights an interaction with G659/T660 in EL3 as predicted from the original study. Additionally, the binding model shows interactions with helices TM3, TM5, TM6 and TM7 forming a putative binding pocket within the RXFP1 TMD (Hu et al., 2016). Further details of this binding model and the identification and optimization of ML290 have been presented recently in a comprehensive review (Agoulnik, Agoulnik, Hu, & Marugan, 2017). Importantly, similar to other small molecule GPCR ligands identified by high-throughput screening, the binding of ML290 to this "allosteric pocket" results in ML290 being a biased agonist (outlined in detail below). Notably, this binding pocket is similar to orthosteric binding pockets in other GPCRs and it can be postulated that the LDLa module also binds to this site. It would therefore follow that, as ML290 is a biased agonist, it is only partially mimicking the LDLa mode of binding and activation of RXFP1.

6. RXFP1 signaling

As outlined in the previous sections the relaxin/RXFP1 signaling axis is ancient, originating in invertebrates, and is associated with multiple important physiological actions. These actions are mediated through RXFP1 expression on multiple cell types in target tissues and importantly, the downstream pathways activated vary significantly depending on the cellular background. Hence relaxin activates pleiotropic signaling pathways involving many key second messengers including cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), nitric oxide (NO) and mitogen-activated protein (MAP) kinase phosphorylation. The detailed signaling pathways downstream of relaxin signaling have been reviewed in detail elsewhere (Bathgate et al., 2013; Bathgate, Hsueh, et al., 2006; Halls, Bathgate, Sutton, Dschietzig, & Summers, 2015; Leo et al., 2016, 2017; Samuel et al., 2017). In this review, we will focus on more recent studies utilizing the small molecule biased RXFP1 agonist ML290 (Xiao et al., 2013) and the cell specific relaxin B-chain mimetic peptide, B7-33 (Hossain et al., 2016). These novel compounds have proven invaluable tools to assess RXFP1 cellular signaling in detail.

6.1. RXFP1 signaling pathways in diverse cellular backgrounds

6.1.1. Recombinant cells HEK-RXFP1

Since the deorphanization of the RXFP1 receptor in 2002 (Hsu et al., 2002), HEK-RXFP1 have been widely utilized to understand relaxin signaling. In HEK-RXFP1 relaxin induces cAMP accumulation through RXFP1 interacting with three diverse G proteins: G_s, G_{oB} and G_{i3}. Following its activation, RXFP1 couples to $G_{\alpha s}$ to increase cAMP, an effect that is negatively modulated by coupling to $G_{\alpha oB}$. Additionally, RXFP1 interactions with $G_{\alpha i3}$ also activate cAMP accumulation via a $G_{\beta\gamma}$ phosphatidylinositol 3-kinase (PI3K)-protein kinase C (PKC)-ζ pathway and stimulation of adenylyl cyclase 5 (AC5) (Halls, Bathgate, & Summers, 2006; Hsu et al., 2000; Nguyen & Dessauer, 2005; Nguyen, Yang, Sanborn, & Dessauer, 2003). Relaxin also increases phosphorylation of mitogen-activated protein kinases in HEK-RXFP1 cells including transient activation of extracellular signal–regulated kinases 1/2 (ERK1/2) and prolonged activation of p38 mitogen-activated protein kinase (p38MAPK) (Hossain et al., 2016; Kocan et al., 2017; Singh, Simpson, & Bennett, 2015). On the other hand, relaxin does not activate cGMP accumulation in this cellular background (Kocan et al., 2017).

As outlined in the section above, B7-33 demonstrates poor affinity for RXFP1 in HEK-293T cells as anticipated by studies on the relaxin-binding mechanism which seems to require the A- and B-chains. This low affinity binding is associated with activation of cAMP in HEK-RXFP1 cells as well as in HEK cells transfected with mouse or rat RXFP1 demonstrating similar efficacy, but markedly lower potency than H2 relaxin (Fig. 7B). In HEK-RXFP1 it is a weak partial agonist of ERK1/2 phosphorylation (Hossain et al., 2016) (Fig. 7D). In contrast, ML290 showed signaling bias in HEK-RXFP1 cells (Kocan et al., 2017). ML290 stimulates cAMP activation with similar efficacy, but significantly lower potency as compared to relaxin (Kocan et al., 2017; Sethi et al., 2016) (Fig. 7A). However, it is an equipotent agonist at the p-p38MAPK pathway and, unlike H2 relaxin, completely lacks the ability to activate ERK1/2 phosphorylation (Fig. 7C) (Kocan et al., 2017). Comparison of the G protein coupling produced by H2 relaxin and

ML290, using real-time kinetic BRET assays, showed that both drugs promoted interactions between RXFP1 and $G_{\alpha s}$, $G_{\alpha o/B}$ and to a lesser extent $G_{\alpha i3}$. The profile of ML290 and H2 relaxin-induced BRET between RXFP1 and $G_{\alpha s}$ was almost identical whereas the profile for ML290-induced BRET for RXFP1-$G_{\alpha oB}$ interactions clearly differed from that observed for H2 relaxin (Kocan et al., 2017).

6.1.2. Native RXFP1 expressing cells

As indicated above, previous studies have demonstrated diverse signaling in response to relaxin in different cell types. In more recent times, in order to unravel the signaling mechanisms related to relaxin's antifibrotic and cardiovascular effects, acute and long-term relaxin signaling pathways have been tested in numerous primary fibroblast and vascular cells.

Long-term signaling and physiological endpoints (48-72 hours) have been a particular focus of studies in fibroblasts aimed at understanding the actions of relaxin on connective tissue metabolism. Such studies have measured the inhibition of fibroblast proliferation and differentiation and the activation of extracellular matrix degradation mediated by the matrix metalloproteases (MMPs) consequently resulting in reduced collagen deposition. The antifibrotic properties of H2 relaxin have been particularly studied in human cardiac fibroblasts, human renal myofibroblasts and rat renal myofibroblasts. Some fibroblasts have been studied in more detail than others, but in all fibroblasts relaxin is able to inhibit the actions of TGF-β1, one of the most important pro-fibrotic cytokines, including suppression of TGF-β1-induced expression of α-SMA (marker of fibroblasts differentiation) (Masterson et al., 2004; Samuel et al., 2004). In human cardiac fibroblasts, H2 relaxin inhibited aberrant myofibroblast differentiation and collagen deposition by disrupting the TGF-β1/Smad2 (Samuel et al., 2014) and TGF-β1/Smad3 signaling axes (Sassoli et al., 2013). Following 48-hour stimulation, H2 relaxin also increased anti-fibrotic markers such as MMP2 and MMP9 and up-regulated protein expression of neuronal nitric oxide synthase (nNOS) (Sarwar, Samuel, Bathgate, Stewart, & Summers, 2015). Similarly, H2 relaxin was shown to inhibit the actions of TGF-β1 including TGF-β1-induced expression of α-SMA, myofibroblast differentiation, Smad2 phosphorylation and collagen deposition in human (Heeg et al., 2005) and rat renal myofibroblasts (isolated from injured rat kidney) (Chow et al., 2014; Mookerjee et al., 2009; Wang et al., 2016). Furthermore, in rat renal myofibroblasts, the anti-fibrotic effects of relaxin including inhibition of the TGF-β1/pSmad2 axis, TGF-β1-induced myofibroblast differentiation and collagen deposition and promotion of collagen-degrading MMP2 and MMP9 activity, was demonstrated via a NO and cGMP dependent pathway (Chow et al., 2014; Mookerjee et al., 2009; Wang et al., 2016).

Recent studies have been focused on dissecting the acute signaling pathways associated with the inhibition of the profibrotic TGF-β1 effects by H2 relaxin. Both cGMP accumulation and ERK1/2 phosphorylation have been identified to contribute towards long-term (48-72 hours) signaling linked to the anti-fibrotic actions of H2 relaxin. Short-term stimulation (30-40 min) in human cardiac fibroblasts increases cGMP accumulation in a concentration dependent manner with no changes in cAMP accumulation. H2 relaxin also increases ERK1/2 phosphorylation (within 10 min) in a concentration dependent manner and this p-ERK1/2 activation has been shown to be downstream of $G_{i/o}$ proteins and PI3-Kinase (Kocan et al., 2017; Sarwar et al., 2015).

The discovery of ML290 and B7-33 has provided valuable tools to dissect downstream actions of RXFP1, especially the association of cGMP and p-ERK1/2 stimulation with the long term antifibrotic effects. B7-33 stimulates p-ERK1/2 within 10 minutes following stimulation in rat renal myofibroblasts and demonstrates similar potency to H2 relaxin (Fig. 7F). Additionally, long term (48-72 hours) treatment with B7-33 increases MMP-2 expression to a comparable extent to H2 relaxin in human cardiac fibroblasts (Fig. 7J) as well as rat renal myofibroblasts (Hossain et al., 2016). In contrast, ML290 exhibits no activity at the p-ERK1/2 pathway in human cardiac fibroblasts (Fig. 7E) however it induces cGMP with a weaker activity as compared to H2 relaxin (Fig. 7G). Interestingly, although ML290 does not activate p-ERK1/2 in human cardiac fibroblasts, it still exhibits similar anti-fibrotic effects as H2 relaxin, including increased MMP-2 expression (Fig. 7I) and inhibition of TGF-β1-induced Smad2 and Smad3 phosphorylation (Kocan et al., 2017). In human cardiac fibroblasts, both H2 relaxin and ML290 decreased p-Smad2 and p-Smad3 levels (Fig. 7H) and this effect is only present in TGF-β1-stimulated fibroblasts (Kocan et al., 2017). The fact that ML290 stimulates cGMP accumulation without ERK1/2 activation, may indicate that cGMP may be involved with both relaxin and ML290 actions, but by different mechanisms (Kocan et al., 2017). Both ligands are able to activate soluble guanylyl cyclase (sGC) and cGMP accumulation, but only H2 relaxin can activate the RXFP1-p-ERK1/2-nNOS-NO-sGC-dependent pathway.

Interestingly, the above mentioned signaling pathways contributing towards the antifibrotic effects of H2 relaxin, including stimulation of p-ERK1/2, nNOS and MMPs as well as inhibition of TGF-β1, α-SMA and Smad2 in rat renal myofibroblasts *in vitro* and in mouse kidney tissues *in vivo*, were all blocked by the AT_2R antagonist PD123319 [S-(+)-1-[(4-(dimethylamino)-3-methylphenyl) methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1Himidazo [4,5-c]pyridine-6-carboxylic acid di (trifluoroacetate)] (Chow et al., 2014). Moreover, the antifibrotic actions of H2 relaxin in a model of kidney fibrosis induced by unilateral ureteric obstruction were completely absent in AT_2R knockout, but not wild-type, mice. These finding suggested that relaxin requires the AT_2R to abrogate renal interstitial fibrosis. This may explain the fact that H2 relaxin exhibits its antifibrotic effects under pathological conditions as AT_2R expression is dramatically increased in disease/injury tissues and is very low under normal physiological conditions (Matsubara, 1998). Notably, the actions of B7-33 in rat renal myofibroblast were also blocked by an RXFP1 antagonist or the AT_2R antagonist (PD 123319) (Hossain et al., 2016). Therefore, the renal antifibrotic actions of H2 relaxin and B7-33 require both RXFP1 as well as the AT_2R.

In summary, the distinct signaling profiles of ML290 and B7-33 have provided a better understanding of RXFP1 signaling in fibroblasts. Studies with ML290 show that the anti-fibrotic actions through RXFP1 can be mediated by cGMP activation independent of ERK activation. The potent actions of B7-33 in fibroblasts, but not in other cells, indicates that RXFP1 has a unique pharmacological profile in fibroblasts, possibly due to the formation of a complex with the AT2R. Further studies will be required to prove if this action indeed involves a RXFP1-AT_2R heteromer or signaling complex or is mediated by another mechanism.

Relaxin has a major role in many of the cardiovascular changes associated with pregnancy (Conrad, 2011) and the understanding of this role has led to the use of relaxin for the treatment of cardiovascular disease. Hence in both males and females relaxin directly acts on the blood vessels, kidney and heart and its effects on the cardiovascular system include increases in plasma volume, cardiac output and heart rate as well as decreased vascular resistance and blood pressure (Conrad et al., 2004; Conrad & Novak, 2004; Debrah et al., 2006; Debrah, Conrad, Danielson, & Shroff, 2005). NO plays a major role in RXFP1 signaling in the cardiovascular system and its generation is activated both acutely and chronically by relaxin. A number of studies have highlighted that the physiological actions of relaxin are mediated through the NO pathway including; inhibition of lipopolysaccharide-induced adhesion of neutrophil to coronary endothelial cells (Nistri, Chiappini, Sassoli, & Bani, 2003); inhibition of the activation of human neutrophils (Masini et al., 2004); and increased coronary blood flow in rat and guinea pig hearts (Bani-Sacchi, Bigazzi, Bani, Mannaioni, & Masini, 1995). Importantly relaxin also influences NO signaling by increasing the activity and expression of three types of NOS: endothelial (eNOS), inducible (iNOS) and neuronal (nNOS) (Baccari et al., 2004; Baccari, Nistri, Vannucchi, Calamai, & Bani, 2007; Bani et al., 1999, 2002).

Recent studies have focused on the understanding of the role of NO in H2 relaxin signaling and the distinct pathways associated with the actions of relaxin on acute and chronic vasodilation. The acute effects of relaxin in vascular cells include cAMP and cGMP activation, ERK1/2 phosphorylation and increased NO synthesis (Conrad & Novak, 2004; Nistri & Bani, 2003). Short-term H2 relaxin-mediated responses (up to 1 hour) occur via a $G_{\alpha i}$/PI3K/cAMP/Akt/eNOS/NO-dependent mechanism in human subcutaneous and rodent renal and mesenteric arteries and in human coronary artery and aortic endothelial cells (McGuane et al., 2011). In human primary umbilical vascular cells, including human umbilical vein endothelial cells (HUVEC), human umbilical vein smooth muscle cells (HUVSMC) and human umbilical artery smooth muscle cell (HUASMC), 30 min stimulation with H2 relaxin increases cAMP and cGMP accumulation as well as ERK1/2 phosphorylation in a concentration-dependent manner (Sarwar et al., 2015). p-ERK1/2 activation by relaxin in HUVEC, HUVSMC and HUASMC was shown to be downstream of $G_{\alpha i/o}$ and PI3-Kinase. Interestingly, H2 relaxin stimulation demonstrated biphasic or bell-shaped concentration response curves in a number of the signaling pathways including cAMP and cGMP in HUVECs, HUVSMCs and HCFs, but not in HUASMCs (Sarwar et al., 2015). The mechanism of this bell-shaped response was investigated in HUVSMC, where $G_{\alpha i/o}$ and lipid raft disruption altered the relaxin–stimulated cAMP and cGMP concentration-dependent responses from bell-shaped to sigmoidal. It was therefore suggested that the bell-shaped cAMP and cGMP responses resulted from $G_{\alpha oB}$ inhibitory coupling at high concentrations. In contrast, in HUASMC, the concentration-response relationships for cAMP and cGMP are sigmoidal involving only stimulatory actions of $G_{\alpha S}$ and $G_{\alpha i3}$ as the inhibition of these G proteins reduced the response, but did not affect the shape of the concentration-response curve (Sarwar et al., 2015). Interestingly, this phenomenon of relaxin-stimulated bell-shaped concentration responses has been observed in signaling assays in recombinant and primary cells (Kocan et al., 2017; Sarwar et al., 2015), in physiological response in animal studies (Danielson & Conrad, 2003) and most importantly, in clinical endpoints in the PRE-RELAX AHF clinical trial (Teerlink et al., 2009). It remains to be tested if this same mechanism of G protein switching is responsible for the bell-shaped responses seen in all these studies.

Chronic actions of relaxin in vascular cells include increased NOS expression and NO generation that in turn stimulates expression of MMPs and activates endothelin (ET) ET_B receptors (Jeyabalan, Shroff, Novak, & Conrad, 2007). A long-term H2 relaxin treatment (24-48 hours) in human primary umbilical vascular cells including HUVEC, HUVSMC and HUASMC, increases expression of nNOS consequently stimulating MMP2 and MMP9 to potentially mediate its vascular remodeling actions. MMP2 and MMP9 activate ET_B receptors, that also increases NO production and may be linked to the vasodilatory effects of relaxin (Sarwar et al., 2015).

ML290 acute signaling has been also assessed in human vascular cells including HCAEC, HUVEC, HUASMC and HUVSMC and demonstrated biased signaling similar to its actions in HEK-RXFP1 cells and fibroblasts. Hence, ML290 had no p-ERK1/2 activity in any of the human primary umbilical vascular cells. ML290 increased p38MAPK phosphorylation in a concentration dependent manner in smooth muscles cells (HUASMC and HUVSMC), but not in endothelial cells (HCAEC and HUVEC) (Kocan et al., 2017). Additionally, ML290 demonstrated similar efficacy to H2 relaxin in stimulating cAMP and cGMP in all cells but was more potent at activating cGMP. The fact that ML290 shows bias towards cGMP versus cAMP signaling in human primary vascular cells may have implications for the clinical relevance of ML290 as a vasodilator as cGMP regulates vascular tone in smooth muscle (Tsai & Kass, 2009). Whether the biased signaling of ML290 in vascular cells, especially the lack of pERK1/2 activation, will impact on the potential cardiovascular actions of relaxin is currently unknown but will be aided by studies in the recently developed humanized RXFP1 mouse (Kaftanovskaya et al., 2017).

The actions of B7-33 on vascular cells was recently investigated (Marshall et al., 2017). Firstly, vascular function of the mesenteric artery, small renal artery and abdominal aorta in male rats was assessed 3 hours following an acute bolus injection of B7-33 or H2 relaxin. It was reported that B7-33, similarly to H2 relaxin, enhanced bradykinin-mediated endothelium-dependent relaxation in the rat mesenteric artery by increasing endothelium-derived hyperpolarization. Both B7-33 and H2 relaxin had no effect on relaxation of the small renal artery or aorta. Secondly, B7-33 effects in a model of vascular disease were compared to H2 relaxin and again replicated the vascular actions of H2 relaxin. Hence, B7-33 prevented endothelial dysfunction characteristic of preeclampsia induced by placental trophoblast conditioned media *ex vivo* in mouse mesenteric arteries. As equimolar doses of B7-33 reproduced the beneficial vascular effects of H2 relaxin, the authors suggested that B7-33 should be considered as a cost-effective vasoactive therapeutic in cardiovascular diseases (Marshall et al., 2017). The signaling pathways activated by B7-33 in vascular cells have not been studied. The distinct signaling pathways activated by B7-33 and ML290 compared to H2 relaxin in different cell backgrounds are summarized in Table 1.

7. Conclusions

In this review, we have summarized some of the more recent advances in the relaxin-RXFP1 field, especially in relation to therapeutic targeting. We have also focussed on the significant advances in our knowledge of the evolution of this signaling system. It is now clear that the relaxin-RXFP1 axis plays an important biological role in some invertebrate species. There is an *rxfp1*-like gene in many invertebrates and all vertebrates together with *rln-like* genes. Further studies on the biology of these ancient relaxin-RXFP1 systems in models like *Drosophila* and zebrafish may provide important insight into the processes that shaped the human relaxin-RXFP1 system and shed light on the potential CTRP8-RXFP, and the relaxin-glucocorticoid receptor interactions.

There have been great advances made in the understanding of the relaxin-RXFP1 interaction and the signaling pathways activated in different cell types. The complex mode of relaxin binding and LDLa-mediated activation suggests that the design of peptide mimetics would be very difficult. However, the discovery of the single chain relaxin analog B7-33 has demonstrated that there is much to learn about the relaxin-RXFP1 interaction and there are clearly cell-specific mechanisms of receptor interaction. This is of particular relevance to

Table 1
Summary of the relative activities of ML290 and B7-33 as compared to H2 relaxin at diverse signaling pathways in HEK-RXFP1, human cardiac fibroblasts, rat renal myofibroblasts and human vascular cells. Note: +++, indicates similar full agonist activity as H2 relaxin; ++/ +, lower potency, efficacy or both as compared to H2 relaxin; -, no effect; ND, not determined; *, partial agonist as compare to H2 relaxin.

		RELAXIN	ML290	B7-33
HEK-RXFP1	cAMP	+++	+	+
	cGMP	-	-	**ND**
	ERK1/2	+++	-	+*
	p38MAPK	+++	++*	**ND**
human cardiac fibroblasts	cAMP	-	**ND**	**ND**
	cGMP	+++	++	**ND**
	ERK1/2	+++	-	**ND**
	Smad2/Smad3	+++	+++	**ND**
	MMP2/MMP9	+++	+++	+++
rat renal myofibroblasts	cAMP	-	**ND**	**ND**
	cGMP	+++	**ND**	**ND**
	ERK1/2	+++	**ND**	+++
	Smad2/Smad3	+++	**ND**	**ND**
	MMP2/MMP9	+++	**ND**	+++
human vascular cells	cAMP	+++	+	**ND**
	cGMP	+++	++	**ND**
	ERK1/2	+++	-	**ND**

clinical targeting as, if relaxin utilizes distinct receptor complexes in different cell types, it complicates drug targeting. Additionally, if these complexes involve other GPCRs such as the AT_2R as demonstrated in fibroblasts, it is possible that standard of care co-drug treatments could inadvertently block or alter relaxin action. Comparative studies using relaxin, ML290 and B7-33 in different pathophysiological models will be essential to determine the presence or absence of distinct signaling complexes in different tissues and cells. Such studies will also highlight the clinical potential for ML290 and B7-33.

Finally, from a therapeutic perspective, the recent failure of the Phase IIIb AHF trial leaves more open questions than answers. It remains to be seen if data from the trial will reveal answers to why serelaxin showed positive results in Phase IIIa, but not in Phase IIIb. Information on the individual patients, including the additional drugs they were being treated with, both during and after hospitalization, could be relevant in the context of the drug effects on receptor complexes mentioned in the previous paragraph. It is not yet known whether the trials in hepatic impairment (Kobalava et al., 2015), renal impairment (Dahlke et al., 2016), and patients with compensated cirrhosis (Snowdon et al., 2017) will continue. Meanwhile, there continue to be multiple publications highlighting the utility of relaxin for the treatment of fibrosis in numerous animal models. ML290 and B7-33 should provide valuable tools to determine the mechanism of relaxin action in fibrosis as well as the vasodilatory and organ protective actions of relaxin.

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Conflict of Interest Statement

RADB and MAH are inventors on the patent application PCT/AU2015/050184, Modified Relaxin B chain peptides. All other authors declare that there are no conflicts of interest.

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