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Expression and purification of the leptin receptor extracellular domain

Miriam Sánchez Sáez

Thesis mentor: Anna Rull Aixa

Internship supervisor: Assistant Professor Dr. Andreas Boland, Anna Höfler

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RESUMEN

El receptor de la leptina sigue siendo un reto en el campo de la biología estructural debido a la falta de datos sobre la estructura de alta resolución. El dominio extracelular del receptor de leptina se une a la leptina y está formado por seis dominios distintos que interactúan para producir la señalización; sin embargo, sólo tres de ellos, el dominio similar a la inmunoglobulina, el segundo dominio de homología del receptor de citoquinas y el dominio de fibronectina tipo 3 son estrictamente necesarios para este proceso.

El objetivo de este trabajo de fin de grado fue desarrollar y purificar construcciones alternativas del dominio extracelular del receptor de leptina para comprobar su estabilidad y a su vez desarrollar un protocolo de expresión y purificación que permita obtener niveles de proteína suficientes para futuros ensayos cristalográficos.

Para ello, se utilizó un sistema de expresión en células eucariotas que ha permitido la expresión de diferentes dominios de la región extracelular del receptor de leptina unidos a etiquetas de purificación para su aislamiento. Algunas de las proteínas recombinantes se expresaron con bajos rendimientos, sin embargo, es necesario mejorar el protocolo de purificación.

ABSTRACT

The structural biology field is still grappling with the leptin receptor, which has resulted in a scarcity of high-resolution structure data. The extracellular domain of the leptin receptor binds to leptin and is made up of six distinct domains that interact to produce signaling; however, only three of them, the immunoglobulin-like domain, the second cytokine receptor homology domain, and the fibronectin type 3 domain, are indispensable for this process.

The goal of this internship was to develop and purify alternative constructions of the extracellular domain of the leptin receptor to test their stability and also to arrive to an expression protocol to obtain sufficient protein levels for future crystallographic assays.

For this purpose, a eukaryotic cell expression system that allowed the expression of different domains of the leptin receptor's extracellular region linked to purification tags to be isolated was used. Some of the recombinant proteins were expressed with low yields, however, the purification protocol needs to be improved.

ABBREVIATIONS

LR: Leptin receptor

ECD: Extracellular domain

FN3: Fibronectin type III domain

CRH: Cytokine receptor homology domain

NTD: N-terminal domain

IGD: Immunoglobulin-like domain

YFP: Yellow fluorescent protein

DPA: Day after proliferation arrest

Hb: Honey bee melittin secretion signal

G-CSF: Granulocyte colony-stimulating factor

IL6: Interleukine-6

CNTF: Ciliary neurotrophic factor

JAK: Janus kinase

STAT: Signal transducer and activator of transcription

LEP-R: Liganded leptin receptor complex

BEVS: Baculoviral expression system

MCS: Multiple cloning site

dNTPs: Deoxyribonucleotide triphosphate

SEC: Size exclusion chromatography

1. INTRODUCTION

1.1 Leptin

Mature leptin is a non-glycosylated 16-kDa peptide hormone of 146 amino acids produced mainly in the white adipose tissue by the adipocytes although it is expressed in other tissues and/or organs like skeletal muscle, stomach, ovaries, or mammary glands (Tissue expression of LEPR - Summary - The Human Protein Atlas 2021; Lin y Li 2007; Guerra et al. 2007; Karlsson et al. 1997).

It has a dual role as a hormone and a cytokine, linking the neuroendocrine and the immune system (Pérez-Pérez et al. 2017) and acts as a major regulator for food intake and energy homeostasis (Zhang et al. 2005).

Leptin acts by binding to its membrane receptor, also known as leptin receptor (LR), which is present in a wide variety of tissues, even though its mainly found in the brain and its peripheral tissues (Couce et al. 1997; Harvey 2003).

1.1.1 Leptin structure

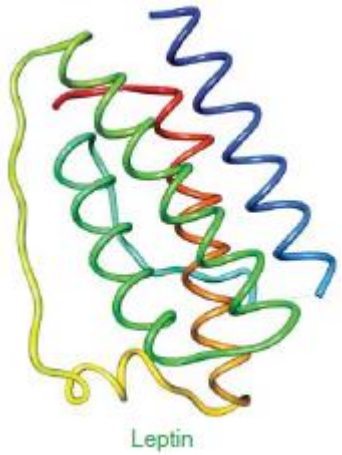


Figure 1. Leptin structure. [Image extracted from Peelman et al. 2014]

Leptin has four anti-parallel helices arranged up-up-down-down in a typical four-helical bundle cytokine configuration. The parallel helices are linked by two long crossover loops AB and CD, while the antiparallel helices are connected by a short BC loop.

G-CSF, the IL6 family of cytokines, and ciliary neurotrophic factor (CNTF) have structures that are very similar to the leptin receptor. Despite the lack of sequence similarity, these related structures have identical inter-helical angles and crossover loops. Leptin is categorized as a member of the long-chain cytokine family, together with these structural relatives; even though the helices of leptin are, on average, one or two turns shorter.

This family of cytokines interacts with their receptors through three different binding sites I-III. Mutations located in this region in leptin demonstrated that site II is important for leptin binding, while site III is crucial for receptor activation without affecting the binding (Peelman et al. 2004).

1.1.2 Leptin and disease

Deficiencies in leptin or its receptor lead to morbid obesity and insulin resistance (Ramos-Lobo y Donato 2017). However, numerous studies have shown that leptin plays a much broader role in the body, including neuronal growth and plasticity, memory and cognition, glucose homeostasis reproduction, and metabolic programming (Park y Ahima 2015; Zeltser 2015; Pereira et al. 2019).

1.2. Leptin receptor

The LR belongs to the class I cytokine receptors; single membrane receptors with one or more cytokine receptor homology domains (CRH) containing the WSXWS motif belong to this family (Peelman et al. 2014).

There have been six different isoforms of the LR identified so far, all of which share a similar extracellular ligand-binding domain at the N-terminus but differ in the intracellular domain and thus in their physiological function.

Isoform B, also known as LR long form, is a 1,162-residue protein with a 302-aa intracellular domain that is thought to be the only isoform capable of JAK/STAT signaling. This receptor is strongly expressed in the hypothalamus' specific nuclei, where it plays a role in body weight control (Fei et al. 1997; Mercer et al. 1996). However, it can be present in a wide variety of cell types.

Isoform E is the only one without a transmembrane domain, suggesting that it represents a soluble LR (Schaab y Kratzsch 2015) formed solely by the extracellular domain (ECD). This soluble fragment is formed in humans by proteolytic ectodomain shedding and regulates leptin bioavailability (Ge et al. 2002).

1.2.1 Leptin receptor structure

This receptor showed the highest structural similarity to the interleukin 6 (IL6) signaling receptor chain glycoprotein 130, the granulocyte colony-stimulating factor (G-CSF) receptor, and the leukemia inhibitory factor (LIF) (Zabeau et al. 2003) and uses JAK2 and STAT3 for its principle signaling pathway (Morris et al. 2018).

1.2.1.1 Extracellular domain

The LR's extracellular domain topology is complex, with six distinct domains: an undefined N-terminal domain (NTD), a first CRH domain (CRH1), an immunoglobulin-like domain (IGD), a second CRH domain (CRH2), and two membrane-proximal FN3 domains (Peelman et al. 2014).

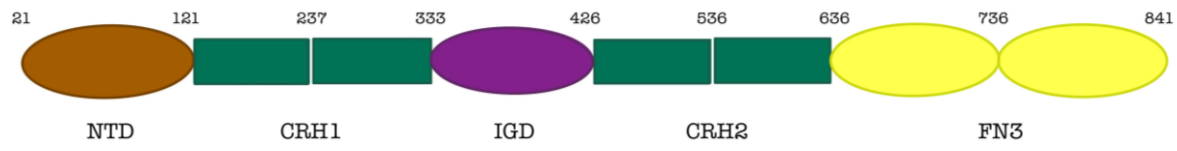


Figure 2. Scheme of the leptin receptor extracellular domain. This scheme shows the different domains, with their boundaries showed in amino acids, that form the extracellular domain of the full-length leptin receptor. It also corresponds to the organization of the isoform E.

The NTD has an unknown function, it is very labile and, together with the CRH1 domain, they are not required for LR signaling. The deletion of each of the four other regions, on the other hand, fully eliminates signaling (Zabeau et al. 2004; Fong et al. 1998). Since none of the other domains exhibit any observable leptin binding, the CRH2 domain is the only high-affinity binding domain for leptin (Mancour et al. 2012; Fong et al. 1998).

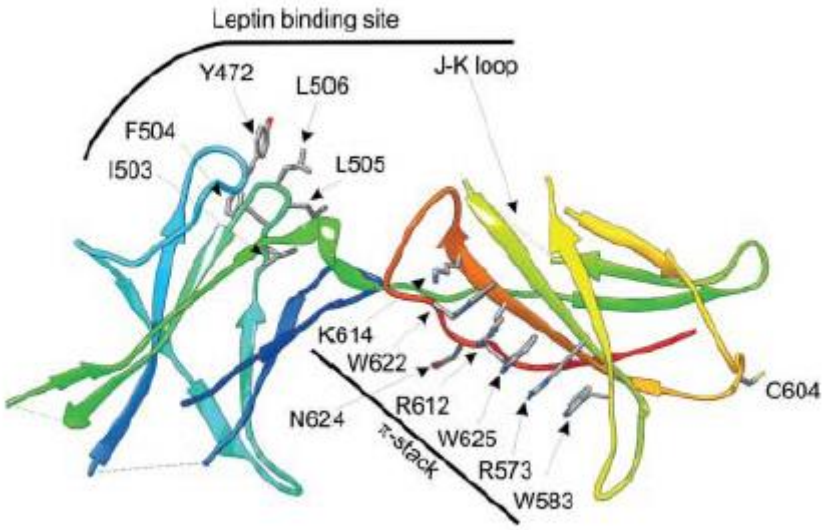


Figure 3. Crystal structure of the CRH2 domain. Residues that are predicted to interact with leptin are indicated.

[Image extracted from Peelman et al. 2014].

For the development of an activated LR complex and downstream signaling, the interaction between the LR IGD and leptin's binding site III is important (Zabeau et al. 2019).

In this case, its function reminiscent the role of these domains in other cytokine receptors, like gp130 or G-CSF, where it interact with the binding site III of the ligand, already bound to another receptor chain. The presence of these analogs in leptin suggests that the LR uses a similar site III-IGD interaction.

The FN3 domain seems essential for signaling, due to its regulation in the orientation of the cytoplasmic tails in a manner that favors signaling (Zabeau et al. 2005).

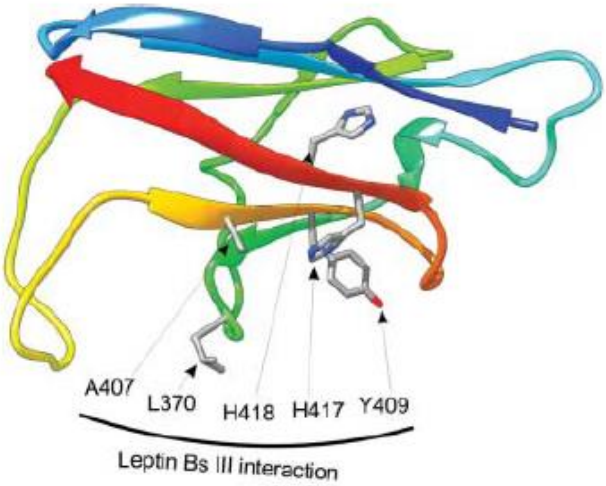


Figure 4. Model of the IGD domain indicating the residues that could interact with the leptin binding site III.

[Image extracted from (Peelman et al. 2014)].

1.2.1.2 Post-translational modifications (PTMs)

Nine disulfide bridges were found for the twenty-eight cysteines within the human LR ECD, and also the CRH2 crystal structure suggests 2 additional attainable disulfide bridges (Carpenter et al. 2012). The LR is additionally heavily glycosylated, leading to a 30-70 kDa increase in apparent molecular weight on SDS-PAGE; N-glycosylation is predominant, however, some O-glycosylation is also present (Haniu et al. 1998).

The following is that the distribution of experimentally determined N-glycosylation sites across domains: six within the NTD, three in CRH1, two in immunoglobulin D, two in CRH2, and five in the membrane-proximal FN3 domains. The LR's relationship with leptin is impaired by N-glycosylation, as deglycosylation of recombinant ECD decreases leptin binding by 80% (Kamikubo et al. 2008). This effect is likely mediated by glycosylation of the IGD or membrane proximal FN3 domains, rather than glycosylation of the CRH2 domain.

1.3 Eukaryotic cell expression

The eukaryotic cells are a well-known model for protein expression. The main advantage is that post-translational modifications can be obtained and many proteins need

post-translational processing to mature, and most of those modifications are required for the proteins' biological functions (Churgay et al. 1997; Klenk 1996).

1.3.1 Insect cells

Spodoptera frugiperda insect cells are a common eukaryotic cell model, which has been previously used for the characterization and purification of human leptin (Churgay et al. 1997).

The Sf9 insect cell line is a clonal isolate originated from the parental *Spodoptera frugiperda* cell line IPLB-Sf-21-AE, and it is the primary choice for the expression of recombinant proteins from baculovirus expression systems (Sf9 Cells - Morphology | Thermo Fisher Scientific - CH 2021).

This model can be easily cultured in suspension cultures in shake flasks but also adherent cultures due to its firm attachment to surfaces and its small and regular size.

1.3.2 Baculoviral expression system

The baculovirus transfer vectors enable multigene applications. They are accompanied by modified recipient baculovirus that has been engineered towards improved protein production (Trowitzsch et al. 2010).

The baculoviral expression system (BEVS) has become a common eukaryotic expression system in its various shapes and forms. However, it has been modified for a variety of other applications, including the production of virus-like particles (Biotech 2011).

1.3.2.1 pACEBac1 transfer vector

The pACEBac1 transfer vector contains a homing endonuclease-based multiplication module that helps facilitate a modular combination of heterologous genes, in their respective gene expression cassettes with a minimum requirement for unique restriction sites (Biotech 2011).

This acceptor vector contains the DNA elements required for integration into the baculovirus via Tn7 transposition. DH10 *E.Coli* cells contain the recipient baculovirus and also the transposase required for Tn7 transposition.

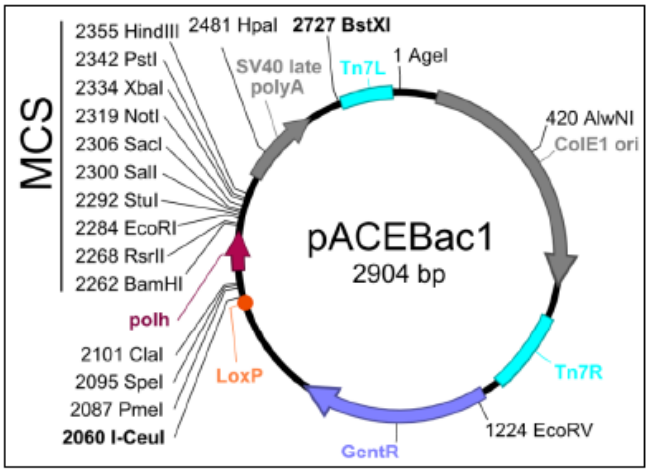


Figure 5. Schematic representation of the pACEBac1 acceptor vector

[Image extracted from (Biotech 2011)]

The pACEBac1 transfer vector carries a ColE1 replication origin to keep high the number of plasmid copies. Multiple cloning sites (MCS), transposition elements (Tn7L, Tn7R), and a gentamycin resistance marker can be also found in the acceptor vector, as well as polh promoters and SV40 terminators.

Using the incomplete inverted LoxP sites in the vector the genes can be recombined via Cre-lox recombination but it can also be done using the unique restriction sites in the MCS (Biotech 2011).

1.3.2.2 DH10MultiBac baculoviral DNA

In DH10 MultiBac baculoviral DNA the genes *v-cath* and *chiA* genes were disrupted which results in reduced virus-dependent proteolytic activity and reduced cell lysis, that increase the efficiency of proteins provided significantly.

Via *cre-lox* site-specific recombination the disrupted viral DNA sequence was replaced with a LoxP sequence; however, this LoxP site is not used for introducing the target constructs.

Instead, the genes of interest are transposed into the mini Tn7 attachment site and transferred to the bacmid. The lacZ subunit-coding sequence is disrupted after successful integration. As a result, clones with inserted DNA would have a white phenotype (Biotech 2011).

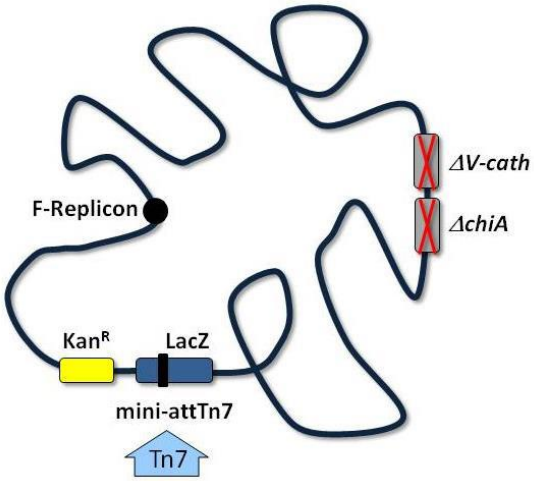


Figure 6. DH10MultiBac baculoviral DNA

[Image extracted from (Biotech 2011)]

1.3.3 Secretion signal

Glycosylations in proteins can be produced by going through the secretory pathway, which can be induced by linking the recombinant protein to secretion signals. It has been proven (Tessier et al. 1991) that there is an increase in the yields of secretion of the system, by using signal peptides of insect origin to direct the secretion of a foreign protein with the use of Sf9 insect cells instead of the native secretion signal.

In this case, the addition of an N-terminal sequence encoding the honeybee melittin signal peptide fused to the desired protein allowed a secretion of over five times more quantity than the wild-type peptide. This sequence has been inserted in several baculovirus transfer vectors to increase efficiency (SnapGene 2021b, 2021a).

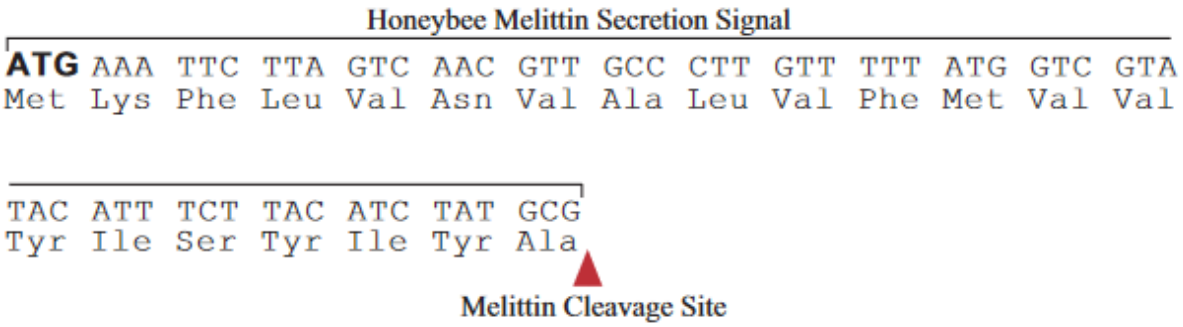


Figure 7. DNA and peptide sequence of the honeybee melittin secretion signal

[Image extracted from (SnapGene 2021a)]

The melittin signal sequence is cleaved upon secretion after Ala in the signal sequence, marked with a red arrow as melittin cleavage site in Figure 7.

2. HYPOTHESIS AND OBJECTIVES

Previous research regarding the characterization of this protein's structure at high-resolution using crystallography has not been successful. With the use of smaller constructs, an improvement in crystallization is expected.

Therefore, the purpose of this internship was to create and purify various constructs of the leptin receptor's extracellular domain to verify their stability and to obtain a protein expression and purification protocol that in last term could be used to get a high-resolution structure by using crystallography.

3. MATERIALS AND METHODS

3.0.1 Chemicals

The following chemicals were used for the experiments: HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) from *Fluorochem*; desthiobiotin from *IBA-Lifesciences*; NaCl, agarose, KCl, Na_2HPO_4, kanamycin sulfate, tetracycline hydrochloride, KH_2PO_4, and glycerol from *Carl Roth*; imidazole from *Aldrich*; Tris from *Biosolve*; acetic acid from *Carlo Erba*; EDTA (Ethylenediaminetetraacetic acid), BSA (bovine album serum), gentamycin sulfate and urea from *PanReac applichem*; isopropanol and ethanol from *ThermoFisher Scientific*; and Tween20 from *Sigma Lifesciences*.

3.0.2 Buffers

The buffers showed in Table 1 were used during the experiments.

Table 1. Description of buffers used during this research

Buffers	Composition
1X PBS	*137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2P0_4*
1X TAE	*40 mM Tris, 20 mM acetic acid and 1 mM EDTA*
1X TBS	*20 mM Tris and 150 mM NaCl*
Base buffer	*20 mM HEPES and 50 mM NaCl pH 7.2*
Pellet lysis buffer	*20 mM HEPES and 50 mM NaCl 7M urea pH 7.2*
Strep-chromatography elution buffer	*20 mM HEPES 50 mM NaCl 2.5 Mm desthiobiotin pH 7.2*
Blotting buffer 10x	*25 mM Tris, 192 mM glycine*
Blotting buffer 1x	*10x blotting buffer 20% ethanol*
Blocking solution	*TBS 0.05% Tween20 4% BSA*
Equilibrated buffer Ni-beads	*20 mM HEPES and 50 mM NaCl 10 mM imidazole pH 7.2*
Elution buffer Strep-beads	*Buffer BXT 1x (IBA Lifesciences) diluted in base buffer*

3.1 Cloning

The vector used for the baculoviral expression is pACEBac1, which contains the DNA elements required for integration into the baculovirus via Tn7 transposition and a gentamycin resistance marker (Sari et al. 2016; Biotech 2011).

3.1.1 Primers design

The eukaryotic cell expression protocol was performed by using pACEBac1 as a vector inserting the leptin receptor extracellular domain constructs. The insert primers were designed leaving a 20 nucleotides overhang and included the purification tags. The honeybee melittin secretion signal was included in the backbone. The newly designed primers were evaluated using *neB calculator*.

3.1.2 PCR Amplification

All the samples were amplified using 0.5 µM of each primer and 0.2 mM deoxyribonucleotide triphosphate (dNTPs from *Carl Roth*), 1 U Phusion Polymerase (*ThermoFisher Scientific*), 0.4 ng/µL template DNA, and 1x Phusion HF or GC buffer (*ThermoFisher Scientific*) giving a total of 50 µL. The elongation time of the PCR cycles was adjusted to 1 min/kb. The results from the PCR were visualized using *Biotium* GelGreen Nucleic Acid Gel Stain in a 1 % agarose in 1x TAE (Tris-acetate-EDTA) buffer in a Dark reader transilluminator from *Clare Chemical Research*. The molecular weight was compared using a *ThermoFisher Scientific* GeneRuler 1 kb DNA Ladder.

3.1.3 Gel extraction

The samples were run in an agarose gel (1 % agarose in TAE 1x buffer) then the *Macherey-Nagel* PCR clean-up gel extraction protocol was followed. The concentration of the DNA fragment was measured using a DS-11 Spectrophotometer from *Denovix Inc*.

3.1.4 Gibson cloning

50 ng of the recipient plasmid and 3x molar ratio of the recombinant DNA were mixed. The amount of Gibson Assembly Master mix from *New England Biolabs* corresponding to a 1:1 concentration was added. Then the sample was incubated at 50 ºC for 1 h.

3.1.5 Chemical transformation of DH5α competent cells

100 ng of plasmid were added to 50 µL DH5α *E.Coli* competent cells and incubated for 20 min on ice. Then a heat shock at 42 ºC for 45 s and incubation of 3 min on ice was performed. After this, 500 µL of SOC medium were added and incubated for at least 1 h at 37 ºC at constant movement. The samples were centrifuged at 3,000 g for 3 min and the supernatant was discarded by decanting. Afterward, the pellet was resuspended with the leftover supernatant and was plated, on gentamycin containing LB agar plates and incubated at 37 ºC overnight. Colonies were picked and inoculated in tubes containing 3 mL LB medium with 7 µg/mL gentamycin, they were left incubating overnight at 37 ºC and constant movement.

3.1.6 Mini-preparation for sequencing

The liquid cultures were centrifugated at 2,500 g for 10 min. Then the DNA was extracted using the NucleoSpin Plasmid Easy Pure kit from *Macherey Nagel*. The concentration of double-stranded DNA was measured using a DS-11 Spectrophotometer from *Denovix Inc*.

3.2 MultiBac expression system and virus production

The protein expression was performed using a eukaryotic expression system using baculovirus transfer vectors, in this case, pACEBac1, for Sf9 insect cells, that allows

obtaining viruses containing a plasmid that encodes the protein of interest and introduces the vector inside the insect cell for protein production.

3.2.1 Transformation of DH10 MultiBac competent cells

This transformation was performed in DH10 MultiBac *E.Coli* competent cells as described in section 3.1.5. In this case, 7 µg/mL gentamycin / 40 µg/mL IPTG (Isopropyl β-d-1-thiogalactopyranoside) / 100 µg/mL BLuOGal (halogenated indolyl-galactoside) LB-agar plates and 3 mL LB medium with 50 µg/mL kanamycin, 7 µg/mL gentamycin, and 10 µg/mL tetracycline tubes for liquid cultures were used.

3.2.2 Bacmid preparation

First, the liquid cultures were centrifuged at 2,500 g for 10 min at 4 ºC and the supernatant was discarded. The pellet was resuspended in 300 µL buffer A1 (*Macherey-Nagel*), it was then lysed with 300 µL buffer A2 (*Macherey-Nagel*) and neutralized using 300 µL buffer A3 (*Macherey-Nagel*).

The sample was then centrifuged for 10 min at 18,750 g and the supernatant was transferred in another tube which was centrifuged for another 7 min at 18,750 g. The supernatant resulted was transferred to another tube and the DNA was precipitated using 700 µL 100 % isopropanol and centrifuged for 10 min at 18,750 g. Afterward, the DNA leftovers were precipitated with 200 µL 70 % ethanol and another centrifugation for 5 min at 18,750 g. Finally, the ethanol was discarded and 50 µL of 70 % ethanol was added.

3.2.3 Insect cells transfection

The ethanol was removed in a laminar flow cabinet from the bacmid preparation from section 3.2.2. and the DNA pellet was dried for 10 min. 20 µL of sterile water and 200 µL *Gibco* Sf-900 III SFM medium were added to resuspend.

Then 10 µL of the FUGENE transfection reagent mix from *Promega* was added and incubated for 15 minutes at room temperature. Finally, half of the mixture was added dropwise to $1*10^6$ Sf9 insect cells per well in a 6 well plate, following the organization showed in Figure 8, and incubated at 27 ºC.

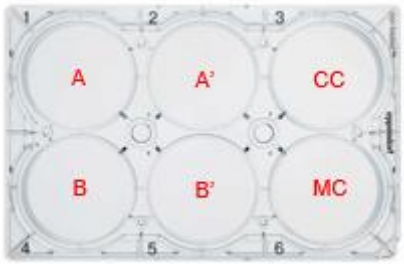


Figure 8. Plate organization for the transfection and Vo harvesting. CC is the cell control that does not contain transfection mix and MC is the medium control, only containing medium

3.2.4 V0 harvesting and V1 amplification

72 h after transfection, 6 mL of the medium, which is the Vo, was taken and stored in the dark at 4 ºC. Then 25 mL of medium containing $1*10^6$ cells/mL Sf9 were transfected with 2.5 mL Vo. It was left to grow at 27 ºC and constant movement and diluted if the concentration is higher than $2.00*10^5$ cell/mL.

3.2.5 Cell and V1 collection

24 h after the Day after Proliferation Arrest (DPA), the cells were transferred to a tube and centrifuged at 900 g for 20 min. The V1 is harvested by collecting the medium, and the pellet is kept for future analysis at cold temperature (-20/-80 ºC). For subsequent expressions, $1.5 * 10^6$ insect cells were infected directly with 1.5 mL V1 and the supernatant or the pellet was collected 24 h after the DPA.

3.3 Protein purification

3.3.1 Non-secreted protein purification

10 mL base buffer, containing ½ tab of proteases inhibitors (Complete Protease Inhibitor Cocktail Tablets from *Roche*) to prevent proteolysis and 0.025 U/mL DNases (SuperNuclease from *SinoBiological*) to avoid DNA contamination were added to a cell pellet of 25 mL and dissolved in constant movement at 4 ºC. After dissolving, 10 mL base buffer was added and the sample was sonicated for 2-3 min at intensity 3, 30 % duty cycle using a *Branson* Sonifier 450. Finally, it was centrifuged at 32,000 g for 1 h at 4 ºC and the supernatant was kept at 4ºC.

3.3.2 Secreted protein purification

The insect cell culture was centrifuged at 800 g for 20 min and the supernatant was kept at 4 ºC. Another centrifugation at 15,000 g for 20 min before analysis was performed in case of precipitation. 2.4 mL BioLock (*IBA-Lifesciences*) per liter of medium was added and it was filtered using a 0.2 µm filter.

3.3.3 Strep-beads purification

A manually packed Strep-beads (Strep-beads XT Superflow from *IBA-Lifesciences*) column was washed twice with base buffer and the lysate/medium was then used to resuspend the 0.3 mL of column bed volume (CV). The mix was incubated at 4 ºC at constant movement for 1-2 h.

The sample was loaded back into the column and washed twice with base buffer to remove the non-specifically adsorbed proteins. Subsequently, the bound Strep-tagged proteins were eluted with 200 µL Strep-beads elution buffer which contains biotin for competitive displacement.

3.3.4 Ni-beads purification

A manually packed 0.3 mL Ni-beads column (Ni-beads Sepharose Excel from *GE Healthcare*) was equilibrated twice. The lysate imidazole content was adjusted to 10 mM to prevent unspecific binding and it was used to resuspend the beads and incubate them in constant rotation at 4 ºC for 1-2 h.

The sample was loaded back into the column and was washed twice with equilibrated buffer to remove the non-specifically adsorbed proteins. Subsequently, the trapped His-tagged proteins were eluted using base buffer with different imidazole concentration (50 mM, 150 mM, and 500 mM).

3.3.5 Affinity chromatography with pre-packed StrepTactin columns

4 x 5 mL StrepTactin columns (*Strep-Tactin Superflow cartridges, QIAGEN*) were placed in an equilibrated ÄKTA Romeo chromatography system from *Cytiva Lifesciences*. The sample, previously filtered, was loaded on the column and washed until the UV signal reaches almost 0. Finally, the proteins were eluted using Strep-chromatography elution buffer.

3.3.6 Concentration with centrifugal Filters

Successive centrifugations at 2,500 g, 4 ºC for 5 min using the Amicon 10 kDa Ultra Centrifugal Filters from *Merck Millipore* were performed until the desired concentration avoiding protein precipitation.

3.3.7 Size exclusion chromatography

The sample was loaded on the Superose 6 increase 3.2/300 *GE Healthcare* analytical column on an equilibrated ÄKTAmicro from *GE Healthcare*, the proteins were eluted using the base buffer.

3.4 Protein analysis

3.4.1 Electrophoresis [SDS-PAGE]

3x loading dye was added to the samples and boiled afterward at 95 ºC for 10 min. The pellet samples were prepared with a step of lysis in pellet lysis buffer and centrifugated at 20,000 g or 15 min at 4 ºC collecting the supernatant. Then the samples were loaded in a Mini-PROTEAN® TGX™ Precast Protein Gel, 10-well, *Bio-Rad* using a PageRuler Prestained Protein Ladder from *ThermoFisher Scientific* for molecular weight analysis.

The SDS-PAGE gel was left to dye, until the protein bands were visible, covered in Der Blaue Jonas Instant blue from *Lubio Science* or following the Pierce Silver Stain Kit protocol from *ThermoFisher Scientific*.

3.4.2 Western blot

SDS-PAGE was performed as described in section 3.4.1 and then electroblotted onto a nitrocellulose membrane.

Membranes were blocked in blocking solution for 1 h at constant movement, they were exposed to anti-His (H1029 *Sigma-Aldrich*. Monoclonal Anti-polyHistidine antibody produced in mouse) or anti-Strep (GT661 *Sigma-Aldrich*. Monoclonal Anti-Strep Tag antibody produced in mouse) antibodies at dilution 1/3,000 or 1/10,000 respectively in blocking solution overnight.

Membranes were washed and incubated with anti-mouse IgG conjugated to a fluorophore (*LI-COR*, IRDye Goat anti-Mouse IgG Secondary Antibody) diluted 1/10,000 in the blocking solution for 1 h at constant rotation. After a series of washes with blocking solution, the protein bands were visualized using *LI-COR* Image Studio Lite at 700 nm. The size of the protein band was determined using a PageRuler Prestained Protein Ladder from *ThermoFisher Scientific*.

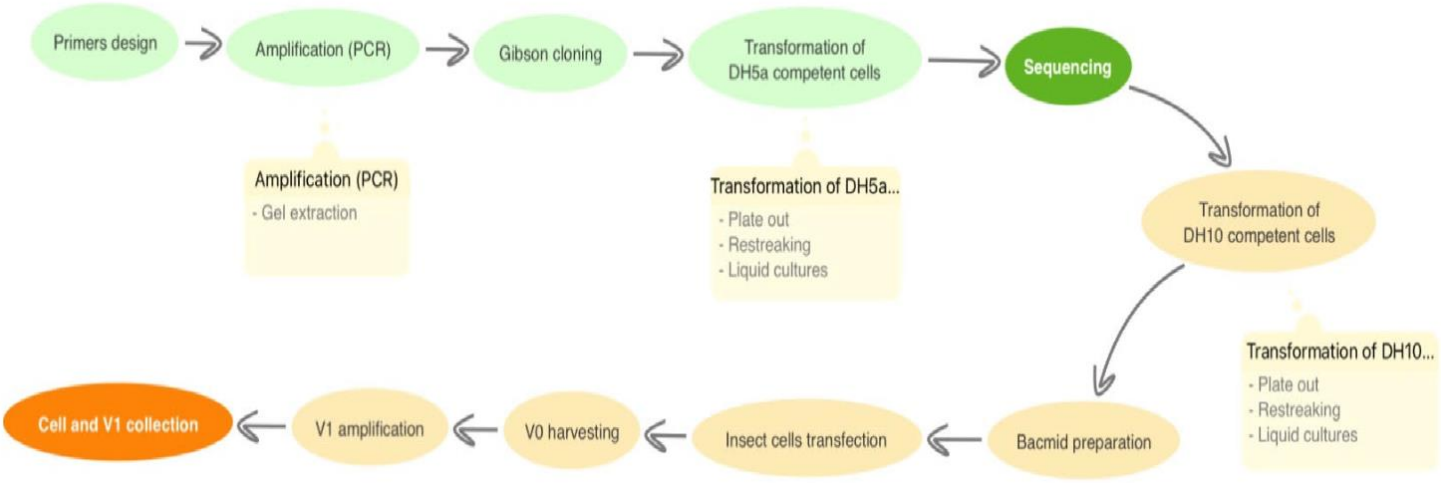


Figure 9. Eukaryotic expression scheme. Steps corresponding to the cloning section are shown in green. Steps corresponding to the multiBac expression system and virus production are shown in orange.

4. RESULTS

Following the baculoviral protocol, using Sf9 insect cells, truncated constructs of the leptin receptor extracellular domain containing the IGD, CRH2, and FN3 subdomains, which are the minimal requirement for leptin signaling, were expressed and purified. The constructs, obtained from the leptin receptor extracellular domain (Figure 2), were expressed in insect cells using the pACEBac1 acceptor vector, including 2 Strep-Tags and 5 histidines, for later purification steps.

4.1 IGD-CRH2 construct

Due to the lack of findings with longer segments, the usage of shortened constructs is intended to create a good candidate for crystallography. In this case, a construct that included two of the most important domains for leptin signaling, the IGD and CRH2 domains (Figure 10), which had a total size of 304 amino acids and are comparable to 34.86 kDa was used (Supplementary figure 1).

Affinity purification using streptavidin beads was used for this construct, which was then evaluated using SDS-PAGE (Figure 11A). A Western Blot was performed on the sample with anti-Strep as the main antibody (Figure 11B).

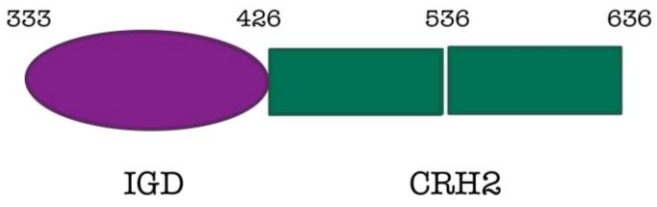


Figure 10. Scheme of the IGD-CRH2 construct. The boundaries indicated in amino acids correspond to the expected domain borders shown in Peelman et al. 2014.

As a result, a faint protein band in elution 2 of the SDS-PAGE after Strep-beads affinity purification (Figure 11A) was seen, which was comparable to others bands in the washout and flow-through. But, using Western Blot with anti-Strep as the main antibody

(Figure 11B), the construct can not be differentiated due to contamination with yellow fluorescent protein (YFP).

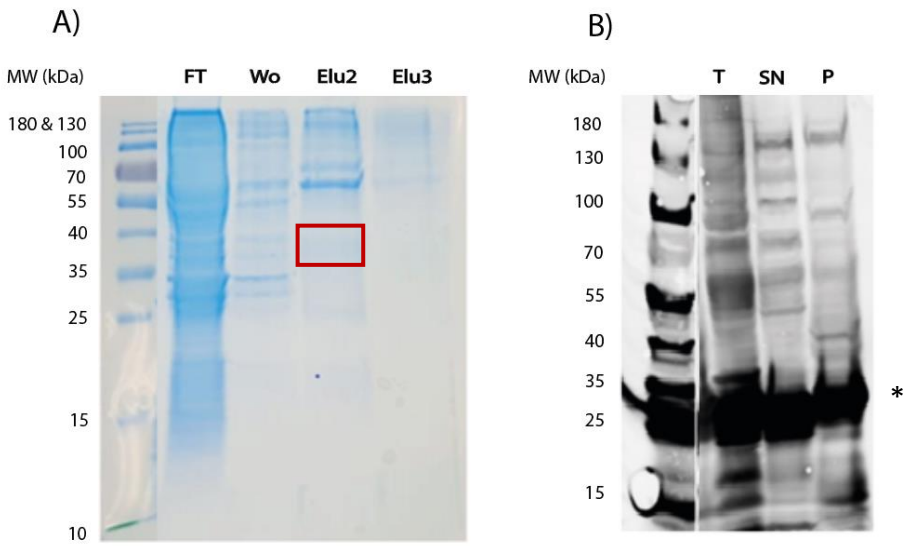


Figure 11. Analysis of the protein purification of the construct IGD-CRH2. A) SDS-PAGE results after Strep-beads purification B) Western blot results obtained by using anti-Strep antibody and visualized employing chemiluminescence. YFP contamination is indicated with an asterisk. Presumed IGD-CRH2 is marked in red. Key: FT, unbound flow-through; Wo, washes; Elu 2-3, eluate fractions; T, total sample; SN, supernatant; P, pellet.

4.2 IGD-CRH2-FN3 construct

After performing assays with the IGD-CRH2 construct, the FN3 domain was included in the next construct because it has also been shown to be required for leptin signaling. Hypothetically, it may aid in leptin receptor stability by allowing the formation of an extra intramolecular disulfide bridge (Carpenter et al. 2012).

The IGD-CRH2-FN3 domains (Figure 12) have a size of 404 amino acids, which is comparable to 46.31 kDa in the recombinant protein (Supplementary figure 2).

This construct was isolated using Strep-beads and analyzed using SDS-PAGE (Figure 13A), as well as a Western blot with an anti-Strep antibody (Figure 13C). The sample after Strep-affinity purification was subsequently purified using Ni-beads and analyzed with SDS-

PAGE (Figure 13B). A Western blot using an anti-His antibody was performed for the Strep-beads purified sample (13D).

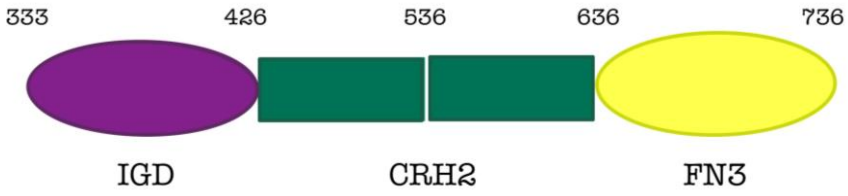


Figure 12. IGD-CRH2-FN3 scheme. This construct is composed of the IGD and the CRH2 domain and one FN3 domain. The boundaries, annotated in amino acids, corresponding to the predicted domain borders shown in Peelman et al. 2014.

In the gel after Strep-beads purification (Figure 13A), a band that correlates with the size of the construct IGD-CRH2-FN3 was observed together with other multiple bands indicating that it was not a pure sample. So, a Ni-beads purification (Figure 13B) was performed to further purify it. Two Western blots were also performed using the Strep-beads purified sample, using anti-Strep (Figure 13C) or anti-His (Figure 13D) antibodies. The same band of around 45 kDa was observed in the different SDS-PAGE and bands around 37 kDa were observed in Western blot. The YFP contamination can also be observed in the gels in Figure 13A-13C.

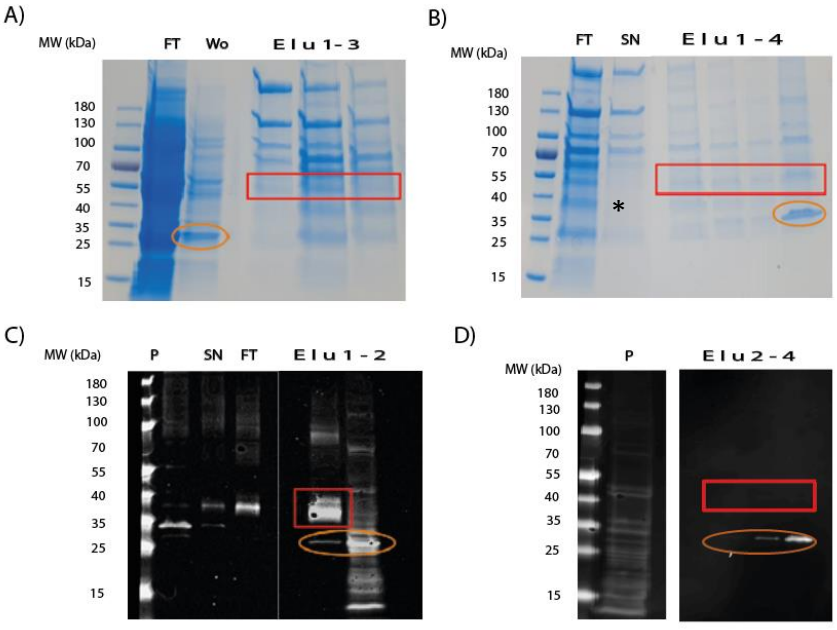


Figure 13. Analysis of the protein purification of the construct IGD-CRH2-FN3. A) SDS-PAGE results after Strep-beads purification B) SDS-PAGE results after Strep-beads and subsequent Ni-beads purification C) Western blot results obtained using anti-Strep antibody after the Strep-beads purification. D) Western blot results obtained using anti-His antibody after the Strep-beads purification. YFP contamination is marked in orange. Presumed IGD-CRH2-FN3 is marked in red. The band marked with an asterisk was sent for MS.

Key: FT, unbound flow-through; Wo1, washes; Elu, eluate fractions; SN, supernatant; P, pellet.

The band, in Figure 13B flow-through, corresponding to a molecular mass around 37 kDa, was analyzed by mass spectrometry (MS) and the sample has a 41% sequence coverage with the full-length leptin receptor extracellular domain (Figure 14), which proves that the purified band is the IGD-CRH2-FN3 construct.

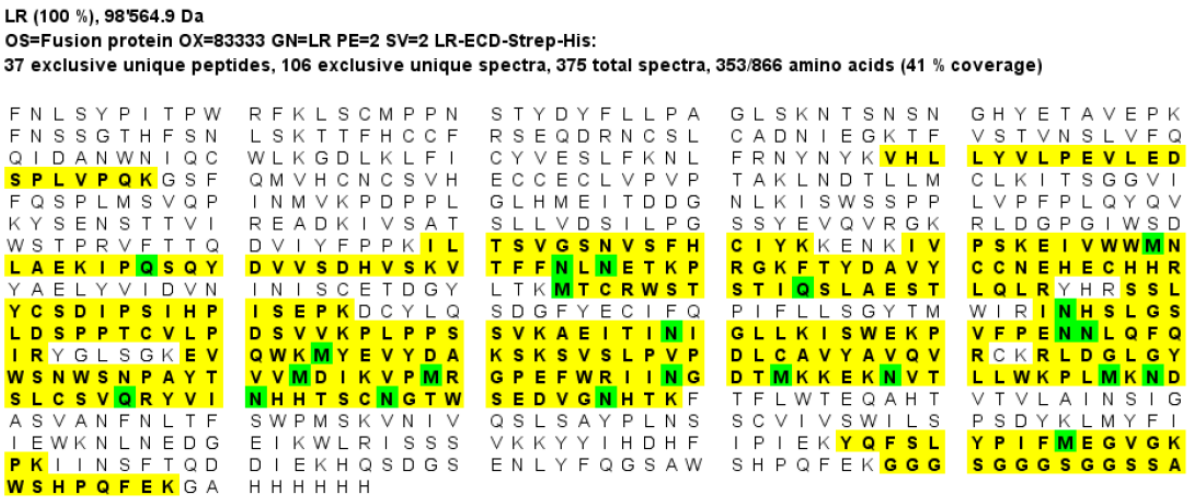


Figure 14. Sequence coverage of the recombinant human leptin receptor protein using an IGD-CRH2-FN3 sample. Green indicates the modified amino acids. This shows the number of amino acids in the leptin receptor that were found in the construct sequenced in the MS study.

Then the fusion of a honeybee melittin secretion signal was tested for both constructs, which might help to improve the protein expression. The secretion promotes glycosylation transfer, which appears to be important for leptin binding, and it has the potential to improve stability.

4.3 Hb-IGD-CRH2 construct

The IGD-CRH2 construct was the first to be tested using the honeybee melittin signal (Figure 15; supplementary figure 3). The medium containing the secreted protein was concentrated using centrifugal filters and examined using SDS-PAGE (Figure 16A) and after, a Streptavidin Affinity Chromatography (Figure 16B) was performed.

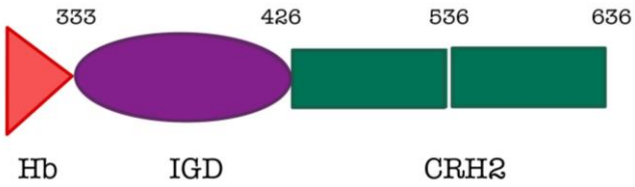


Figure 15. Hb-IGD-CRH2 scheme. A melittin secretion signal (Hb), the IGD, and the CRH2 domain constitute this construct. The boundaries used, annotated in amino acids, are the expected borders shown in Peelman et al. 2014.

The concentrated filtered medium and the pellet, for verification, were loaded on the SDS-PAGE (Figure 16A), however, the result was too smeary to be conclusive. A blurry wide band, which is positioned in the elution fraction in the analysis after affinity chromatography with StrepTactin, was observed (Figure 16B). This band has a higher molecular weight than expected. The expected size was 34.86 kDa and a band can be seen around 37 kDa.

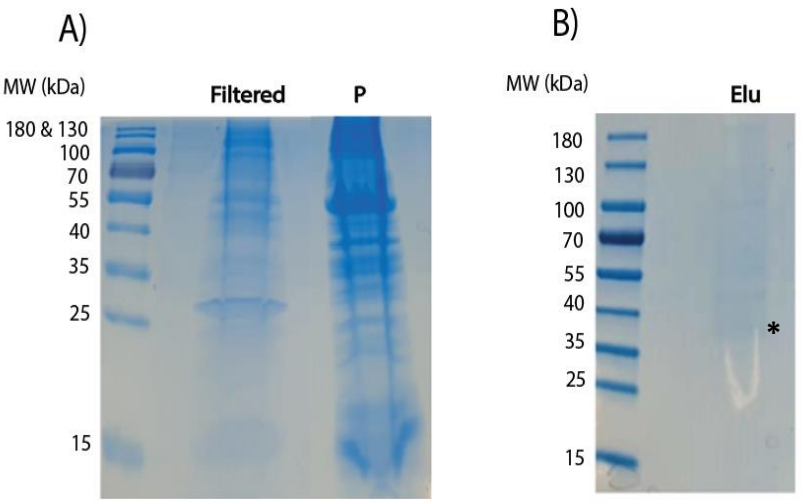


Figure 16. Analysis of the protein purification of the construct Hb-IGD-CRH2.
A) SDS-PAGE results after centrifugal concentration
B) SDS-PAGE results after centrifugal concentration and streptavidin affinity chromatography. Presumed IGD-CRH2-FN3 is marked with an asterisk.

Key: Elu, eluate fractions; P, pellet.

4.4 Hb-IGD-CRH2-FN3 construct

The final construct, the Hb-IGD-CRH3-FN3 (Figure 17A; supplementary figure 4), was designed de novo, using the same limits as the other constructs (Peelman et al. 2014). The primers were designed (Supplementary table 1) and PCR amplification of the backbone and

insert were performed (Figure 17B-C). The plasmid was finished by joining them together using a Gibson cloning procedure.

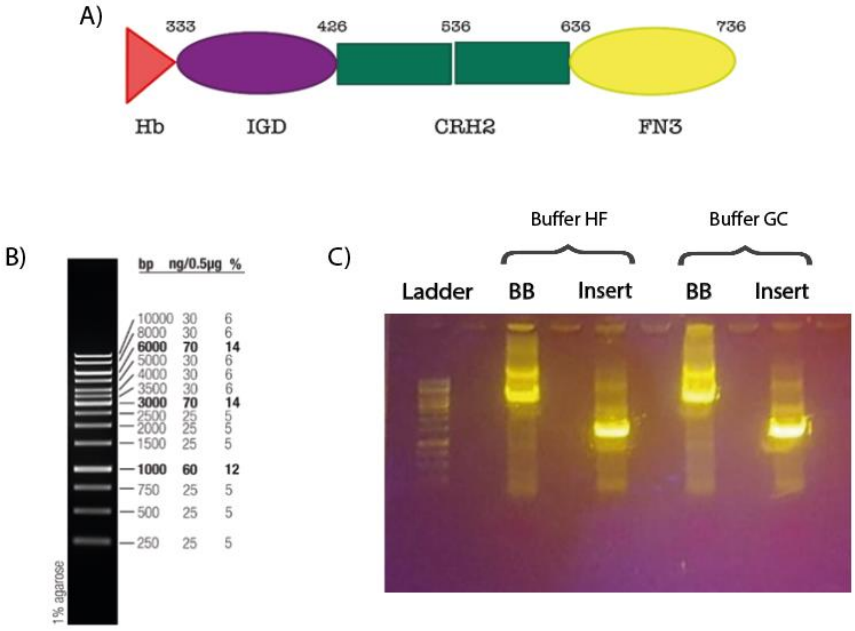


Figure 17. Hb-IGD-CRH2-FN3 scheme. A) Scheme of the construct Hb-IGD-CRH2-FN3 which is composed of a melittin secretion signal (Hb), the IGD, the CRH2 domain, and the FN3 domain. The boundaries, annotated in amino acids, corresponding to the predicted domain borders shown in Peelman et al. 2014. B) GeneRuler 1 kb DNA ladder extracted from *ThermoFisher Scientific* user guide. C) The amplification was performed independently for the backbone and the insert using buffer HF and buffer GC. Key: BB, backbone.

After expression in insect cells, the sample, consisting of secreted proteins, was purified through Strep-affinity chromatography (Figure 18A). Samples were taken from the cellular fraction (P1), the supernatant, but also the precipitation fraction (P2), which could contain our precipitated construct, before performing the chromatography. The precipitation in the medium was obtained after overnight storage at 4 ºC.

Afterward, the sample was concentrated and analyzed on size exclusion chromatography (SEC) (Figure 18B-C).

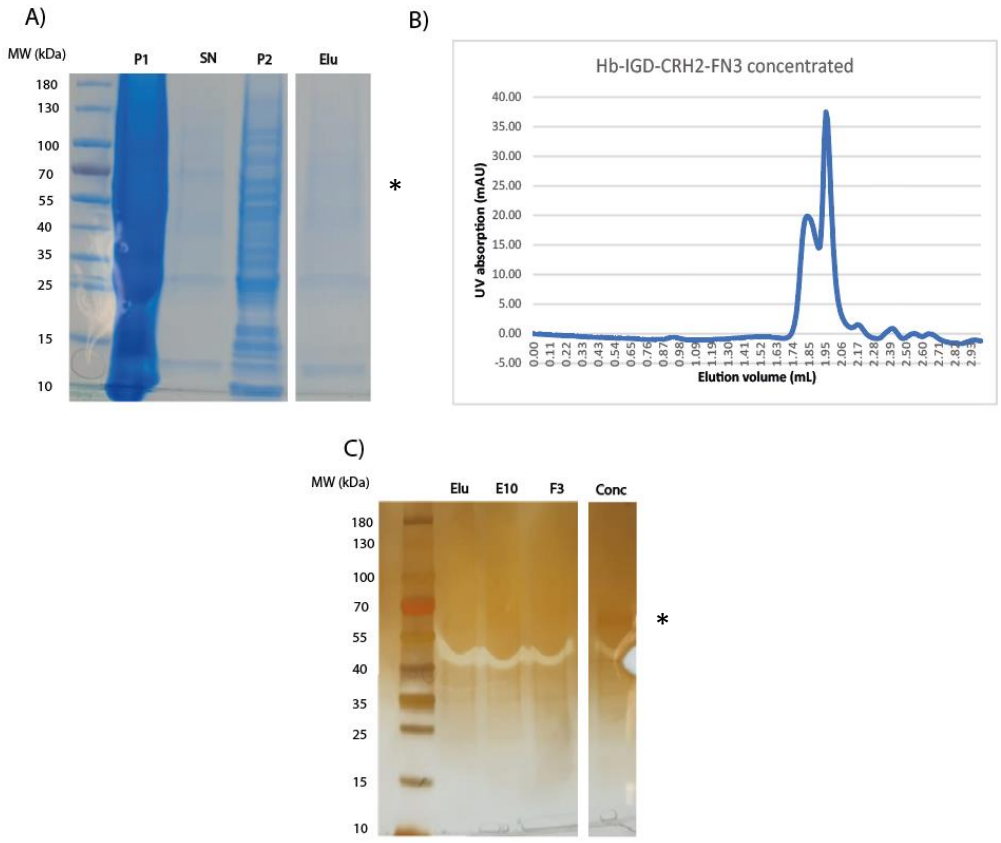


Figure 18. Analysis of the protein purification of the construct Hb-IGD-CRH2-FN3. A) SDS-PAGE results after affinity chromatography with StrepTactin B) Chromatogram obtained from the sample Hb-IGD-CRH2-FN3 concentrated with centrifugal filters. The peaks correspond to the elutions E10 and F3 respectively. C) SDS-PAGE after centrifugal concentration, size-exclusion chromatography, and stained using silver stain. Presumed Hb-IGD-CRH2-FN3 is marked with an asterisk.

Key: P1, cellular pellet; SN, supernatant; P2, precipitation pellet; Elu, eluate fractions; Conc, concentrated sample; E10 and F3, size exclusion fractions.

In the SDS-PAGE after affinity chromatography with StrepTactin (Figure 17A), two faint blurry bands, corresponding to a molecular weight of 55 kDa and 70 kDa, were observed in the elution.

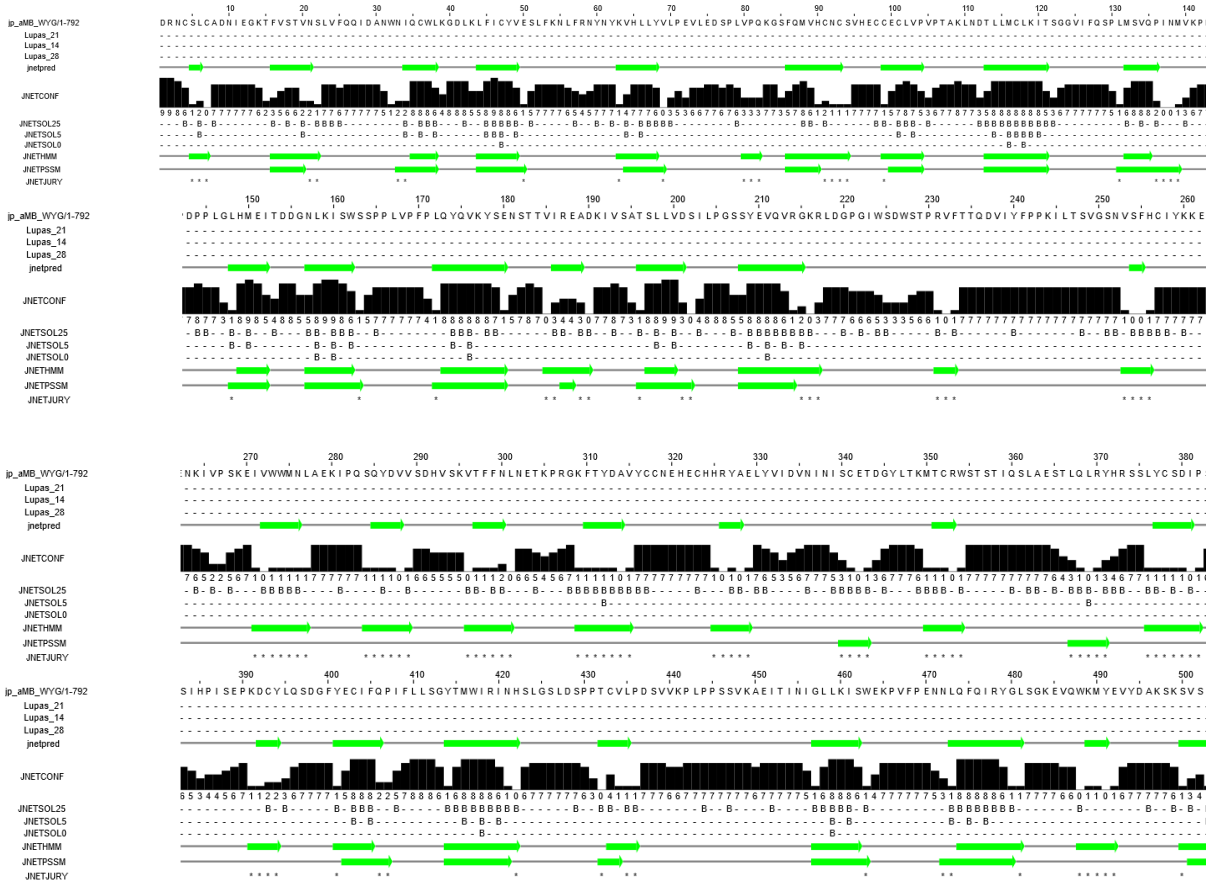
Even though there was a YFP band in the previous SDS-PAGE, the low void volume/precipitation in the chromatogram indicated that a pretty pure sample was injected (Figure 17B).

Then an SDS-PAGE from the two peaks that can be observed in the chromatogram using silver staining was performed (Figure 18C). A band, around 65 kDa can be appreciated in the concentrated sample. However, due to the high background from the staining and the less concentrated material, there is no visible band in the SEC elutions.

After the lack of information from the SDS-PAGE, the first peak, with a maximum of 20 mAU has been hypothesized to correspond to the IGD-CHR2-FN3 after comparison with other proteins, like aldolase and ferritin, showed in the column manual. The other peak, which can be detected on SDS-PAGE, is most likely YFP or another impurity.

4.5 New designs

Based on the results obtained, the primer design was revised and compared with a prediction of the secondary structure using JPred (Figure 18), and the predicted tertiary structure using Swiss Model (Figure 19). Both predictions were made using the UniProt sequence for the leptin receptor (P48357) to be as accurate as possible.



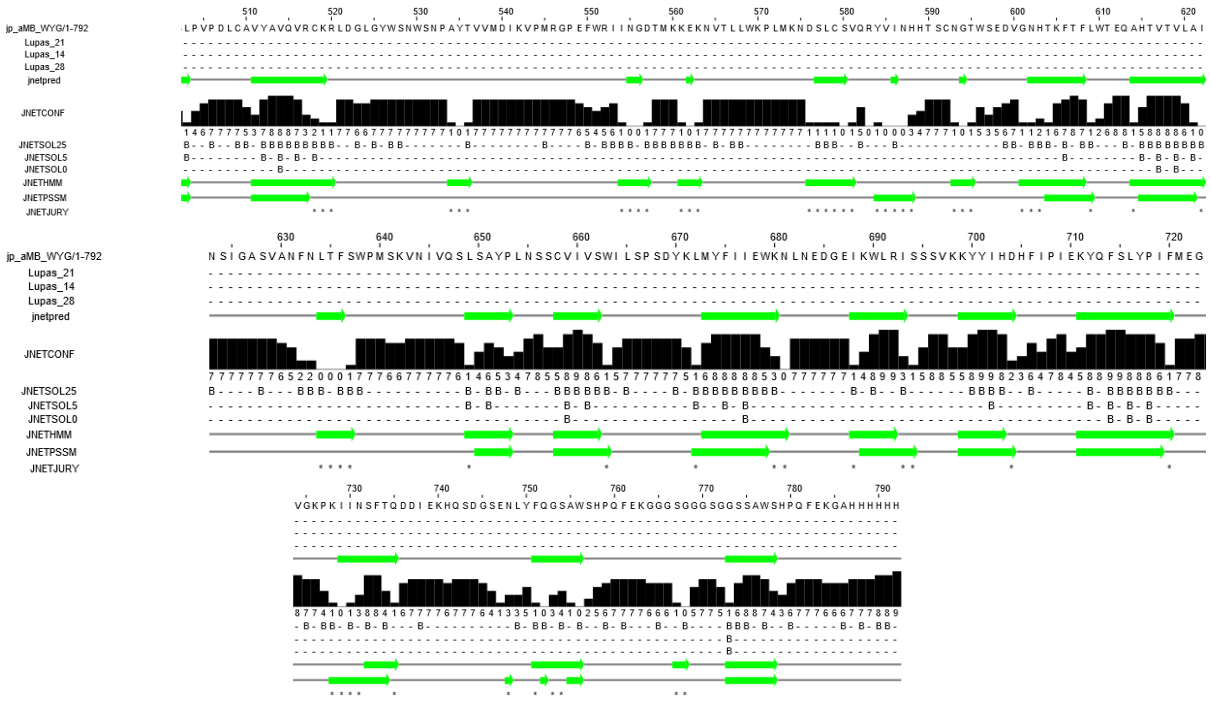


Figure 19. Secondary structure prediction of the LR-ECD obtained by using JPred

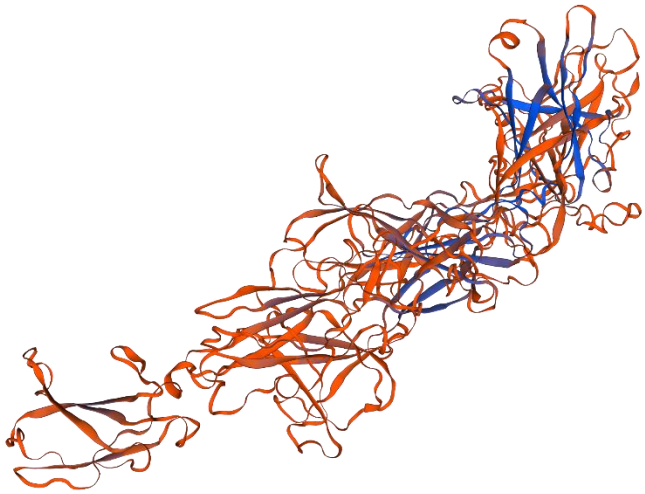


Figure 20. Tertiary structure prediction of the LR-ECD obtained by using Swiss-Prot

For the tertiary structure, a fragment of the human fibronectin (1FNF), the CRH2 leptin receptor domain (3V60), and the human interferon-alpha/beta receptor 1 (IFNAR1) were used as models. These fragments were chosen due to the homology with the leptin receptor (Mancour et al. 2012; Peelman et al. 2014) and the sequence identity.

Once the prediction was made, new primers were redesigned for all the constructs (Supplementary Table 2) to avoid possible beta-sheets and to preserve the original protein conformation instead of using the domain boundaries.

5. DISCUSSION

Small-scale expression systems, using Sf9 insect cells, were used to express truncated LR recombinant proteins including just the minimal required domains for the correct leptin biding.

Concretely, a small expression of the IGD-CRH2-FN3 construct (Figure 13) was achieved, corroborated by the presence of the leptin receptor in the MS analysis (Figure 14). However, this bands shows a smaller molecular mass, around 37 kDa, than expected. This difference is expected to be due to unspecific protein cleavage despite the addition of protease inhibitors.

In the gel regarding the secreted version of this construct (Figure 18C) a blurry band with a higher molecular weight than expected, 65 kDa, can be observed. This increase in the molecular weight was expected, which will be attributable to the different glycosylation patterns. However, this hypothesis was not tested during this project and it must be confirmed in future analysis. This can be tested by performing staining procedures, mainly based on gel stains specific for glycoproteins or affinity-based procedures like the use of antibodies.

However, in the IGD-CHR2 construct (Figure 11) and its secreted version (Figure 16) a clear expression can not be seen, this can be due to the lack of the intramolecular disulfide bridge that is formed in the FN3 domain. This will cause a decrease in the protein stability and therefore can increase its degradation.

Overall, and supported by a previous publication (Carpenter et al. 2012) and previous research performed by my internship supervisor, Ph.D. candidate Anna Höfler, it can be assessed that there is a decrease in the protein expression after depletion of any subdomain of the leptin receptor extracellular domain. It is hypothetically due to an increased degradation by the cell machinery or a decrease in the solubility due to a decrease in the protein stability or changes in the tridimensional structure.

In the analysis with His-tag purification and anti-His as primary antibody (Figures 13C-D), there is no specificity binding and it was not used for purification in the secreted constructs. It is hypothetically due to the lack of a sixth histidine due to a point mutation. This hypothesis is supported by the fact that MS showed that the protein did not bind to the Ni-beads as it was only detected in the flow-through (Figure 14). So, even though the protein expression was achieved, the purification protocol has still to be established to get a high-resolution structure by using crystallography.

6. CONCLUSION

Due to the increase in leptin-related diseases, such as metabolic syndrome and obesity, the leptin receptor holds great importance; however, there is no high-resolution structural data available to date which would be helpful for the research of new therapeutic approaches and drug development.

A eukaryotic cell expression method was employed in this research to create shortened LR recombinant proteins with only the minimum domain necessary for leptin binding. This will allow crystallography to be performed and a high-resolution structure to be obtained.

All the domains, including the ones that are not strictly necessary for leptin signaling, seem to have a specific role in protein stability and/or solubilization which appears to make it difficult to obtain high yields of expression of these constructs. This can mean that the recombinant proteins are not stable in a cell-free context.

One proposed methodology is the use of non-neutralizing antibodies which showed good results previously in this protein (Carpenter et al. 2012). However, the protein is denaturalized during this process, which can lead to an artificial structure.

Also, even though glycosylations seem to be essential and increase the stability of the leptin receptor, glycosylations might impair crystallization, therefore a step of deglycosylation might need to be performed.

The construct IGD-CHR2-FN3 and its secreted version were successfully expressed and purified and, although the quantity of protein was not enough for crystallography, hypothetically it could be achieved with a bigger scale expression, in the order of liters, but also with the optimization of the protein purification protocol.

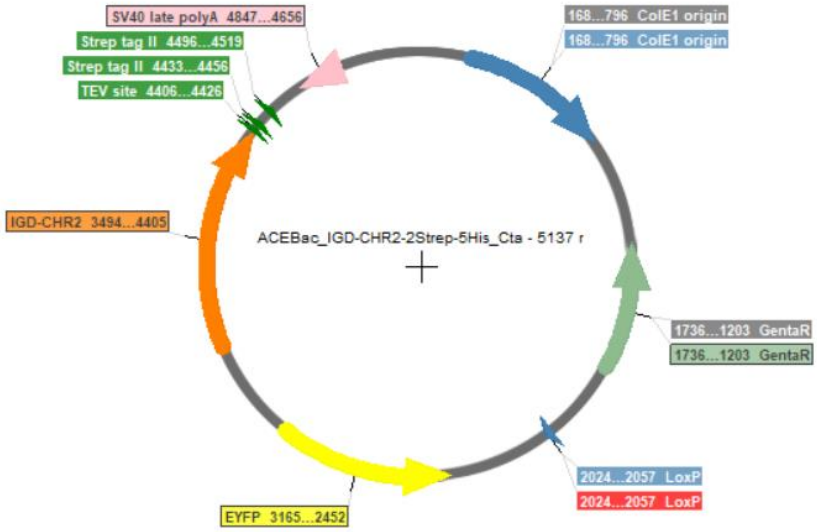
7. ACKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to Dr. Andreas Boland for giving me the opportunity to do an internship in his laboratory in the molecular biology department. I would also like to thank all the members of the group, Ph.D. candidate Lina Poulain, Dr. Jun Yu, Dr. Pierre Raia, and Mr. Yvan Pfister with whom I have been working on a daily basis and who made my stay a very pleasant one.

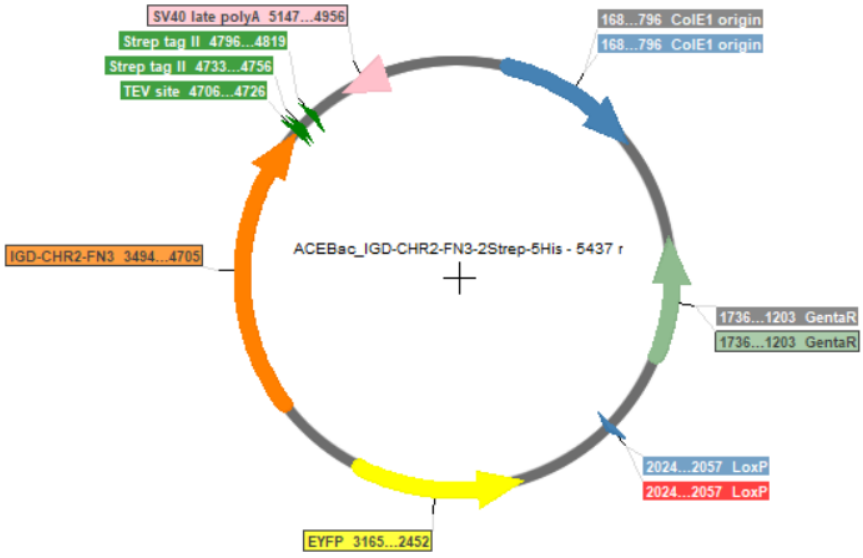
I would also like to express my deep gratitude to Ph.D. candidate Anna Höfler for guiding me all the way through the course of this internship. Her support, help, and dedication have been indispensable for the realization of this thesis.

Finally, I would like to thank Dr. Anna Rull for her help and advice during the writing of this thesis, as well as her corrections and suggestions.

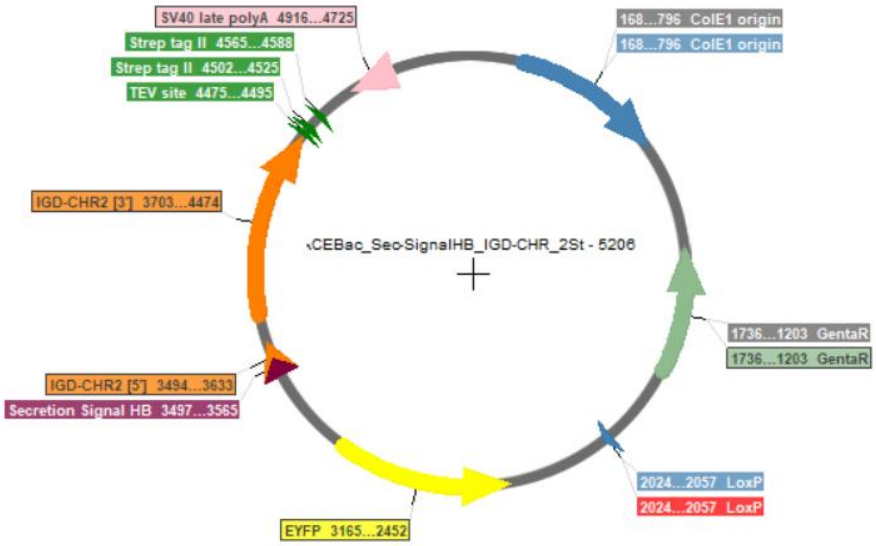
8. SUPPLEMENTARY MATERIAL



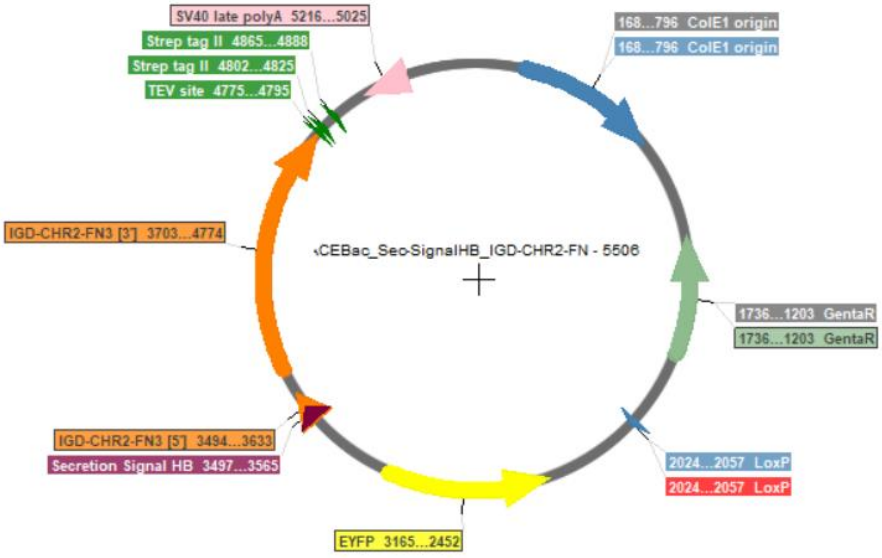
Supplementary figure 1. Plasmid map from the insert encoding the recombinant protein IGD-CRH2 in pACEBac1 transfer vector



Supplementary figure 2. Plasmid map from the insert encoding the recombinant protein IGD-CRH2-FN3 in pACEBac1 transfer vector



Supplementary figure 3. Plasmid map from the insert encoding the recombinant protein Hb-IGD-CRH2 in pACEBac1 transfer vector



Supplementary figure 4. Plasmid map from the insert encoding the recombinant protein Hb-IGD-CRH2-FN3 in pACEBac1 transfer vector

Supplementary Table 1. The sequence of primers designed to express the construct Hb-IGD-CRH2-FN3

Primers		Sequence (5'-3')	Annealing temperature (ºC)	Length (nt)
Hb-IGD-CRH2-FN3 Primer 1	Forward	CTT ACA TCT ATG CGG ATG GAA GCG TGA TCT ACT TCC CAC CTA AG	80	44
Hb-IGD-CRH2-FN3 Primer 2	Reverse	GAT TCG AAA GTC CGA TTC GAT	60.1	22
pBac1 Hb included Primer 1	Forward	GAT CGA ATC GGA CTT TCG AAT	60.1	22
pBac1 Hb included Primer 2	Reverse	GCT TCC ATC CGC ATA GAT GTA AG	62.9	23

Supplementary Table 2. The sequence of primers designed to express the constructs with new boundaries

Primers		Sequence (5'-3')	Annealing temperature (ºC)	Length (nt)
IGD-CRH2_V2 Primer 1	Forward	GTC CCA CCA TCG GGC GCG GAT GGG CCC TGG AAT TTG G	84.6	37
IGD-CRH2_V2 Primer 2	Reverse	ACC TTG GAA GTA GAG GTT CTC AGG GCC TCT CAT GGG CAC CTT	81.9	42
Hb-IGD-CRH2_V2 Primer 1	Forward	CTT ACA TCT ATG CGG ATG GAA GCA TGG GCC CTG GAA TT GG	79.8	41
IGD-CRH2-FN3_V2 Primer 1	Reverse	ACC TTG GAA GTA GAG GTT CTC GCT GGG GCT CAG AAT CCA	80.0	39

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