

1 **Supporting Information**

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5 **Engineering and characterization of a long half-life relaxin receptor RXFP1 agonist**

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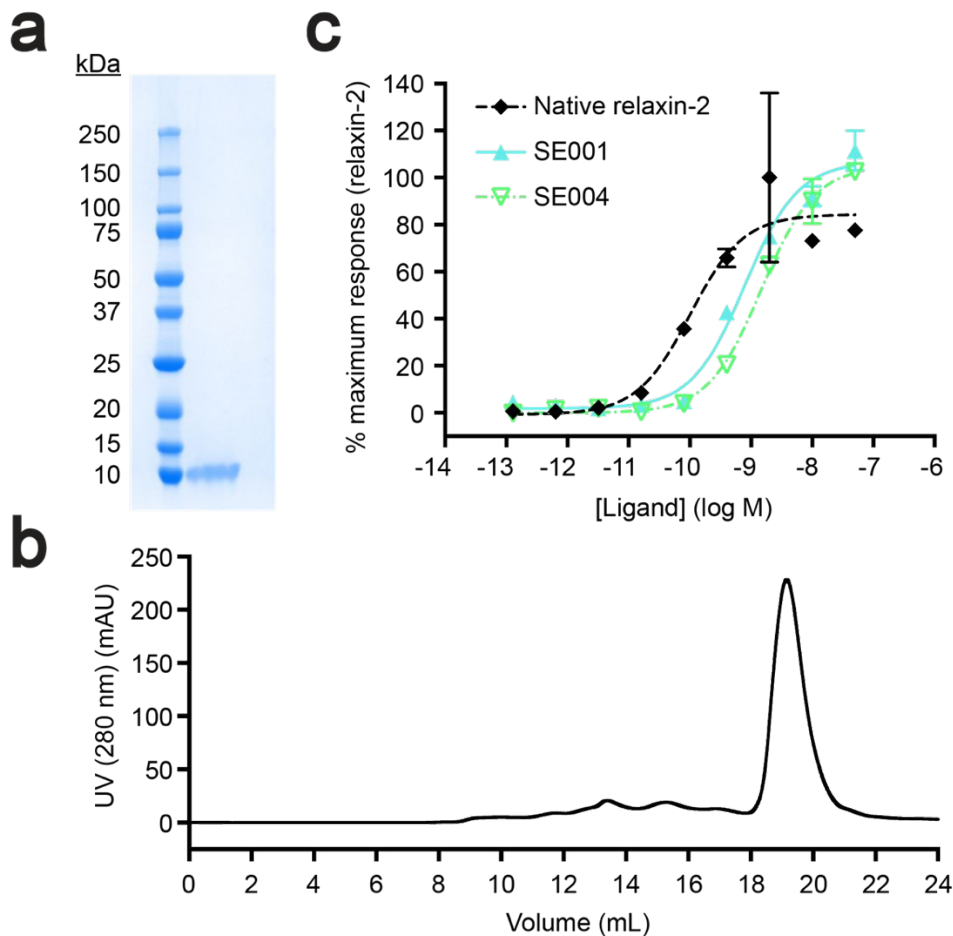
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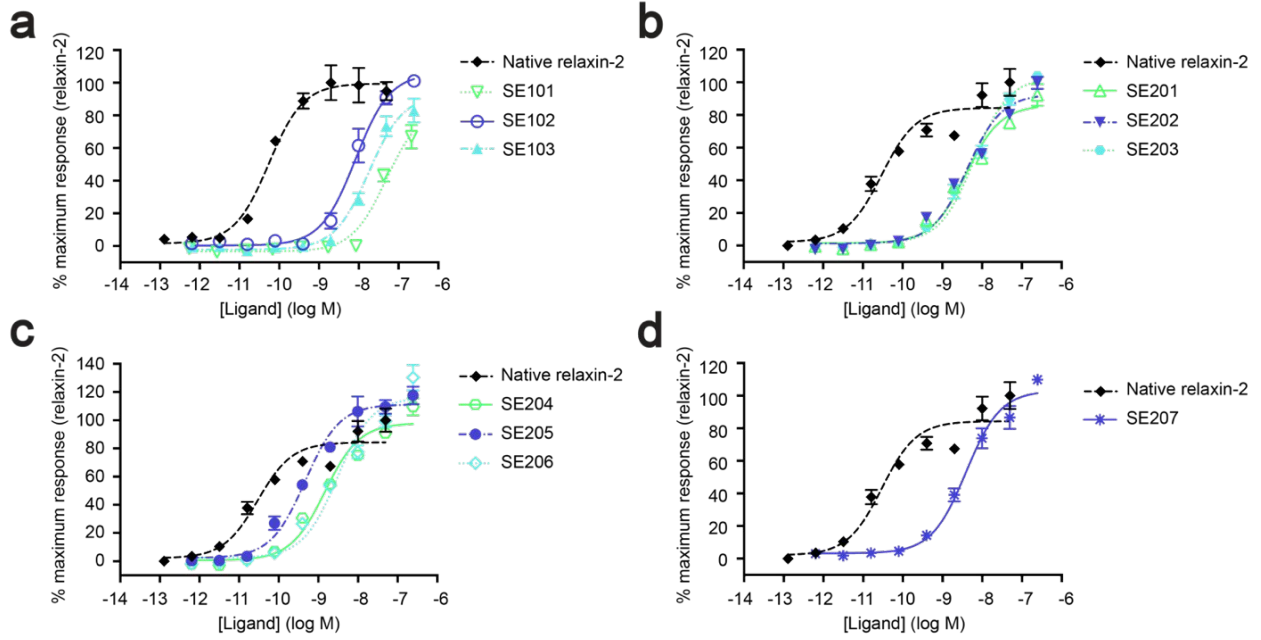
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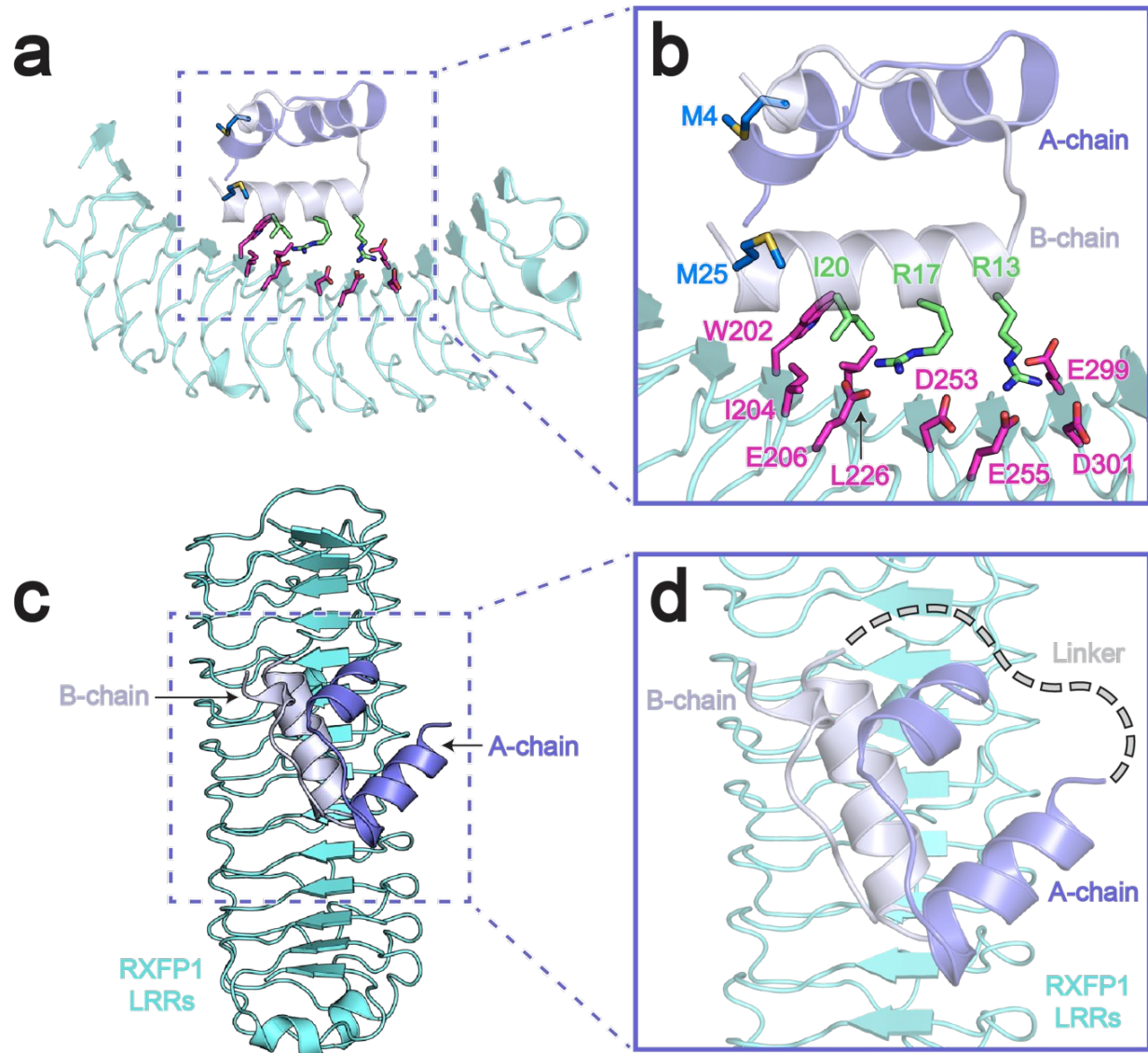


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 21 **Figure S1: Purification and characterization of single-chain relaxin-2.** **a**, Coomassie-stained
 22 SDS-PAGE gel for His-tagged single-chain relaxin-2 (SE001). **b**, Size exclusion
 23 chromatography profile for His-tagged single-chain relaxin-2 (SE001). **c**, CRE-SEAP G_s
 24 signaling assay with human RXFP1 for His-tagged single-chain relaxin-2 (SE001) and His-
 25 tagged, protein C-tagged single-chain relaxin-2 (SE004). Data are normalized to the native
 26 relaxin-2 response and are mean \pm s.e.m. from technical triplicates.
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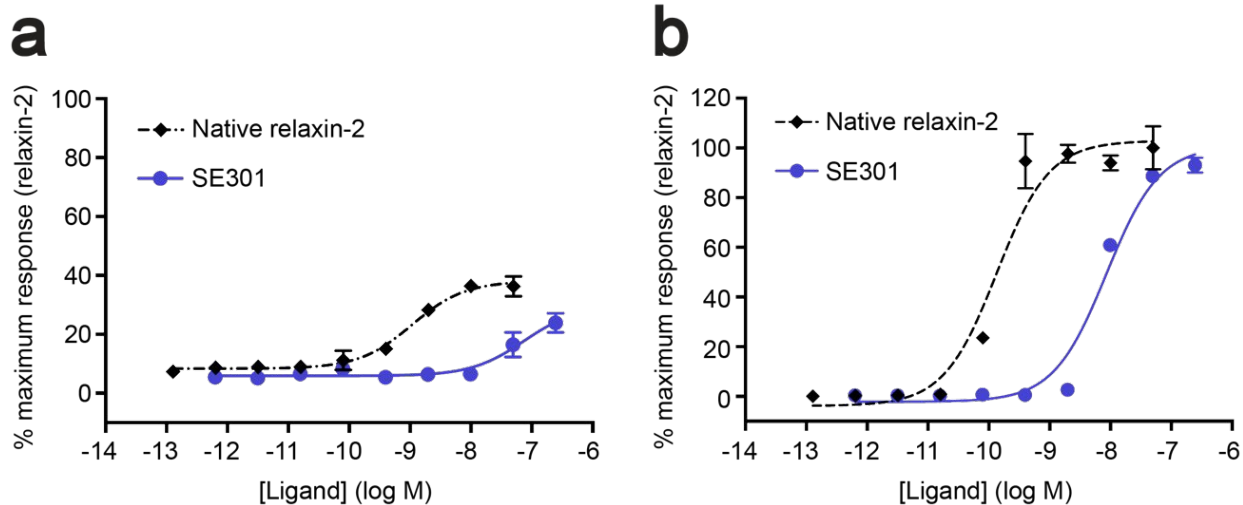
29 **Figure S2: Optimizations of Fc-relaxin-2 fusions.** a-d, CRE-SEAP G_s signaling data for
 30 human RXFP1 using native relaxin-2 compared to SE101–SE103 (a), SE201–SE203 (b),
 31 SE204–SE206 (c), and SE207 (d). Data are normalized to the native relaxin-2 response at human
 32 RXFP1 and are mean \pm s.e.m. from technical triplicates.



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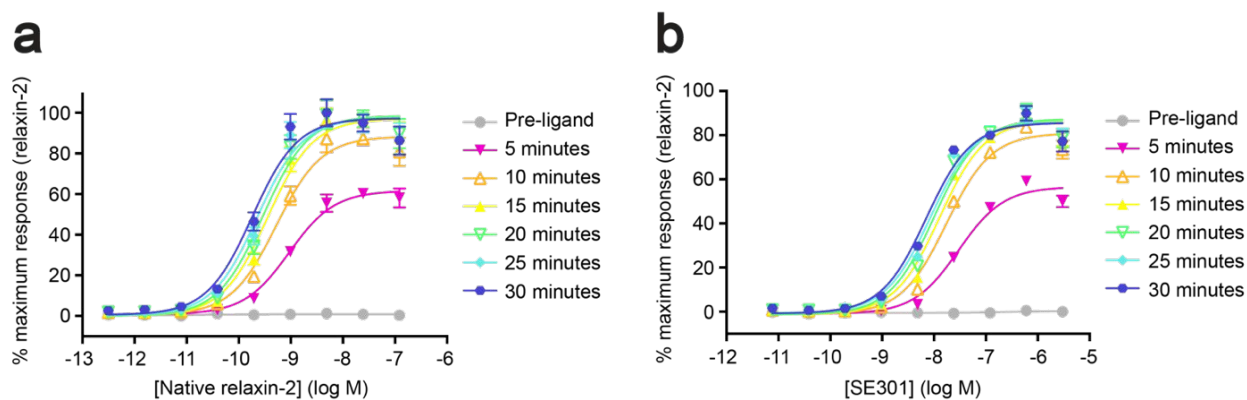
34 **Figure S3: Sites of relaxin-2 engineering in Fc-relaxin-2 fusion constructs.** **a,c**, Docking
 35 model of relaxin-2 bound to the leucine-rich repeats (LRRs) of RXFP1's ectodomain ¹. **b**, Details
 36 of the relaxin-2–LRR interface. In magenta are RXFP1 residues involved in the binding
 37 interface, in green are the “relaxin-binding cassette” residues of relaxin-2's B-chain, Arg13,
 38 Arg17, and Ile20, and in blue are the Met residues mutated to Lys in SE301. The Trp28 residue
 39 was not included in the docking model. The model shows that the Met residues on the relaxin-2
 40 B-chain are not positioned near the binding interface. **d**, Based on the model, the position of the
 41 single-chain relaxin-2 “mini-C” linker likely does not interfere with the binding of relaxin-2 to
 42 RXFP1.

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 45 **Figure S4: SE301 signaling activity at human RXFP2 and mouse RXFP1. a,** CRE-SEAP G_s
 46 signaling assay data for SE301 compared to native relaxin-2 at human RXFP2. Data are
 47 normalized to the native relaxin-2 response at human RXFP1 and are mean \pm s.e.m. from
 48 technical triplicates. **b,** CRE-SEAP G_s signaling assay data for SE301 compared to native human
 49 relaxin-2 at mouse RXFP1. Data are normalized to the native human relaxin-2 response at mouse
 50 RXFP1 and are mean \pm s.e.m. from technical triplicates.

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 54 **Figure S5: SE301 versus native relaxin-2 activity in the G_s GloSensor cAMP assay. a-b,**
 55 GloSensor G_s signaling assay data for native relaxin-2 (a) and SE301 (b) with measurements
 56 taken before ligand addition, and 5, 10, 15, 20, 25, and 30 minutes after ligand addition. Data are
 57 normalized to the native relaxin-2 response at human RXFP1 and are mean \pm s.e.m. from
 58 technical triplicates.

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61 **Table S1: CRE-SEAP G_s signaling assay EC₅₀ and E_{max} data for Figure 1c**

62 †Mean ± s.e.m., n=3 technical replicates.

Ligand	pEC ₅₀	E _{max} (%)
Native relaxin-2	10.0 ± 0.06	100 ± 2.0
SE301	8.2 ± 0.05	110 ± 2.5
Fc N297Q	ND	ND

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64 **Table S2: CRE-SEAP G_s signaling assay EC₅₀ and E_{max} data for Figure S1c**

65 †Mean ± s.e.m., n=3 technical replicates.

Ligand	pEC ₅₀	E _{max} (%)
Native relaxin-2	10.0 ± 0.2	100 ± 6.9
SE001	9.1 ± 0.1	126 ± 3.3
SE004	8.8 ± 0.1	124 ± 2.9

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72 **Table S3: CRE-SEAP G_s signaling assay EC₅₀ and E_{max} data for Figure S2**

73 †Mean ± s.e.m., n=3 technical replicates.

Ligand	pEC ₅₀	E _{max} (%)
Native relaxin-2	10.3 ± 0.1	100 ± 3.1
SE101	7.3 ± 0.1	86 ± 7.4
SE102	8.1 ± 0.1	106 ± 3.9
SE103	7.7 ± 0.1	93 ± 4.5
Native relaxin-2	10.5 ± 0.1	100 ± 3.5
SE201	8.4 ± 0.1	102 ± 3.1
SE202	8.4 ± 0.1	110 ± 3.3
SE203	8.2 ± 0.1	122 ± 3.0
SE204	8.8 ± 0.1	116 ± 3.3
SE205	9.3 ± 0.1	132 ± 3.1
SE206	8.5 ± 0.1	138 ± 4.9
SE207	8.4 ± 0.1	122 ± 3.3

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78 **Table S4: CRE-SEAP G_s signaling assay EC₅₀ and E_{max} data for Figure S4**

79 †Mean ± s.e.m., n=3 technical replicates.

Ligand	pEC₅₀	E_{max} (%)
Native relaxin-2, human RXFP2	9.0 ± 0.1	38 ± 1.7
SE301, human RXFP2	7.1 ± 0.3	30 ± 5.7
Native relaxin-2, mouse RXFP1	9.9 ± 0.1	100 ± 4.2
SE301, mouse RXFP1	8.1 ± 0.1	98 ± 3.7

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81 **Table S5: GloSensor G_s signaling assay EC₅₀ and E_{max} data for Figure S5**

82 †Mean ± s.e.m., n=3 technical replicates.

Ligand	pEC₅₀	E_{max} (%)
Native relaxin-2, Pre-ligand	ND	1 ± 0.2
Native relaxin-2, 5 minutes	9.0 ± 0.1	63 ± 1.7
Native relaxin-2, 10 minutes	9.3 ± 0.1	91 ± 2.7
Native relaxin-2, 15 minutes	9.4 ± 0.1	100 ± 2.9
Native relaxin-2, 20 minutes	9.5 ± 0.1	101 ± 2.9
Native relaxin-2, 25 minutes	9.6 ± 0.1	100 ± 3.2
Native relaxin-2, 30 minutes	9.7 ± 0.1	100 ± 3.1
SE301, Pre-ligand	ND	0 ± 0.5
SE301, 5 minutes	7.5 ± 0.1	58 ± 1.6
SE301, 10 minutes	7.8 ± 0.1	83 ± 2.0
SE301, 15 minutes	7.9 ± 0.1	89 ± 2.0
SE301, 20 minutes	8.0 ± 0.1	90 ± 2.2
SE301, 25 minutes	8.1 ± 0.1	89 ± 2.2
SE301, 30 minutes	8.1 ± 0.1	88 ± 2.0

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90 **Table S6: CRE-SEAP G_s signaling assay EC₅₀ and E_{max} data for Figure 3b**

91 †Mean ± s.e.m., n=3 technical replicates.

Ligand	pEC₅₀	E_{max} (%)
Native relaxin-2	9.9 ± 0.05	100 ± 1.7
SE301 Day 0	8.1 ± 0.05	102 ± 2.4
SE301 Day 7	8.2 ± 0.03	104 ± 1.3
SE301 Day 14	8.1 ± 0.1	98 ± 2.4
SE301 Day 21	8.1 ± 0.04	98 ± 2.0
SE301 Day 28	8.3 ± 0.04	108 ± 2.1

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Supplementary references

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- 96 1. Erlandson, S. C. *et al.* The relaxin receptor RXFP1 signals through a mechanism of
97 autoinhibition. *Nat Chem Biol* **19**, 1013–1021 (2023).

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Table S7: Construct sequences.

Name	Sequence
SE001	MKTIIALSYIFCLVFAHHHHHHDSWMEEVIKLCGRELVRAQIAICGMSTWSDAASSHSHSSARQLYSALANKCCHVGCTKRSLARFC
SE004	MKTIIALSYIFCLVFAEDQVDPRLIDGKGSHHHHHHDSWMEEVIKLCGRELVRAQIAICGMSTWSDAASSHSHSSARQLYSALANKCCHVGCTKRSLARFC
SE101	MYRMQLLSICIALSLALVTNSDSWMEEVIKLCGRELVRAQIAICGMSTWSDAASSHSHSSARQLYSALANKCCHVGCTKRSLARFCGSGGSGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK
SE102	MKTIIALSYIFCLVFAADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGKGGSDSWMEEVIKLCGRELVRAQIAICGMSTWSDAASSHSHSSARQLYSALANKCCHVGCTKRSLARFC
SE103	MKTIIALSYIFCLVFAADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGKGGSGGSGGSGGSDSWMEEVIKLCGRELVRAQIAICGMSTWSDAASSHSHSSARQLYSALANKCCHVGCTKRSLARFC
SE201	MKTIIALSYIFCLVFAADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGKGGSDSWMEEVIKLCGRELVRAQIAICGMSTWSDAASSHSHSSARQLYSALANKCCHVGCTKRSLARFC
SE202	MKTIIALSYIFCLVFAADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGKAAADSWMEEVIKLCGRELVRAQIAICGMSTWSDAASSHSHSSARQLYSALANKCCHVGCTKRSLARFC
SE203	MKTIIALSYIFCLVFAADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYQSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP

	QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKPPPDSWMEEVIKLCGRELVRAQIAICGMSTWSDAASSHS SSARQLYSALANKCCHVGCTKRSLARFC
SE204	MKTIALS ^{YIFCLVFA} DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQY ^Q STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSDSW ^K EEVIKLCGRELVRAQIAICGMSTWSDAASSHS SSARQLYSALANKCCHVGCTKRSLARFC
SE205	MKTIALS ^{YIFCLVFA} DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQY ^Q STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSDSWMEEVIKLCGRELVRAQIAICG ^K STWSDAASSHS SSARQLYSALANKCCHVGCTKRSLARFC
SE206	MKTIALS ^{YIFCLVFA} DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQY ^Q STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSDSWMEEVIKLCGRELVRAQIAICGM ST ASDAASSHS SSARQLYSALANKCCHVGCTKRSLARFC
SE207	MKTIALS ^{YIFCLVFA} DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQY ^Q STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSDSWMEEVIKLCGRELVRAQIAICGMSTWSDAAG ^{ANA} NAGARQLYSALANKCCHVGCTKRSLARFC
SE301	MKTIALS ^{YIFCLVFA} DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQY ^Q STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKGGSDSW ^K EEVIKLCGRELVRAQIAICG ^K STASDAAG ^{ANAN} AGARQLYSALANKCCHVGCTKRSLARFC
Fc N297Q	MKTIALS ^{YIFCLVFA} DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW YVDGVEVHNAKTKPREEQY ^Q STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

99 Legend: Signal sequence, His tag, Protein C tag, Relaxin-2 A-chain, Relaxin-2 B-chain, Fc, Mutation