UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Use of Biotinylated BLRP-Tagged Proteins for Genome-Wide Location Analysis

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

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Biology

by

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The Thesis of Cindy Ngo is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Dedication

I would like to dedicate this thesis to my beloveds:

To my mom, my role model and my endless supporter.

To my grandfather for teaching me the values in life.

To my Aunt Tina, Uncle Dai, and brother John for always believing in me.

To my best friends Lisa and Jasmine for taking care of me during my defense quarter.

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List of Abbreviations

ACTH	adrenocorticotropic hormone
Ad4BP	adrenal 4 binding protein
BLRP	biotin ligase recognition peptide
CV-1	monkey kidney cell line
Egr1	early growth response protein 1
Eh1	engrailed homology-1
FSH	follicle stimulating hormone
GH	growth hormone
GnRH	gonadotropin-releasing hormone
GnRH-R	gonadotropin-releasing hormone receptor
GSE	gonadotrope-specific element
GT1-7	hypothalmic GnRH mouse cell line
HPG	hypothalamic-pituitary-gonadal
IHH	idiopathic hypogonadotropic hypogonadism
LH	luteinizing hormone
LβT2	gonadotrope cell line
NF-Y	nuclear factor-Y
PCOS	polycystic ovarian syndrome
Ptx1	pituitary homeobox 1
SF-1	steroidogenic factor 1
Six6	sine oculis homeobox homolog 6

SP1	specificity protein 1
TSH	thyroid-stimulating hormone
VMH	ventral medial hypothalamus
αGSU	α-subunit of glycoprotein hormones

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ABSTRACT OF THE THESIS

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Protein interaction studies using chromatin immunoprecipitation (ChIP) to map transcription factor targets genome-wide has led to an increased understanding of the regulation and function of these transcription factors. However, the current methods are hindered by inefficient purification systems due to the lack of specific antibodies. In this thesis, we developed the BLRP-biotinylation system to mark two specific transcription factors that are critical for the transcriptional regulation of the hypothalamic-pituitary-gonadal (HPG) axis, SF-1 and Six6, to allow for ChIP or ChIP-seq analysis. We

expressed the bacterial BirA biotin ligase in hypothalamic and pituitary cells, which allows the efficient biotinylation of our tagged proteins that contain a small biotin ligase recognition peptide (BLRP) tag. We showed that these BLRP-tagged proteins are expressed and retain functions such as DNA-binding and transcriptional regulation. Taking advantage of the strong noncovalent interaction between biotin and streptavidin, we showed that the biotinylated tagged proteins were efficiently pulled-down with this single step purification method *in vitro*. Lastly, we used the pulled-down fraction for ChIP analysis to study the binding of SF-1 and Six6 on gene targets in gonadotrope LβT2 and hypothalamic GT1-7 cell lines. Therefore, the BLRP-biotinylation system provides a novel method to specifically label and purify proteins, and ultimately allows for the identification of complex protein-DNA and protein-protein interactions *in vitro*.

I

Introduction

Biotinylation system

Several methodologies exist for the study of protein-protein and protein-DNA interaction. Some techniques include yeast two-hybrid strategies and affinity-based purification assays. Examples of problems inherent in some existing tag systems include the limitation of specific antibodies, low affinity, low recovery, too many purification steps, and high background. Some affinity tags may also be too long resulting in modification of the structure of the protein and thus changes in its activity. An important assay for our work is chromatin immunoprecipitation (ChIP). ChIP is a tool that is used to identify the interactions between DNA and proteins in cells. Most importantly, it allows for the mapping of these protein-DNA interactions in the genome. However, an impediment is that the antibodies and purification systems available have low affinity and specificity for detection of a particular transcription factor of interest. Thus, several other *in vivo* bio-tagging systems have been gaining popularity because they circumvent these problems [1, 2].

These biotinylation systems allow the study of protein-DNA, protein-protein interactions by the use of post-translational modification to label these proteins of interest. Of most interest is the BLRP-biotinylation system. The major components of system include: a short biotin ligase recognition peptide (BLRP), *Escherichia Coli* biotin ligase (Bir A), Biotin, and a cleavage site (TEV). The BLRP tag, also known as an Avi-tag, is a 20-aminoacid peptide sequence that is recognized and biotinylated by biotin ligase [3, 4]. The biotin that is ligated to the BLRP tag is a naturally occurring molecule that covalently binds to streptavidin and avidin with a high affinity [5]. The TEV cleavage site is required for the release of the tagged-protein from the streptavidin matrix

for further protein-DNA or protein-protein studies. In short, highly specific biotinylation and quantification can be obtained through the co-expression of BirA and the tagged protein in cells [1]. DNA that binds to the tagged transcription factor can be pulled down using ChIP and sequenced for genome-wide location analysis.

The BLRP system has several advantages over other purification systems. Biotinylation is an attractive approach for protein complex purification due to the very high affinity of avidin/streptavidin for biotinylated templates. The dissociation constant (K_d) value of the interaction is 10^{-15} M, making biotin/avidin the strongest noncovalent interaction known in nature [6]. Furthermore, biotin and avidin form bonds that are so stable and rapid that they are unaffected by the extreme conditions required for efficient purification, such as pH, temperature, organic solvents and other denaturing agents. The system is also highly specific due to the high selectivity of biotin ligases. These enzymes catalyze the covalent attachment of biotin to the lysine chain on the epsilon nitrogen in tagged proteins of interest [7, 8]. The system also uses the fusion to a smaller peptide sequence – a 20 amino acid sequence. This purification tag has the advantage that it can be used for proteins produced in any cell type whereas other tags can be compromised by cell type. For example, polyhistidine tags work poorly in yeast due to abundant proteins that bind to the metal chelate column [7]. This system can be used for a broad range of applications such as Western blot analysis, ELISA, affinity purification, IHC, IP, ChIP, and more. In previous studies by other groups, it has been utilized for purification of multiprotein complexes [6], chromatin immunoprecipitation [9], and microscopic localization [10].

Aside from *in vitro* assays, the system will also allow for BirA-mediated specific biotinylation in transgenic animal experiments *in vivo* [1]. These systems have been used by several groups to express proteins that are biotinylated *in vivo* in a wide range of biological systems, such as mammalian cells [4, 6, 9, 11, 12]. The biological model organism our lab is most interested in is the mouse model. Humans and mice exhibit similar physiology and anatomy and share approximately 99% of their genes [13, 14]. Furthermore, mice are small, have short breeding cycles, and are easy to genetically manipulate to model various existing human diseases [14]. Due to such similarity and ease of genetic manipulation, the findings found in transgenic mouse models could be used to apply to the human model and ultimately provide potential therapeutic interventions of human diseases [15].

This thesis presents the development of the BLRP-biotinylation system to mark two specific transcription factors to allow for ChIP or ChIP-seq analysis for the binding locations in the chromatin of an individual cell type within a complex tissue. It can be used for proteomics for complexes formed by specific transcription factors. In this thesis, we focus on two transcription factors, SF-1 and Six6, which are critical for the transcriptional regulation of the hypothalamic-pituitary-gonadal (HPG) axis.

Hypothalamic-Pituitary-Gonadal (HPG) Axis

The diseases of the human reproductive system are widespread and common. The reproductive system disorders include, but are not limited to, abnormal functioning of glands related to the secretion of sex hormones, genital abnormalities, and infertility.

About 10% of all men and women suffer from infertility [16]. It can be attributed to the insufficient secretion of sex hormones or the impaired development of the internal or external genitalia. An example of a congenital abnormality syndrome includes Kallmann syndrome, a specific form of idiopathic hypogonadotropic hypogonadism (IHH) commonly diagnosed in males [17]. It is a genetic disorder that is characterized by hypogonadism and causes males to fail to complete puberty [18, 19]. An example of a disorder in women would be polycystic ovarian syndrome (PCOS), characterized by hyperandrogenism, insulin resistance, neuroendocrine abnormalities, and chronic amenorrhea [20, 21]. Since the maturation of genitals and puberty are mediated by sex hormones, it is important to look at the hypothalamic-pituitary-gonadal axis (HPG).

In the HPG system, the hypothalamus, pituitary, and gonads work together to mediate the regulation and development of reproductive function in mammals. Specifically, the hypothalamus produces gonadotropin-releasing hormone (GnRH). GnRH neurons extend axons to the median eminence, a part of the hypothalamus that releases GnRH in a pulsatile fashion into the pituitary portal system. GnRH then stimulates the anterior pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which ultimately act on the gonads for further reproductive processes [22]. In females, LH and FSH act on the ovaries to produce steroids, such as estrogen, to trigger processes such as ovulation. In males, LH and FSH act on Sertoli and Leydig cells to stimulate spermatogenesis and the production of testosterone, respectively [23]. Changes in the pulsatile pattern of GnRH release causes abnormal FSH and LH secretion [24]. Thus, GnRH plays a vital role in the reproductive system and is therefore regulated by several positive and negative feedback loops in the

HPG axis. These feedback loops play a role in various cycles by inhibiting or stimulating the release of GnRH into the bloodstream. For example, in the regulation of ovulation and menstrual cycles, estrogen can either inhibit or stimulate GnRH to induce an LH surge that is required for the release of an oocyte in the fallopian tube. On the other hand, circadian rhythms have been reported to regulate the pulsatility of GnRH through a collaboration of several genes such as *Per, Bmal1* and *Clock* [25].

There are five different endocrine cell types that develop in the anterior pituitary. Each cell type produces different hormones: corticotropes produce adrenocorticotropic hormone (ACTH); thyrotropes produce thyroid stimulating hormone (TSH); gonadotropes produce FSH and LH; somatotropes produce growth hormone (GH); and lactotropes produce prolactin [26]. Of most interest are the gonadotropes and their associated genes LH and FSH, which are important regulators of reproduction in mammals. LH and FSH expression in gonadotropes and TSH expression in thyrotropes require the presence of the glycoprotein hormone α subunit. For the expression of the different hormones, the α-subunit gene forms a dimer with a specific β-subunit gene that allows for the differential expression of LH, FSH, or TSH [27]. Mutations in the genes involved in the HPG axis could cause a defect in reproduction. Mutations can occur in the genes coding for GnRH, LH, FSH, and their receptors or interacting transcription factors.

SF-1

A transcription factor that plays a key role in the HPG axis is an orphan nuclear receptor steroidogenic factor 1 (SF-1), also known as Adrenal 4 Binding Protein

(Ad4BP). It is predominantly expressed in the gonadal steroidogenic tissues, adrenal cortex, anterior pituitary and the ventral medial hypothalamus (VMH). It has been shown to regulate genes that are important for steroidogenesis and has also been proposed to play several roles in mammalian ovarian development [28]. SF-1 knockout mice develop an abnormal VMH and lack the expression of gonadotrope-specific markers such as LH, FSH, αGSU, and GnRH-R [29, 30]. Due to the lack of expression of these gonadotropins, target organs are also affected. These mice have testes and ovaries are that are hypoplastic, showing signs of hypogonadism and sterility [31]. Furthermore, male knockout mice are unable to produce testicular androgens and thus have female internal and external genitalia regardless of genetic sex determination [32].

Transfection promoter analyses have shown that SF-1 has a role in regulating the expression of several gonadotrope markers: glycoprotein hormone α subunit (αGSU), LHβ, FSHβ, and GnRH receptor (GnRH-R) promoters. In all sites, SF-1 works with other factors that are required for full basal activity. SF-1 has also been known to interact with other transcription factors such as Ptx1, Egr1, GATA-5, and SP1 [33-36]. The two interacting partners of interest for LHβ gene regulation are Ptx1 and Egr1. Ptx1 is a bicoid-related homeobox transcription factor and is important in the development of somatotropes, lactotropes, and thyrotropes [37]. Egr1 is an early growth response protein 1 that regulates GnRH [38]. Synergistic effects of these transcription factors with SF-1 have been observed in gonadotropes.

Several SF-1 binding elements have been identified. SF-1 has been shown to bind to αGSU at the gonadotrope-specific element (GSE) located at -223 to -197 in the human

gene, a sequence that is conserved in mammalian α-subunit genes [39, 40]. Furthermore, the expression of FSH and LH have been known to co-localize with SF-1 expression in the pituitary [29]. In the LHβ promoter, SF-1 binding sites are located at -137 and -59 bp relative to the transcriptional start site [41, 42] and SF-1 has been found to interact with NF-Y, Egr1, and Ptx1 [34]. In LβT2 cells, Egr1, SF-1, and Ptx1 have shown to have synergistic effects on the activation of the LHβ promoter [43]. In the FSHβ promoter, two SF-1 binding sites were found at -341 bp and -239 bp relative to the transcriptional start site along with a nuclear factor-y (NF-Y) and Ptx1 site [44]. By mutating SF-1 and NFY elements, it was found that NF-Y and SF-1 physically interact with one another to activate FSH expression. In GnRH-R, SF-1 has a binding site on the proximal promoter at -234/-236 [40, 45]. Although several SF-1 binding sites have been identified, there are potential sites for SF-1 that have yet to be discovered due to the limitation of specific commercial antibodies and techniques to pull down SF-1 and its interacting partners. Thus, in this thesis, the BLRP-biotinylation system will be used to confirm SF-1 binding on the above promoters in LβT2 cell lines. We ultimately seek to develop the whole BLRP-biotinylation system for future studies of the interaction of SF-1 with other transcription factors.

SF-1 plays a vital role in the development of the reproductive system. Once the molecular mechanisms of SF-1 are known, there could be a treatment for disorders such as hypogonadotropic hypogonadism. Since the mouse gene encoding SF-1 shares strong homology with its human counterpart, the mouse model will provide information that would help in treating disorders such as hypogonadism, adrenal deficiency, or sex reversal in human patients [46, 47].

Six6

Studies have shown that defects in GnRH neurons lead to a delay or loss of puberty, ovulation, and fertility [22]. Thus, much research has looked at the site of GnRH action, specifically the sites on GnRH that transcription factors can bind to induce or repress GnRH expression. Previous studies have shown that the GnRH regulatory sequence has four conserved homeodomain binding sites (ATTA) that are required for the induction of GnRH and basal promoter activity: enhancer sites -1635 bp and -1622 bp and promoter sites -53 bp and -41 bp [48]. There are dozens of known transcription factors that activate or repress GnRH induction by interacting with these ATTA sites.

An activator of GnRH is Six6, a homeobox-gene that is expressed at the neurula stage in the anterior neural plate and is continues to be expressed in later development in the developing eyes, hypothalamus, pituitary gland, and olfactory placodes [49, 50]. Six6-null mice are characterized by a reduced number of GnRH neurons and reduced fertility in both males and females [51]. This suggests that Six6 plays an important role in the induction of GnRH and ultimately the functionality of the HPG axis in reproduction. Since Six6 directly binds to GnRH to regulate its expression, studies have identified four Six6-binding locations at enhancer sites -1635 bp and -1622 bp and promoter sites -53 bp and -41 bp. Although Six6 can bind to all four ATTA sites on GnRH, only the two ATTA elements located at -41 and -53 bp on the GnRH proximal promoter seem to have an effect on GnRH transcription [51]. When these two promoter sites were deleted in a transient transfection and luciferase assay, there was a loss of GnRH induction [48]. As a

result, it is hypothesized that there are potential co-regulators of Six6 near the promoter ATTA sites that allow Six6 to regulate GnRH transcription. These Six6 binding sites have been shown to be binding sites of Msx1/2 and Dlx1/2/5, suggesting that factors may be interacting partners of Six6 [48].

In addition, Six6-null mice exhibit an irregular development of the pituitary gland, optic nerves and chiasm [52]. Anophthalmia and pituitary anomalies in human are related to Six6 haploinsufficiency [53, 54]. In gonadotropes, Six6 has also been shown to regulate GnRH-R, LHβ, and FSHβ genes. In LβT2 gonadotrope cells, Six6 acts as a repressor for the above genes. Six6 repressive activity is located at -300 to -87 in LHβ, -95 to +1 in FSHβ, and -800 to -600 in GnRH-R (unpublished). Since Six6 activity corresponds to regions with binding elements known to bind Ptx1/2 and Isl1 tissue-specific activators in LHβ and FSHβ, Six6 is hypothesized to interact with these factors to activate promoter activity in these genes. In GnRH-R, it was found that the engrailed homology domain (eh1) was required for repression (unpublished). Since the eh1 motif is known to mediate the recruitment of transcriptional corepressors of the TLE/Groucho family, it is hypothesized that Six6 interacts with these factors for the repression of gonadotrope gene expression [55, 56]. It has been shown that TLE's interact with DNA binding transcription factors to mediate repression by either multimerizing by aggregating with other factors along the DNA template or interacting with histones to modify the chromatin structure [57].

However, little is known about the molecular mechanisms of Six6 and its interacting partners in the hypothalamus and pituitary. This is partially due to the lack of good commercial antibodies that are available. Thus, this thesis seeks to set up the BLRP-

biotinylation system to confirm Six6 binding on known promoters in LβT2 and GT1-7 cell lines. In the long run, we seek to utilize this system to determine whether Six6 acts alone or in cooperation with other transcription factors to induce GnRH and gonadotrope gene expression in GT1-7 and LβT2 cells. If Six6 acts in cooperation with other factors to induce GnRH expression, other proteins, such as co-repressors or co-activators could be pulled down. To determine whether other factors are involved, Six6 was fused with a BLRP-tag, a bacterial biotin ligase recognition peptide sequence, to create a construct that would be used in chromatin immunoprecipitation (ChIP) assays.

Six6 plays a vital role in the reproductive system with its main role focused on fertility. Since Six6 plays a vital role in the induction of GnRH, LHβ, and FSHβ gene expression in the HPG axis, Six6 may play a role in many reproductive disorders in humans such as IHH, Kallman's syndrome, or bilateral anophthalmia. Once the exact mechanism of Six6 action is found, further studies can be done to target genes that are interacting with Six6 to cause reproductive problems. Thus, by identifying Six6 interacting partners, therapeutic intervention will be a possibility.

Cell Models

To study GnRH neurons, GT1-7 cells, an immortalized hypothalamic neuronal cell line was used. The GT1-7 cell line was created from hypothalamic neurons by targeting oncogenesis in transgenic mice carrying the GnRH promoter region linked to a SV40 T-antigen oncogene at the 5' flanking region [58, 59]. These GT1-7 cells showed characteristics found in neuronal cells *in vivo*. These cells display similar morphology,

have mature processes, secrete GnRH in a pulsatile manner, and establish synaptic networks with neighboring neurons [59-61]. In this thesis, the GT1-7 cell model was used to study potential binding sites of Six6 with other transcription factors that regulate the transcription of GnRH in neuronal cells.

To study mature pituitary gonadotropes, our lab has previously generated an immortalized LβT2 cell line. The LβT2 cell line was made from a pituitary tumor induced by targeted oncogenesis in transgenic mice carrying the rat LHβ region linked to a SV40 T-antigen oncogene [62]. The cell line has many characteristics of a mature gonadotrope cell. These cells express markers of a mature gonadotrope such as αGSU, GnRH-R, FSHβ, and LHβ [63]. In addition, it also expresses SF-1, progesterone, and estrogen receptors [64]. Since this cell line has many characteristics of a mature gonadotrope cell, it has been used extensively in our studies, and others, to learn more about the cellular and molecular pathways that are required for normal gonadotrope function. In this thesis, the LβT2 model was used to study the transcriptional regulation and the potential binding sites of SF-1 and Six6 in gonadotropes.

There are many advantages to these immortalized cell lines. These models allow the study of specific cell types, hypothalamic or gonadotrope, and their expression of proteins or genes that is difficult to study *in vivo* due to the presence of many cell types in a given area. In addition, complex neuronal networks found *in vivo* are absent in these cells, which makes it easier to study *in vitro*. Most importantly, these cells can be maintained in a controlled environment with fewer confounding variables, which allow the study of the effects of each transcription factor on gene expression alone. In essence, these immortalized cell lines are powerful tools to study the molecular mechanisms of

specific transcription factors in the hypothalamus and pituitary and their effects on the HPG axis.

Our main objective is to set up a biotinylation method to identify protein-DNA or protein-protein interactions for any protein in the hypothalamus or pituitary. We will do this by confirming the presently known interactions of SF-1 and Six6 with their interacting partners with ChIP assays, utilizing a single purification step that takes advantage of the high affinity of the biotin-streptavidin complex.

II

Materials and Methods

Construction of Recombinant Expression Plasmids

To make recombinant plasmids encoding SF-1 or Six6 with a 20 amino acid peptide BLRP tag and TEV cleavage site, mouse SF-1 or Six6 coding region was amplified by PCR from SF-1 pCMV or Six6 pSG5 expression plasmids [51] using Thermo Scientific Phusion Hot Start III High Fidelity DNA polymerase with forward and reverse primers containing XhoI and PmeI restriction sites respectively (Table 1). PCR of the SF-1 DNA was performed in a Thermal cycler using the following program: 98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 55°C for 30 seconds, 72°C for 40 seconds; 72°C for 10 minutes. PCR of the mouse Six6 coding region was performed with a different annealing temperature of 62°C. The PCR products with restriction sites Pme1 and Xho1 were cloned into the TOPO vector and transformed into One Shot competent cells using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The cloned TOPO plasmids were digested with Xho1 and Pme1 endonuclease enzymes (New England BioLabs, Ipswich, MA) and the SF-1 and Six6 inserts were purified using the QIAquick Gel Extraction protocol (QIAGEN). The inserts were ligated into the BLRP plasmid using T4 DNA Ligase from (New England BioLabs, Ipswich, MA) and subcloned into MAX Efficiency DH5α competent cells (Invitrogen). The BirA expression plasmid and BLRP backbone plasmid were obtained from Dr. Nathanael Spann from the laboratory of Dr. Christopher Glass at University of California, San Diego [65].

Transient transfections and luciferase reporter assays

Mature gonadotrope LβT2 [62] and mature hypothalamic GT1-7 cells [58, 59] created by our lab and monkey kidney CV-1 cells created by Jensen and colleagues [66] were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose (DMEM; Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA) and 1% penicillin-streptomycin at 37°C under a 5% CO_2 atmosphere.

In transfections used for luciferase assays with SF-1, the plasmids used were luciferase reporter LHβ promoter (-1800 bp); pGL3 luciferase reporter with SV40 promoter; Egr1 expression vector with CMV backbone and promoter; SF-1 expression vector with CMV backbone and promoter; BLRP-SF1 expression vector with a pCAGGS backbone and CMV promoter; Ptx1 expression vector with a pCDNA3 backbone and a CMV promoter; and empty vectors. Cloning of Egr1 pCMV, SF-1 pCMV, Ptx1 pCDNA3 expression plasmids have been previously described [41, 43]. The rat Egr1 pCMV and mouse SF-1 pCMV were obtained from Dr. Jacques Drouin and Dr. Bon-chu Chung respectively [34, 67]. The -1800 LHβ luciferase reporter was also previously described [41]. For experiments in CV-1 and LβT2 cells with SF-1, the following plasmids were co-transfected: 200 ng empty pGL3 vector or LHβ (-1800) luciferase reporter plasmid, 100 ng Egr1 pCMV or empty pCMV, 100 ng SF-1 or empty pCMV, 100 ng BLRP-SF1 or empty BLRP, and 50 ng Ptx1 pCDNA3 or empty vector.

In transient transfections used for luciferase assays with Six6 in GT1-7 cells, the luciferase reporter that was used was a pGL3-ATTA-multimer that contains five copies of -48 to -55 bp of the rat GnRH promoter fused to a herpes simplex virus thymidine

kinase (TK) promoter [51]. The following plasmids were co-transfected: 400 ng empty pGL3 vector or pGL3-ATTA-multimer luciferase reporter plasmid, 200 ng pSG5-Six6 (Six6 expression plasmid with pSG5 backbone and SV40 promoter) or empty pSG5; and BLRP-Six6 (Six6 expression plasmid with CMV promoter) or empty BLRP.

In transient transfections used for luciferase assays with Six6 in LβT2 cells, the following plasmids were used: -1800 LHβ-luciferase reporter [41] and Six6-pCDNA3 expression plasmid (containing a CMV promoter). The following plasmids were co-transfected: 200 ng empty pGL3 vector or LHβ (-1800) luciferase reporter plasmid, 200 ng of Six6-pCDNA3 or empty pCDNA3; and BLRP-Six6 or empty BLRP.

For transient transfections in LβT2 and GT1-7 cell lines, cells were seeded at a density of 200,000 cells per well 24 hours prior to transfection in 10% FBS DMEM in 24 well plates at 37°C. CV-1 cells were seeded at a density of 70,000 cells per well in 24-well plates. Cells were transfected with plasmid DNA using PolyJet In Vitro DNA Transfection Reagent (SignaGen Laboratories, Rockville, MD) according to the manufacturer's protocol with a PolyJet to DNA ratio of 3:1. 100 ng of β-galactosidase expression vector was transfected as an internal control for transfection efficiency.

Approximately 18 hours after transfection, LβT2 and GT1-7 cell media was replaced with fresh DMEM with serum and antibiotics while CV-1 cell media was replaced with serum free DMEM with antibiotics (supplemented with 0.1% BSA). Around 48 hours after transfection, cells were rinsed with cold PBS and lysed with 80 μl lysis buffer (100 nM KPO_4, 0.2% Triton X-100). Veritas Microplate luminometer (Turner Biosystems, Sunnyvale, CA) was used to measure luciferase and β-galactosidase activity.

For luciferase activity, 20 μl of cell lysates in each well was injected with 100 μl of luciferase assay buffer (25 mM Tris pH 7.4, 15 mM $MgSO_4$, 65 μM luciferin, 10 mM ATP). For β-galactosidase activity, 20 μl of cell lysates in each well was assayed as instructed by the protocol for the Galacto-light Plus Kit (Applied Biosystems, Foster City, CA).

All transfection experiments for luciferase assays were done at least three times in triplicate and values are presented as the mean ± SEM. To normalize for transfection efficiency, luciferase values were divided by β-galactosidase values. Means were compared using Student's T-Test with an asterisk indicating means significantly different ($P < 0.05$) from empty vector control.

Creation of Stable Cell Lines

Before proceeding to stable transfections, a kill curve for Neomycin (G418) and Puromycin was done in LβT2 and GT1-7 cells and the drug concentrations were determined to be: 275 μg/μl G418 and 1.5 μg/μl Puromycin in LβT2 cells and 500 μg/μl G418 and 1 μg/μl Puromycin in GT1-7 cells.

Once the drug concentrations were known, LβT2 and GT1-7 cells were seeded in 10 cm tissue culture dishes at a density of 3.5×10^6 in DMEM containing fetal bovine serum and antibiotics 24 hours prior to transfection. Cells were co-transfected with the following plasmids: 4 μg BirA and 4 μg BLRP-SF1 or BLRP-Six6 using PolyJet reagent. 18 hours after transfection, the media from the dishes were replaced with fresh 10% FBS DMEM. Approximately 30 hours after transfection, LβT2 cell media was replaced with

10% FBS DMEM supplemented with 275 μg/μl G418 (Invitrogen) and 1.5 μg/μl Puromycin (Gemini Bio-Products, West Sacramento, CA), while GT1-7 cell media was replaced with 10% FBS DMEM supplemented with 500 μg/μl G418 and 1 μg/μl Puromycin. The cell media was replaced every 3-4 days to ensure antibiotic potency.

Conditioned media was used to culture the surviving resistant colonies. To make the conditioned media, old LβT2 cell culture media was collected, sterile-filtered with a 0.22 μm filter, and mixed with 10% FBS DMEM (supplemented with 1% penicillin) at a ratio of 1:4. Since LβT2 cells do not grow well without cells surrounding it, this media (containing proteins that are secreted from LβT2 cells) will help the clones grow in the absence of other cells. Conditioned media containing the antibiotics, Neomycin and Puromycin, are made fresh immediately prior to utilization.

Approximately 5 weeks after drug treatment, once individual resistant clones were big enough to transfer, standard techniques were used to transfer each colony into 48-well plates. Briefly, cloning cylinders were placed around each individual clone. Each colony was washed with PBS, trypsinized with 5% Trypsin in PBS, transferred into 48-well plates, and maintained in conditional media containing the appropriate amount of Puromycin and G418. When colonies in the wells were big enough, they were further transferred into 24-well plates followed by several serial dilutions, and ultimately to 10 cm tissue culture dishes. To freeze these cells, cells were frozen in freezing media (8% DMSO, 10% FBS DMEM) and stored in a nitrogen tank.

To confirm the stable transfection of the BirA and BLRP-SF1 plasmids, the genomic DNA was purified using *Quick-gDNA* MiniPrep (Zymo Research Corp.) and a PCR was done using primers specific for BLRP and BirA plasmids (Table 2). PCR of the

regions specific to BirA and BLRP-SF1 were performed in a Thermal cycler using the following program: 95°C for 5 minutes; 35 cycles of 95°C for 1 minute, 57°C for 1 minute, 72°C for 1 minute; 72°C for 10 minutes.

Western blot analysis

LβT2, GT1-7, and CV-1 cells were plated on 10 cm tissue culture dishes 24 hours prior to transfection with PolyJet Transfection reagent according to the manufacturer's protocol. The plates were transfected with the following plasmid concentrations: 4 μg of BirA and 4 μg of BLRP-SF1 or BLRP-Six6. Approximately 18 hours after transfection, the cell media was replaced with fresh 10% FBS DMEM.

Once the cells reached 90% confluency, they were harvested. At harvest, cell media was aspirated and the cells were rinsed with 5 mL of cold PBS. They were then lysed with 1 mL RIPA lysis buffer (20 mM Tris pH 7.4, 140 mM NaCl, 10 mM NaF, 1% Nonidet P-40, 0.05 mM EDTA, 1 mM EGTA, 1 mM PMSF, and protease inhibitors from Sigma) and spun down at 13,200 rpm for 12 minutes at 4°C. The supernatant was flash frozen in dry ice and ethanol and stored at -80°C for future experiments.

Before proceeding to pull-down assays, NanoLink Streptavidin Magnetic Beads (Solulink) were prepared. The beads were washed twice for 5 minutes in 1 mL 0.1% BSA/TE (bovine serum albumin in TE buffer pH 8), blocked for 30 minutes in 1 mL 0.5% BSA 20 μg/mL Glycogen/TE, washed twice in 1 mL 0.1% BSA/TE, and resuspended in 0.1%BSA/TE to original volume at room temperature. The beads were

left in a rotator at room temperature during incubation and wash periods. 10 μl of the magnetic beads were added to 500 μl of each sample and left on a roller at 4°C overnight.

To elute the IP samples to run the Western blot analysis, the samples were washed with several buffers in the following order: 2x with RIPA buffer, 1x with Washing buffer I (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8), 1x with Washing buffer II (20 mM Tris HCl pH 7.4, 500 mM NaCl, 1% Triton X-100, 2 mM EDTA), 1x with washing buffer III (10 mM Tris HCl pH 7.4, 250 mM LiCl, 1% Nonidet P-40, 1% Deoxycholate, 1 mM EDTA pH 8), and 1x TE buffer (pH 8). All samples were incubated in each wash buffer for 5 minutes on a rotator in a 4°C cold room. After each wash, the samples were left on a magnetic separation rack (New England BioLabs, Ipswich, MA) for 2.5 minutes and the wash buffer was aspirated and replaced with the next washing buffer. The beads were then boiled with 15 μl of 2x SDS-Lysis buffer with Biotin from Invitrogen (3 mM final) for 5 minutes at 100°C. They were washed 1x with 10 μl of 2x SDS-Lysis buffer with Biotin (3 mM final) and pooled together. The 20 μl of the whole cell lysate was also boiled in 5x SDS-Lysis with Biotin (3 mM final) for 5 minutes.

The samples were run on a 10% SDS polyacrylamide gel (acrylamide:bis-acrylamide ratio of 29:1) and electroblotted for 60 minutes at 400 mA onto a nitrocellulose membrane (Millipore, Billerica, MA) in transfer buffer (1x Tris-glycine-sodium dodecyl sulfate 20% methanol). After the transfer, the membranes were blocked overnight at 4°C in blocking buffer (5% BSA, 0.1%Tween20, in PBS). On the second day, the membranes were incubated overnight at 4°C with rabbit anti-Avi tag antibody

(GenScript) diluted 1:5000 in blocking buffer. On the third day, the blots were washed 3 x 10 minutes in T-PBS (0.1% Tween20, 1x PBS) and incubated at room temperature for 1 hour in a horseradish peroxidase-conjugated goat anti-rabbit antibody (sc-2004; Santa Cruz Biotechnology) diluted 1:10000 in blocking buffer. GAPDH-horseradish peroxidase (ab9842; Abcam) diluted at 1:5000 was used as a loading control. The blots were then washed 3 x 10 minutes in T-PBS. The bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). BenchMark Pre-Stained Protein Ladder (Invitrogen, Carlsbad, CA) was used as a size marker.

Chromatin Immunoprecipitation (ChIP)

LβT2 or GT1-7 cells were plated on 10 cm tissue culture dishes 24 hours prior to transfection. The plates were transfected with PolyJet Transfection reagent with the following plasmid concentrations: 4 μg of BirA and 4 μg of BLRP-SF1 or BLRP-Six6. Approximately 18 hours after transfection, the cell media was replaced with fresh 10% FBS DMEM. Once the cells reached 90% confluency, they were harvested. All samples were done in duplicates for the comparison of TEV cleavage and no TEV cleavage.

At harvest, cells were cross-linked with 11% formaldehyde solution (11% formaldehyde, 0.1 M NaCl, 1 mM Na-EDTA pH8, 0.5 mM Na-EGTA, 50 mM Hepes pH 8) for 10 minutes on a gentle shaker at room temperature. To stop the crosslink reaction, 1.25 M Glycine was added to each plate and incubated at room temperature on a shaker for 5 minutes. Each plate was then washed with 5 mL ice-cold PBS, aspirated, and lysed with 750 μl of hypotonic solution (20 mM Tris pH 7.4, 10 mM NaCl, 1 mM $MgCl_2$, 10

mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, and protease inhibitors from Sigma). The solution was spun down at 5000 rpm for 5 minutes at 4°C and the supernatant was removed. The pellet was flash frozen in dry ice and ethanol and stored at -80°C to be used for ChIP analysis.

The nuclear fraction pellet was resuspended in sonication buffer (50 mM Hepes pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% NaDOC, 0.1% SDS) and left on ice for 10-20 minutes. The nuclear extracts were sonicated to about 300-500 base pairs using Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, CT) 5x for 25 seconds at a duty cycle of 10% and power output of 1.0. To check the size of the sonicated fragments, 10 μl of the supernatant was run on an agarose gel. All samples were kept on ice during the whole process. After sonication, 10% of the supernatant was removed for input and ChIP dilution buffer (20 mM Tris/HCl pH 7.4, 100 mM NaCl, 2 mM EDTA pH 8, 0.5% Triton X-100) was added to all samples. Each sample was precipitated with 15 μl of Solulink Nanolink Streptavidin Microsphere Beads (Solulink Inc., San Diego, CA) and left on a rotator at 4°C overnight. The magnetic beads were prepared in the same manner as mentioned earlier.

All samples were washed twice with each washing buffer at 4°C in the following order: Washing buffer I, Washing buffer II, Washing buffer III, and TE buffer. After the last wash, the beads from each sample were incubated in TEV buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT) from Invitrogen for 5 minutes at room temperature. The buffer was aspirated and TEV enzyme buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT, 12.4 μl of AcTEV Protease) or control TEV buffer were added to the

appropriate samples and incubated at room temperature for 1-2 hours. The eluate was saved and the beads were washed with ChIP elution buffer (50 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.1 mM PMSF, 5 mM EDTA) for 20 minutes at room temperature and pooled with the eluate. Elution buffer was added to all samples (no TEV cleavage, TEV cleavage, and 10% input) to the appropriate volume. All samples were treated with RNAse A and incubated at 37°C for 15 minutes. To reverse crosslink the samples, NaCl was added to each sample for a final concentration of 300 mM and was left to incubate at 65°C overnight.

Quantitative PCR was used to analyze the DNA from the 10% input and the immunoprecipitated DNA from the TEV cleavage using primers for specific sequences on FSHβ**,** LHβ, and αGSU promoters (Table 2). Primers specific to GnRH promoter (Table 2) were used as a negative control. Each PCR reaction was carried out in duplicate. A standard curve of the input was used to quantify the immunoprecipitated DNA samples. To normalize the values, each value was divided by the values from the negative control GnRH promoter. The fold induction was determined by dividing the normalized TEV cleavage value by its respective No TEV cleavage values. QPCR of the DNA with GnRH-promoter and FSHβ primers were performed using IQ5 real-time PCR machine (Bio-Rad Laboratories) with the following program: 95°C for 5 minutes; 40 cycles of 95°C for 45 seconds, 58.9°C for 45 seconds, 72° C for 45 seconds; 55° C for 30 seconds. QPCR of the DNA with αGSU and LHβ primers were performed with the following program: 95°C for 5 minutes; 40 cycles of 95°C for 45 seconds, 60.4°C for 45 seconds, 72° C for 45 seconds; 55° C for 30 seconds. iQ SYBR Green supermix (Bio-Rad Laboratories) was used for the qPCR reactions.

Statistical Significance

Data were expressed as means ± SEM and evaluated by Student's two-tailed t-test as indicated in figure legends. A P-value of less than 0.05 was used to determine significance.

III

Results

Verification of BLRP-tagged recombinant plasmids

To develop the BLRP-biotinylation system, we first constructed SF-1 and Six6 BLRP-tagged plasmids. That is, coding sequences of the transcription factors of interests, SF-1 and Six6, were inserted into a BLRP vector that contained a CMV promoter. Since the BLRP vector contained PmeI and XhoI restriction sites, the primers that were designed to encode SF-1 and Six6 contained PmeI and XhoI restriction sites (Table 1; Figure 1, A and B).

The original uncut BLRP vector had a length of 6113 bp. Once SF-1 coding region was ligated into the BLRP vector, the BLRP-SF1 plasmid had a total length of 7507 bp, indicated by a slower migration of the band of the uncut BLRP vector (Figure 2A, lane 1) compared to the faster migration of the band of the uncut BLRP-SF1 vector (Figure 2A, lane 2). When the BLRP-SF-1 plasmid was digested with PmeI and XhoI, the expected 1408 bp SF-1 insert and 6099 bp BLRP vector fragments were observed (Figure 2A, lane 3). Two additional restriction endonucleases, AccI and BstEII, were used to confirm the ligation of the SF-1 coding sequence. AccI had two restriction sites on the BLRP-SF1 plasmid. When AccI was used to digest the plasmid, the two expected bands with lengths 4827 bp and 2680 bp were observed along with a third band at the top with the linear size due to the incomplete digestion of the plasmid (Figure 2A, lane 4). BstEII had one restriction site. When BstEII was used, a fragment of 6113 was observed along with a fragment of the incomplete digestion of the plasmid (Figure 2A, lane 5). For further confirmation of the plasmid, the samples were sequenced using CMV primers from the CMV promoter and then blasted for confirmation of the SF-1 coding region in

the BLRP vector (data not shown). Digestion with restriction enzymes and DNA sequencing shows that SF-1 was indeed ligated into the BLRP vector.

Once Six6 coding region was ligated into the BLRP vector, the BLRP-Six6 plasmid had a total length of 6861 bp, seen by the slower migration of the uncut BLRP-Six6 plasmid (Figure 2B, lane 2). When the BLRP-Six6 plasmid was digested with PmeI and XhoI, the expected 762 bp Six6 insert and 6099 bp BLRP vector fragments were observed (Figure 2B, lane 3). When AccI was used to digest the plasmid, the two expected 4681 bp and 2180 bp bands were observed along with a third band of the incomplete digestion of the plasmid at the top (Figure 2B, lane 4). When BstEII was used, a band of 6861 was observed along with a second band of the incomplete digestion of the plasmid at the top (Figure 2B, lane 5). The second band seems to be a nicked plasmid. For further confirmation, the plasmids were sequenced with primers from the CMV promoter and blasted for Six6 coding region (data not shown). The restriction enzyme digestion and DNA sequencing show that Six6 was inserted into the BLRP vector.

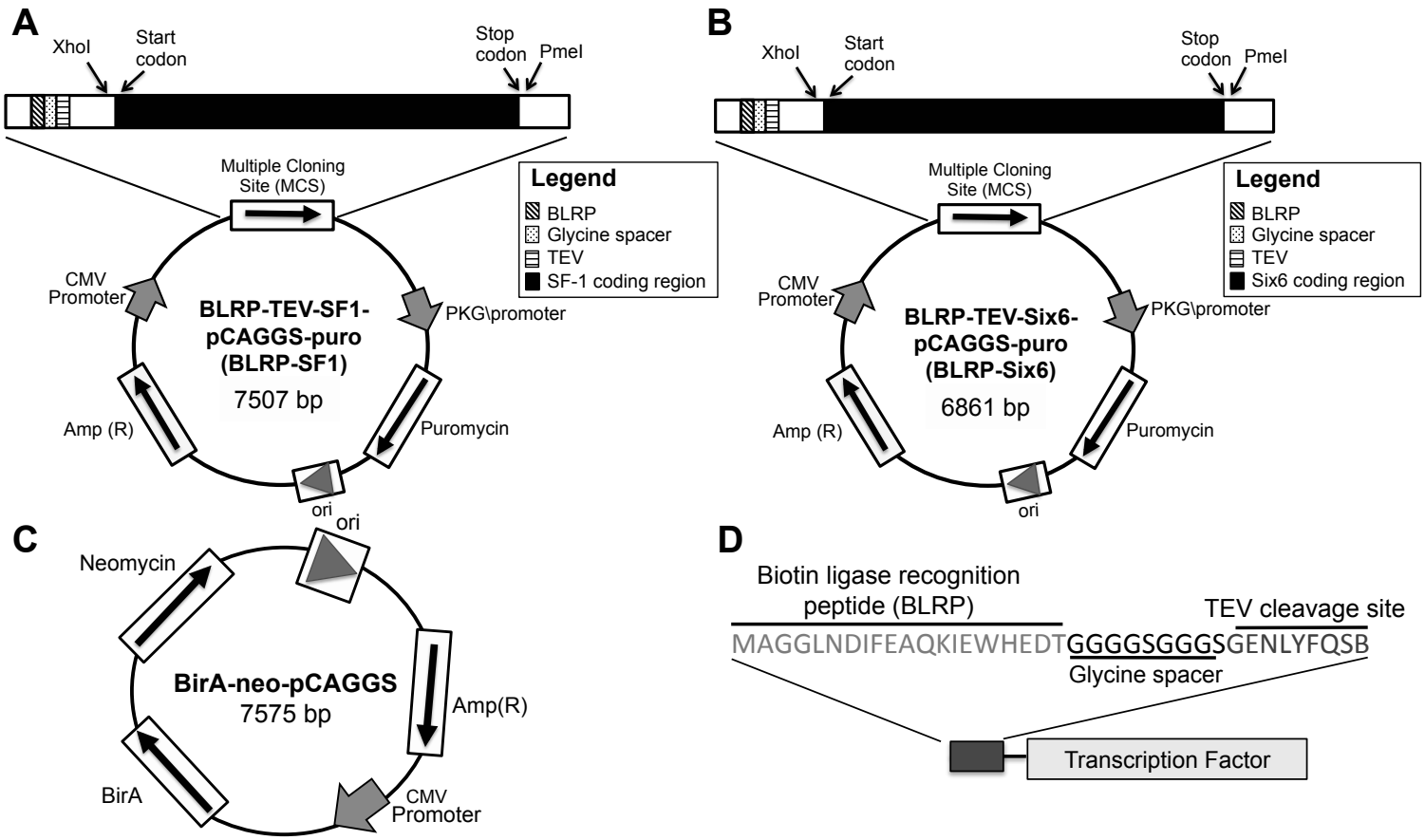

Figure 1. Recombinant BLRP-tagged constructs. SF-1 (A) and Six6 (B) coding regions were ligated into a BLRP vector with restriction sites for XhoI and PmeI. (C) The BirA plasmid was used in co-transfections with the BLRP-tagged proteins to encode enzyme biotin ligase. BLRP and BirA vectors have a resistance for Puromycin and Neomycin respectively, which can be used as a selectable marker for screening purposes. (D) Once expressed, the transcription factors will have a 20 amino-acid BLRP peptide sequence, a glycine spacer, and a TEV cleavage site. Adapted from figure by Dr. Christopher Glass.

Table 1. Primers used for the cloning of SF-1 and Six6 inserts into the BLRP vector. The forward primers included an XhoI restriction site while the reverse primers included a PmeI restriction site with both encoding the coding regions of SF-1 or Six6. Restriction sites are underlined, start codons are bolded, and stop codons are bolded and italicized.

SF-1	Forward (XhoI primer): GTCTCGAG**ATG**GACTATTCGTACGACG Reverse (PmeI primer): GTGTTTAAACC***TCA***AGTCTGCTTGGC
Six6	Forward (XhoI primer): CTCTCGAGTCG**ATG**TTCCAGCTGCC Reverse (PmeI primer): CTGTTTAAAC***TCA***GATG TCGCACTCACTGT

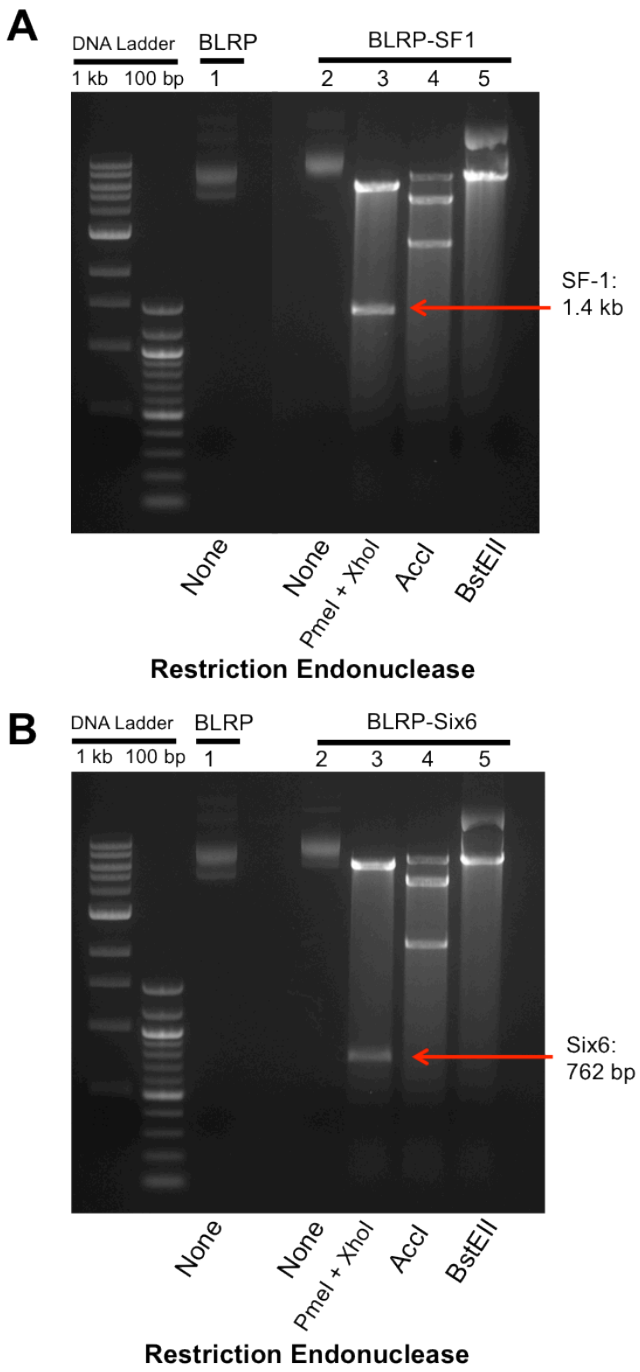

Figure 2. Inserts are properly ligated into the BLRP vector. To check for the ligation of SF1 (A) and Six6 (B) inserts into the BLRP vector, PmeI, XhoI, AccI, and BstEII restriction enzymes were used to check for the correct DNA fragments. Lane 1 is the BLRP vector without the insert. Lane 2 is the BLRP-SF1 (A) or BLRP-Six6 recombinant plasmid (B). Lanes 3-4 contains the indicated restriction enzymes used to cut the BLRP recombinant plasmids.

Recombinant plasmids are expressed in LβT2 cell lines

Having confirmed the ligation of the coding regions into the BLRP vector, we next determined whether the BLRP-tagged plasmids are expressed in their respective cell lines using pull-down assays and Western blot analysis. The bands were detected with an anti-Avitag antibody. An Avi-tag is a biotin-acceptor peptide that acts as a substrate for the attachment of biotin using the BirA biotin ligase enzyme. As mentioned earlier, the transcription factor, when expressed, should contain a BLRP tag (a variant of an Avi-tag) that gets biotinylated by BirA biotin ligase enzyme encoded by the BirA plasmid (Figure 1D).

Since streptavidin beads bind to biotin, the biotinylated BLRP-SF1 proteins will be covalently bound to the magnetic beads at a high affinity. BirA and the BLRP-SF-1 plasmids were transiently co-transfected in monkey kidney CV-1 cells and gonadotrope LβT2 cells (Figure 3). SF-1 has a molecular weight of 53 kDa. However, since we created a recombinant protein with a BLRP tag, and due to the biotinylation that occurs in cells, the expected molecular weight of the biotinylated BLRP-SF1 proteins will be approximately 59 kDa. Thus, only cells that were co-transfected with the BLRP-SF1 and BirA plasmids should have the BLRP-SF1 protein that will be able to be pulled down with the beads. The results were as expected, indicated by the 59 kDa sized-bands in the Western blots using cell lysates from CV-1 and LβT2 cells (Figure 3, A and B, lane 8). CV-1 cells served as a positive control because these cells do not express endogenous SF-1. The results prove that BLRP-SF1 is indeed expressed and can be pulled down with streptavidin beads for further experiments in LβT2 cells. Due to the low transfection

efficiency in LβT2 cells, the amount of protein that was pulled down was not as much as the amount in CV-1 cells (Figure 3, A and B, lane 8) as seen from the thickness of the bands.

A similar experiment was done using BLRP-Six6 in LβT2 cells. Wild-type Six6 has a molecular weight of 28 kDa. However, with the addition of the BLRP tag and biotin, the molecular weight should be around 34 kDa. When LβT2 cells were co-transfected with BLRP-Six6 and BirA plasmids, BLRP-Six6 was able to be enriched using streptavidin beads (Figure 4, lane 8). Thus, the data show that the BLRP-Six6 plasmid is able to express the protein in LβT2 cells. A similar experiment with BLRP-Six6 in GT1-7 cells is currently being done.

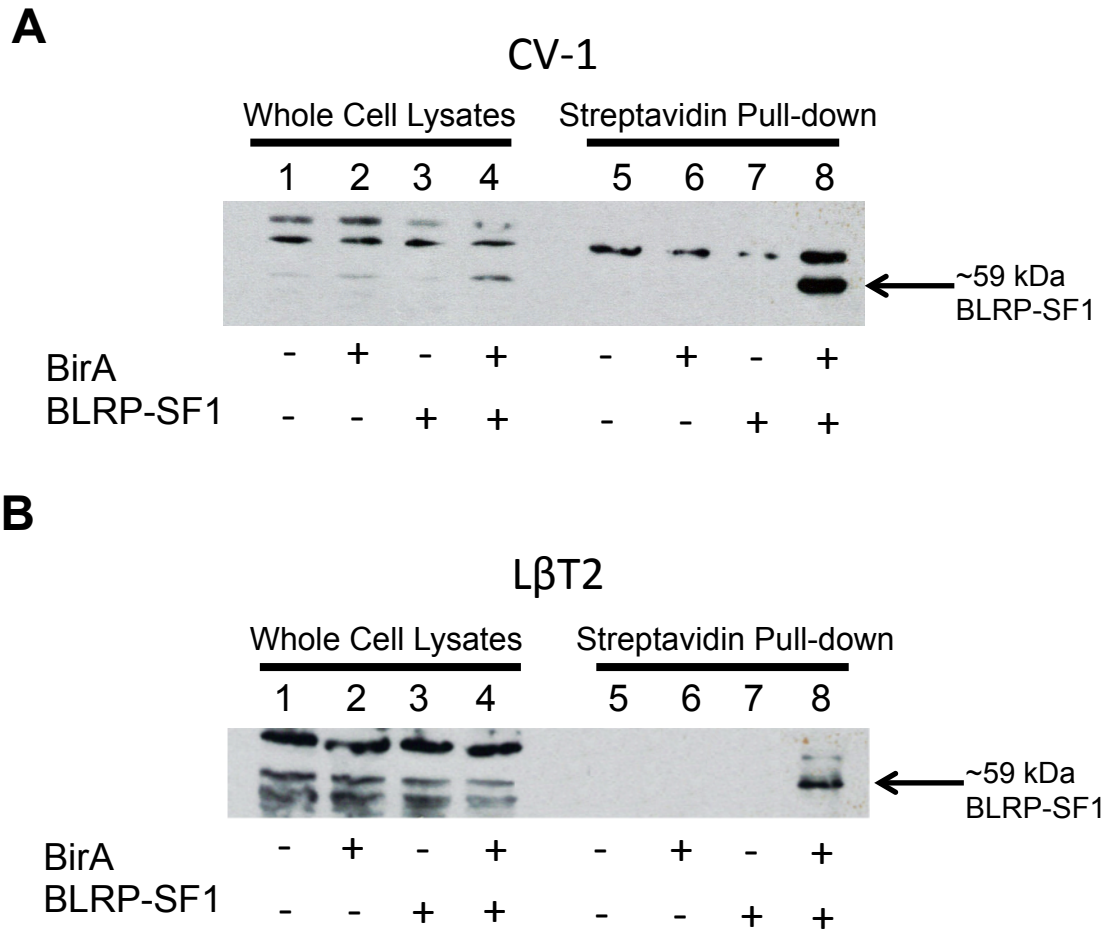

Figure 3. BLRP-SF1 is pulled down with Streptavidin beads. CV-1 (A) and LβT2 (B) cells were transfected with BirA and BLRP-SF1 plasmids. Lanes 1-4 contain cell lysates without the treatment with Streptavidin beads. Lanes 5-8 contain cell lysates subjected to immunoprecipitation with Streptavidin beads. Lanes 1 and 5 serve as the negative control since it was not transfected with either plasmids. Lanes 2 and 6 contain the BirA plasmid. Lanes 3 and 7 contain only the BLRP-SF1 plasmid. Lanes 4 and 8 contain both BirA and BLRP-SF1 plasmids. Pull-down of the BLRP-SF1 protein is seen at approximately 59 kDa. The bound proteins were visualized with an anti-Avi-tag antibody. An Avi-tag is a biotin-acceptor peptide that contains the same sequence for the recognition of biotin ligases as the BLRP-tag.

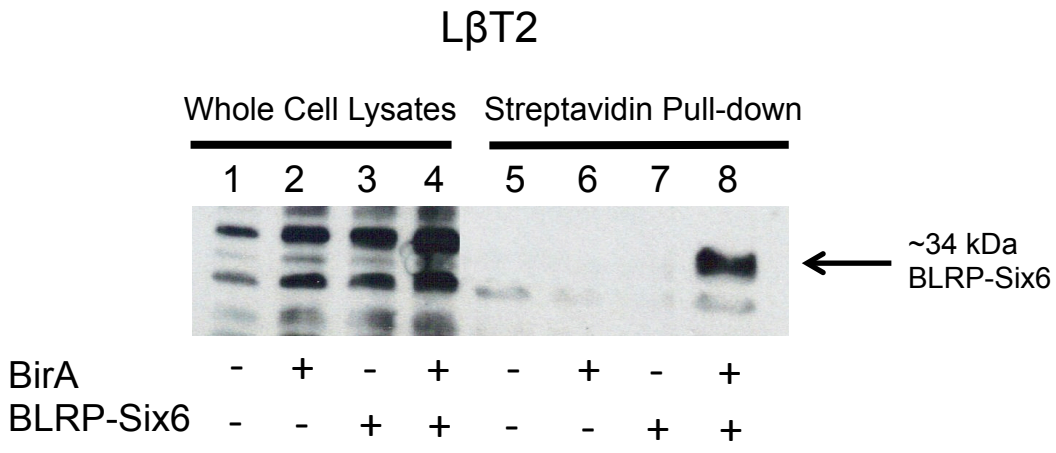

Figure 4. BLRP-Six6 protein is pulled down with Streptavidin beads. LβT2 cells were transfected with BirA and BLRP-Six6 plasmids. Lanes 1-4 contain cell lysates without the treatment with Streptavidin beads. Lanes 5-8 contain cell lysates subjected to immunoprecipitation with Streptavidin beads. Lanes 1 and 5 serve as the negative control since it was not transfected with either plasmids. Lanes 2 and 6 contain the BirA plasmid. Lanes 3 and 7 contain only the BLRP-Six6 plasmid. Lanes 4 and 8 contain both BirA and BLRP-Six6 plasmids. Pull-down of the BLRP-Six6 protein is seen at approximately 34 kDa. The bound proteins were visualized with an anti-Avitag antibody. An Avi-tag is a biotin-acceptor peptide that contains the same sequence for the recognition of biotin ligases as the BLRP-tag.

Recombinant plasmids express functional SF-1 and Six6 proteins

Earlier, we showed that the BLRP-tagged constructs were properly ligated (Figure 2) and were able to express proteins (Figures 3 and 4). Next, we tested whether the expressed proteins are functional.

To test the function of BLRP-SF1 protein, LβT2 cells were co-transfected with LHβ -1800 bp reporter luciferase plasmid and CV-1 cells were used as a positive control (Figure 5A). The LHβ luciferase promoter was used because SF-1 has been shown to regulate the promoter during gonadotrope maturation [41]. Unlike gonadotrope cells, CV-1 cells do not express GnRH receptors, SF-1, Ptx1, or Egr1 [43, 68]. Thus, this allowed us to see the affect of each of the transcription factors, alone and in combination, on the LHβ promoter without the influence of endogenous SF-1, Ptx1, or Egr1 [43]. The combination of Egr1, SF-1, or Ptx1 caused a synergistic induction of the LHβ promoter -1800 bp in CV-1 cells (Figure 5B). In CV-1 cells, the interaction among the three showed LHβ induction 1.5 times higher using the BLRP-SF1 plasmid than an established SF-1 expression plasmid, suggesting that the BLRP plasmid can express functional SF-1 for transcription activation compared to its established counterpart (Figure 5B).

Next, we tested the same experimental conditions in mature gonadotrope LβT2 cells to see whether the BLRP-SF1 plasmid can be expressed in gonadotropes. When the same experimental conditions were performed in LβT2 cells, luciferase/β-gal induction patterns were different from the results found in CV-1 cells (Figure 5C). Egr1, the established and BLRP plasmids, alone in LβT2 cells exhibited a 19-fold induction as compared to the 1.2-fold induction in CV-1 cells, confirming that Egr1 is abundant in this

cell line. In addition, SF-1 alone exhibited a 6-9-fold induction higher in LβT2 cells than CV-1, confirming that SF-1 is already present in LβT2 cells. Compared to the data obtained from CV-1 cells, LβT2 cells exhibited an approximately 10-fold increase in LHβ transcription activity when Egr1, Ptx1, and SF-1 were all present. Thus, when comparing the effects of BLRP-SF1 in combination with Ptx1 or Egr1 with its established SF-1 counterpart, the LHβ promoter induction was fairly similar indicating that the BLRP-SF1 plasmid could indeed be expressed in LβT2 cells and retains wild-type functions.

Next, we tested whether the BLRP-Six6 plasmid was functional. LβT2 cells were co-transfected with an LHβ -1800 bp luciferase reporter (Figure 5A) or empty vector and pSG5-Six6 or BLRP-Six6 plasmids. We used this luciferase reporter because promoter truncations and mutagenesis analyses from our lab shows that Six6 regulates the LHβ promoter (unpublished). Similar to the established Six6 expression plasmid, the BLRP-Six6 plasmid was able to repress the LHβ promoter activity in LβT2 cells (Figure 6A), indicating that the BLRP-Six6 plasmid has normal transcriptional activity.

To test the function of BLRP-Six6 protein in GT1-7 cells, similar experiments were performed. In GT1-7 cells, Six6 was shown to induce GnRH promoter activity [51]. The conditions that were previously described were used to compare the established Six6 expression plasmid with the BLRP-Six6 plasmid [51]. GT1-7 cells were co-transfected with an ATTA-multimer luciferase reporter or empty vector and an established Six6 expression plasmid (pSG5-Six6) or BLRP-Six6. The reporter plasmid had a five-copy repeat of the rat regulatory GnRH region located at -44 to -35 bp (ctcATTAaat) and has

been shown to be required for GnRH basal promoter activity [51] (Figure 6B). As shown in Figure 4, both the BLRP-Six6 plasmid and its established counterpart induced an approximately threefold increase in the activity of the ATTA multimer GnRH promoter, indicating that the BLRP-Six6 plasmid can be expressed and retains transcriptional activity. While Six6 acted as a repressor on the LHβ promoter in LβT2 cells, it acted as an activator on the GnRH promoter in GT1-7 cells.

Therefore, our luciferase assays have shown that both BLRP-tagged plasmids, SF-1 and Six6, are able to express function proteins.

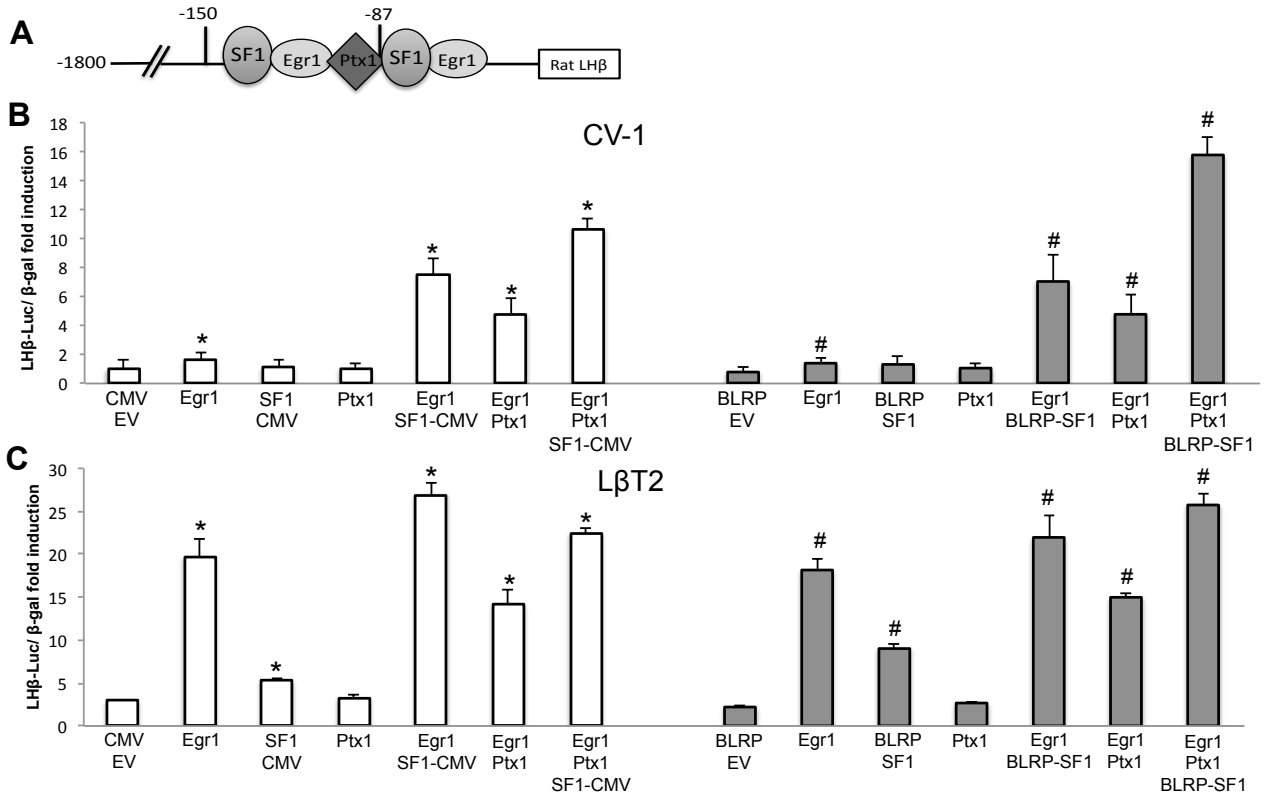

Figure 5. BLRP-SF1 recombinant plasmid retains transcriptional activity. (A) Luciferase reporter, -1800 bp LHβ promoter contains currently known binding sites for SF-1, Egr1, and Ptx1, all of which are known to act together to induce LHβ promoter activity. CV-1 cells (B) and LβT2 cells (C) were transiently transfected with the -1800 bp LHβ promoter luciferase reporter with Egr1, SF-1, or Ptx1 alone or in combination as indicated above. White bars indicate experiments using CMV empty vector (EV) or CMV-SF1. Gray bars indicate experiments using BLRP empty vector or BLRP-SF1. Luciferase assays were used to assess the transcriptional activity of the BLRP-SF1 plasmids on the LHβ promoter. To normalize for transfection efficiency, luciferase values were divided by β-galactosidase values. All transfection and luciferase experiments were done at n = 5 in triplicate and values are presented as the mean ± SEM. Means were compared using Student's T-Test with * and # indicating means significantly different ($P<0.05$) from CMV control and BLRP control respectively.

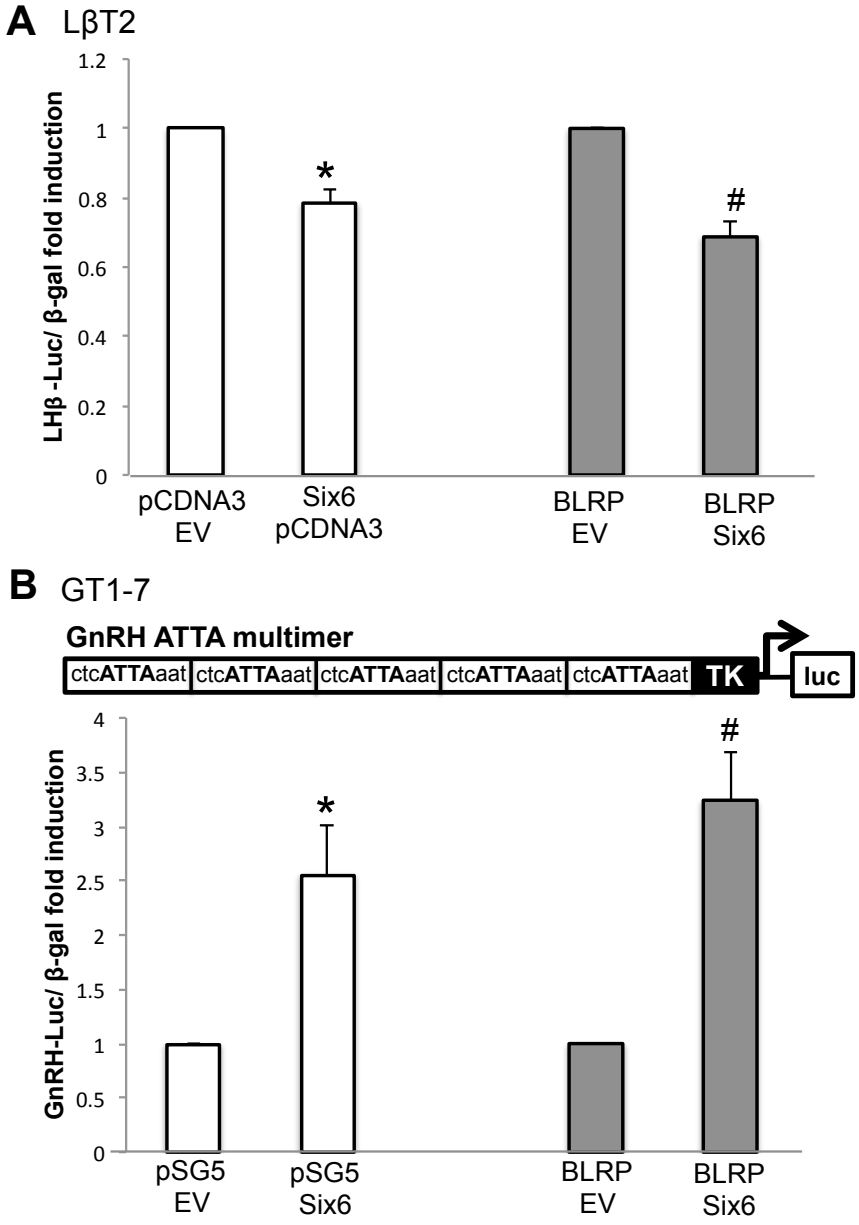

Figure 6. BLRP-Six6 recombinant plasmid retains transcriptional activity. (A) LβT2 cells were transiently transfected with a -1800 bp LHβ promoter luciferase reporter, pCDNA-Six6, BLRP-Six6, or empty vector (EV). (B) GT1-7 cells were transiently transfected with a GnRH multimer luciferase reporter, pSG5-Six6, BLRP-Six6, or empty vector. Values are presented as the mean ± SEM n = 3. Means were compared using Student's T-Test with * and # indicating means significantly different ($P < 0.05$) from pSG5 control and BLRP control respectively.

LβT2 stable cell line co-transfected with BirA and BLRP-SF1

Due to the low transfection efficiency of LβT2 and GT1-7 cell lines, we attempted to create a stable cell line that expresses both plasmids. The BirA and BLRP plasmids had different antibiotic resistance genes, Neomycin and Puromycin respectively, which allowed for us to select the colonies that acquired both plasmids and are able to express the genetic material. To create stable cell lines for better expression of the biotinylated BLRP-SF1 proteins, LβT2 cells from our lab were co-transfected with BirA and BLRP-SF1 and treated with the appropriate drug concentrations. After isolating the colonies that survived both drugs, the colonies were cultured in different sized-well plates until they were ready to be transferred into 10 cm tissue culture dishes.

To verify that these LβT2 cells actually have the genes, the genomic DNA was purified and genotyped with primers specific to regions of the BirA and BLRP vectors (Table 2). The primer sequences encompassed regions upstream and downstream of SF-1 codon region in the BLRP-SF1 plasmid to avoid a false positive from the DNA amplification of endogenous proteins in the cells. When a PCR was done using BirA primers with clone C5A-BLRP-SF1, the expected 967 bp PCR product was seen (Figure 7A, lanes 3-5). When a PCR was done using BLRP primers, the expected 1815 bp PCR product was seen (Figure 7B, lanes 3-5). Thus, this confirms that we have successfully created a stable LβT2 cell line that expresses both BirA and BLRP-SF1.

The same procedure was done with BLRP-Six6 in LβT2 and GT1-7 cell lines. However, presently, we are unsuccessful at maintaining these cells.

Table 2. Primers used to genotype to confirm BLRP-SF1 and BirA co-transfection in LβT2 stable cell line. The sites were specific to the BLRP and BirA vectors and did not include just the SF-1 coding region to avoid a false positive from endogenous SF-1 in LβT2 cells. The BLRP primers encode a 1815 bp region that consists of the BLRP and SF-1 coding regions. The BirA primers encode a 967 nt BirA coding region.

Name	**Primer Sequence (5' → 3')**
BLRP-TEV-pCAGGS-puro plasmid	Forward: CGGCTTCTGGCGTGTGAC Reverse: TTTTATTAGCCAGAAGTCAGATG
BirA-neo-pCAGGS plasmid	Forward: ATGAAGGATAACACCGTG Reverse: CTTATTTTTCTGCACTACGC

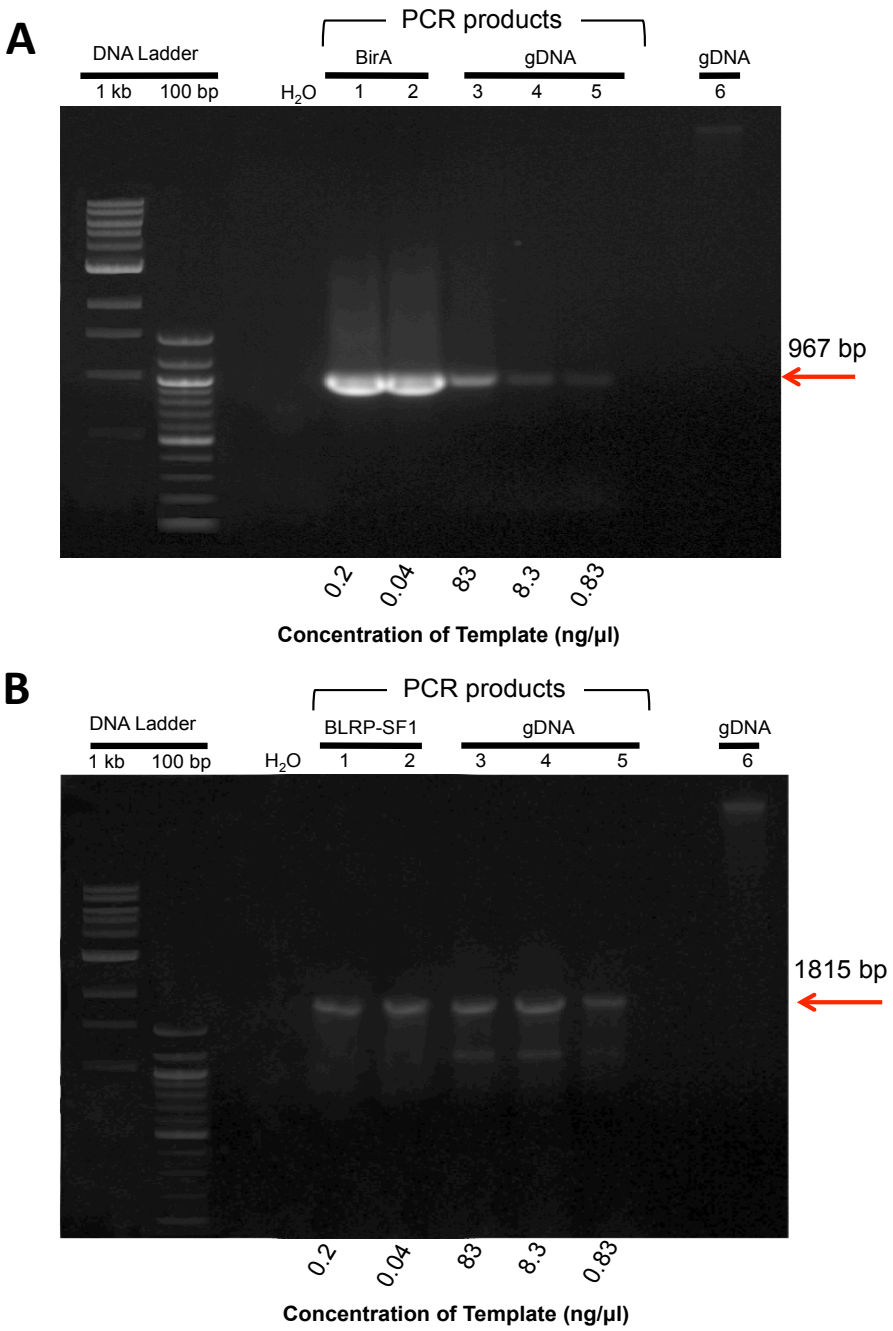

Figure 7. Genotyping of LβT2 stable cell genomic DNA (gDNA) for BirA and BLRP-SF1. Primers specific to BirA (A) and BLRP-SF1 (B) coding regions were used and amplified by PCR. 1 µl of DNA template was used for each PCR reaction and the concentrations are indicated below the gel. Lanes 1-5 represent PCR products. Lanes 1 and 2 contain BirA or BLRP-SF1 plasmids as a template (control). Lanes 3-5 contain genomic DNA template purified from clone C5A-BLRP-SF1 as a template. Lane 6 contains only 1 µl of C5A-BLRP-SF1 genomic DNA template.

ChIP analysis

We previously showed that BLRP-SF1 and BLRP-Six6 proteins were expressed (Figures 3 and 4) and retained transcriptional activity (Figures 5 and 6). Next, we wanted to quantitatively measure the two transcription factors' effect on gene transcription using ChIP assays. LβT2 cells were first co-transfected with BirA and BLRP-SF1 or BLRP-Six6, harvested at 80% confluency, and subjected to ChIP assay. Samples were run in parallel with one group receiving the acTEV protease (TEV) while the other one received a control TEV buffer with no enzyme (no TEV). TEV protease is a specific cysteine protease that cleaves between the Gln and Ser of its recognition site (Figure 1D) [69]. Once BLRP-SF1 or BLRP-Six6 was precipitated with streptavidin beads, TEV protease was used to cleave the TEV site of the tag and SF-1 or Six6 were released from the beads. Since the TEV cleavage site is between the BLRP tag and the coding region (Figure 1D), the transcription factor that is released from the bead matrix no longer has a BLRP tag and is then purified and precipitated for further qPCR analysis. Since the LβT2 cell line has been found to express four gonadotrope markers αGSU, GnRH-R, FSHβ, and LHβ, primers specific to SF-1 and Six6 binding sites were used (Figure 8, Table 3). The controls included the 10% input chromatin, which contains the sheared chromatin without the treatment with streptavidin beads since this establishes the background. Another control is the sample with just the transfected BirA, which controls for background due to non-specific binding in the cells. GnRH promoter was used as an internal control for nonspecific pull-down.

As expected, the qPCR data confirm an enrichment of known SF-1 target gene sequences on αGSU, FSHβ, and LHβ promoters (Figure 8). There was a 1.4 fold increase

in SF-1 binding for all three promoters, indicating that SF-1 binds to these locations and plays a role in gene expression in gonadotropes (Figure 9). Using the BLRP-biotinylation system, we were able to verify the currently known binding sites of SF-1 in LβT2 cells.

We were also able to perform a preliminary ChIP experiment with BLRP-Six6 in LβT2 cells. Since unpublished data from our lab show that Six6 is able to bind to LHβ, primers that were specific to this region was used for qPCR's (Table 3). As expected, Six6 gene target, LHβ was enriched. There was a 4.73 fold binding for LHβ (Figure 10). However, we were only able to do n=1 of this experiment in LβT2 cells. More trials are under way.

Once we are able to successfully pull-down and see the expression of BLRP-Six6 in Western blot analysis for hypothalamic GT1-7 cells, we will do similar ChIP analysis.

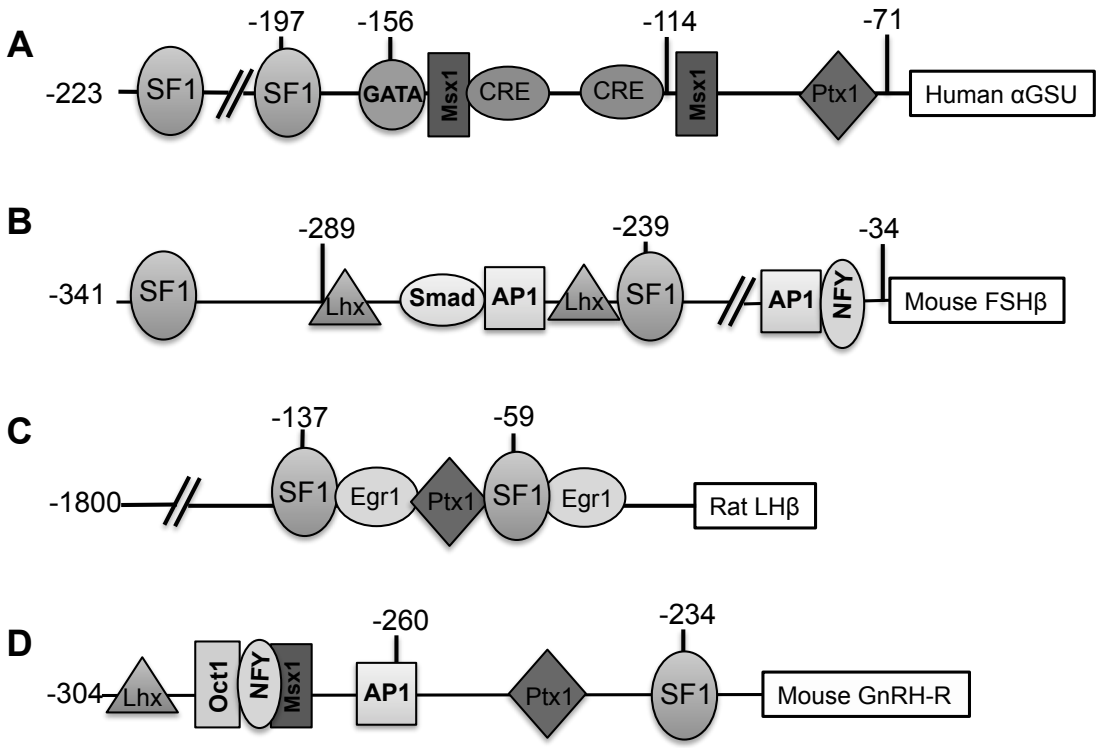

Figure 8. Known SF-1 binding sites. SF-1 has been known to interact with (B) αGSU, (B) FSHβ, (C) LHβ, and (D) GnRH receptor promoters.

Table 3. Primers used for qPCR analysis for ChIP. All αGSU, FSHβ, LHβ, and GnRH-R are expressed in LβT2 cells. The GnRH promoter was used as a control since LβT2 cells do not express it.

Name	**Primer Sequence (5' → 3')**
αGSU promoter	Forward: GAAAATGGCCAAATGCTCTC Reverse: TGTTCCCAGCTGCACATAAG
FSHβ promoter	Forward: GGTGTGCTGCCATATCAGATTCGG Reverse: GCATCAAGTGCTGCTACTCACCTGTG
LHβ promoter	Forward: CGAGTGTGAGGCCAATTCACTGG Reverse: GGGCCCTACCATCTTACCTGGAGC
GnRH promoter	Forward: CAGCAGGTGTTGCAATTACATTCACCATTAAG Reverse: CCTGTTTGGATGTGAAAGTCAAAGGGATCTC
GnRH receptor	Forward: ATCAGAAGTAACAGGGACTCCACTC Reverse: AGGCAGTAGAGAGTAGGAAAAGGAAG

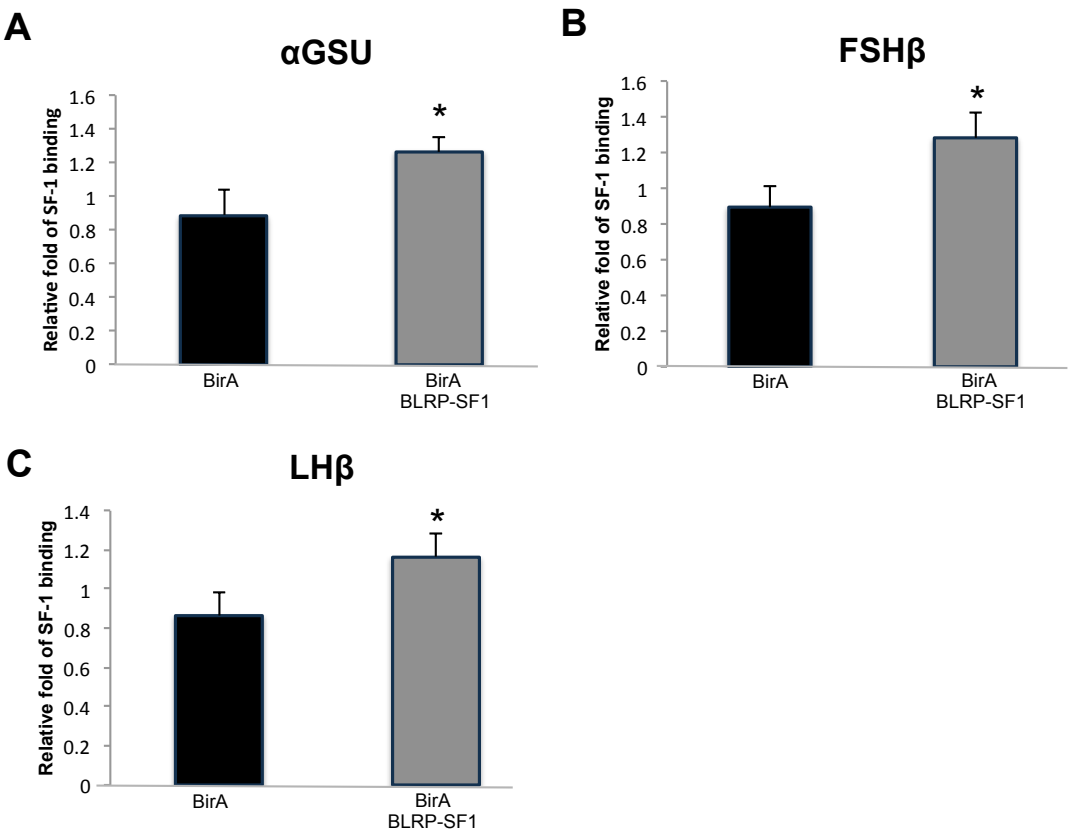

Figure 9. qPCR confirms enrichment of known SF-1 target gene sequences. LβT2 cells were co-transfected with 4 μg of BirA alone or in combination with 4 μg of BLRP-SF1. Cells were harvested at 80% confluency and subjected to ChIP using streptavidin beads. qPCRs were performed with specific primers for (A) αGSU, (B) FSHβ, and (C) LHβ. Samples were done in parallel with one group receiving TEV protease (TEV group) while one received a control buffer (No TEV group). The values are represented as the fold of SF-1 binding relative to TEV values over the no TEV values. An asterisk indicates significant differences ($P < 0.05$) from the control, BirA ($n = 3$).

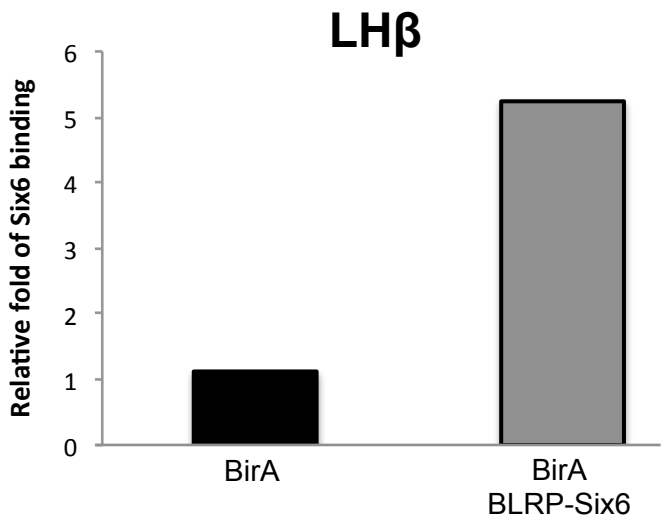

Figure 10. Preliminary qPCR data show enrichment of known Six6 target gene sequences. LβT2 cells were co-transfected with 4 μg of BirA alone or in combination with 4 μg of BLRP-Six6. Cells were harvested at 80% confluency and subjected to ChIP using streptavidin beads. qPCRs were performed with specific primers for LHβ. Samples were done in parallel with one group receiving TEV protease (TEV group) while one received a control buffer (No TEV group). The values are represented as fold of Six6 binding relative to TEV over no TEV values (n = 1).

IV

Discussion

The elucidation of protein-DNA and protein-protein interactions plays an important role in the understanding of biological processes. Several methods have been developed to study such interactions. However, most of the pre-existing methods have several disadvantages. The BLRP-biotinylation system has several distinct advantages over the other conventional systems. The high specificity of biotin ligases and the high affinity of biotin-streptavidin binding allow the circumvention of problems that are associated with conventional methods, such as the lack of commercial antibodies, the abundant purification steps, or the weak interactions between prey and bait substrates. Thus, small peptide tags, such as His-tag [70], strep-tag [71], and biotinylation tag [4], have gained popularity. Despite the benefits of these small tags, the biotinylation tag has the highest affinity of all the currently available tags. In this thesis, we have shown that the BLRP-biotinylation system is a powerful method for the isolation of transcription factors SF-1 and Six6 through a single purification step. Specifically, we utilized this method to demonstrate several known protein-DNA interactions for SF-1 and Six6 in gonadotropes and hypothalamic cells. Since SF-1 and Six6 have no available commercial antibody that worked well, this method allowed for the capture of the proteins on a streptavidin matrix for protein interaction studies such as ChIP or ChIP-seq.

Before proceeding to protein interaction studies, we were able to confirm that the recombinant BLRP-tagged constructs expressed the appropriate proteins. We were able to enrich biotinylated BLRP-SF1 and BLRP-Six6 in LβT2 cells. Unfortunately, the pull-down and enrichment of BLRP-Six6 in GT1-7 cells were unsuccessful. The difference in the BLRP-protein expression between the two cell lines possibly reflects, in part, the difference in transfection efficiency in the two cell lines. Both LβT2 and GT1-7 cells are

known to have low transfection efficiencies [25, 72] while CV-1 cells have a high 60% transfection efficiency as indicated by PolyJet. In figures 3 and 4, the presence of BLRP-SF1 and BLRP-Six6 in the cells shows that the recombinant plasmid that was created was functional. Since CV-1 cells have a higher transfection efficiency than LβT2 cells, the band of BLRP-SF1 in the Western blots in CV-1 cells were much thicker, indicating that there is more protein expression in the CV-1 cell line (Figure 3). Thus, the inability of BLRP-Six6 to be fully expressed in GT1-7 cells may be due to the degradation of proteins or another cell-restricted regulatory mechanism. Furthermore, we were trying to co-transfect two different plasmids into the cells which lowers the transfection efficiency even further. If BirA is not expressed at adequate levels, the BLRP proteins would not be biotinylated and vice versa.

While we were able to pull-down BLRP-SF1 and BLRP Six6 in LβT2 cells, we were not able to see the respective bands of the biotinylated products in all of our different cell passages that were used for the pull-down assays and Western analysis. Out of the six passages of LβT2 cells that were used, three of these passages had enough biotinylation of target proteins to be seen on the Western blot. A problem inherent in the transient transfection of these cells could be that the biotinylation efficiency was not consistent, a problem that has been noted by other groups [73, 74]. This might be due to low amounts of BirA and ATP that are present in the cells. Thus, large amounts of BirA and ATP are required for successful in vitro biotinylation. As mentioned earlier, enzyme biotin ligase attaches a biotin group post-translationally to a specific lysine residue with the requirement of ATP, so without ATP, biotinylation would not occur [7]. Even if the targeted BLRP proteins could still be expressed at high levels, a large proportion of them

may be unbiotinylated and thus were not pulled down. Several groups have engineered other forms of BirA expression plasmids to bypass this problem. For example, Wu and colleagues have designed a BirA plasmid with a His tag that allows for the recovery of large amounts of pure BirA [75]. With this purified BirA fraction, they added it to crude extract for biotinylation to occur and then pulled down the labeled targeted proteins with an avidin matrix as was done in this thesis. Another group circumvented the problem of the co-expression of two different plasmids by designing a plasmid that placed the BirA ORF downstream of the gene of interest on the same plasmid [9]. In principle, because the BirA enzyme would be translated near the gene of interest, there would be a more efficient biotinylation of these proteins. We are continuing to find new ways to make this BLRP-biotinylation work well using only transient transfections while creating a stable cell line that co-expresses BirA and our tagged target protein.

After verifying that the recombinant BLRP-tagged constructs expressed the proteins, we showed that these proteins retained function using transient transfections and luciferase assays. BLRP-SF1 caused a synergistic induction of the LHβ promoter with Ptx1 and Egr1 (Figure 5). When the same experimental conditions were used for both CV-1 and LβT2 cells, the LHβ fold induction was much higher in LβT2 cells (Figure 5B). This could be attributed to the fact that there is endogenous SF-1, Egr1, and Ptx1 in the LβT2 cell line. Both Egr1 and SF-1 alone caused a much higher induction in LβT2 cells, with the former having a much greater effect on the LHβ promoter, indicating that both are abundant in gonadotropes. Egr1 has been known to be an important regulator of LHβ gene expression, binding directly to the LHβ promoter or acting in synergy with SF-

1 [34, 76, 77]. Egr1 knockout mice are characterized by the loss of LHβ gene expression in gonadotrope cells despite the presence of other gonadotrope markers such as FSHβ, αGSU, and GnRH-R [76]. Since Egr1 plays an important role in the induction of LHβ in the HPG axis, Egr1 is regulated by GnRH while SF-1 and Ptx1 transcription factors are not [34]. Besides these endogenous transcription factors in LβT2 cells, there are other transcription factors such as specificity protein 1 (SP1) that are important for LHβ expression. Mutation of binding sites upstream of the LHβ promoter showed that SF-1, Ptx1, and SP1 are important for basal transcription [77, 78]. Although LHβ transcription activity, in general, was much higher in LβT2 cells than CV-1 cells due to other endogenous proteins present in the former, the BLRP-SF1 plasmid was shown to be functional.

The case for Six6 is a different story. Six6 has been documented to have binding sites on the enhancers and promoters of GnRH, with the site of action to be on the promoters [51]. Originally, in the transfection assay for BLRP-Six6 in GT1-7 cells, we wanted to test the effect of the BLRP-Six6 plasmid on a luciferase reporter containing both the GnRH enhancer and promoter but failed to see a significant activation as compared to the same experiment done by Larder and colleagues [51]. Even when we used the GnRH ATTA multimer, when the effects of the BLRP-Six6 plasmid were compared to the effects of an established plasmid, the fold induction was not as high (Figure 6). This could be due to the addition of the BLRP-tag on the recombinant protein. Although the tag is small and should not affect the activity of Six6, it could still prevent the binding of Six6 to certain sites that are important for activation. The tag could also be

preventing other factors from binding to Six6 for full activity, which may pose a problem when we isolate BLRP-Six6 to study its interacting partners. This problem was not encountered with the BLRP-SF1 plasmid.

Lastly, we used ChIP to confirm the enrichment of SF-1 and Six6 gene targets. BLRP-SF1 was able to bind to αGSU, FSHβ, and LHβ promoters. BLRP-Six6 was also able to bind to αGSU, FSHβ, LHβ, and GnRH-R promoters. Although both SF-1 and Six6 showed binding on the gonadotrope markers, the relative binding fold of both were low (Figures 8 and 9). This may be due to the aforementioned transfection efficiency problem. Theoretically, the strong covalent binding of streptavidin and biotin would allow us to isolate our recombinant proteins and their interacting partners. Taking advantage of the sensitivity of ChIP analysis, we were hoping that the transfection efficiency problem could be bypassed. Having higher transfection efficiency would allow us to use less cells and would improve the signal-to-noise ratio since cells that were not transfected would cause high noise background.

The low transfection efficiency in GT1-7 and LβT2 cells poses a problem for our experiments. Thus, a stable LβT2 cell line that expresses both BirA and BLRP-SF1 has been created. We are currently attempting to stably transfect BirA and BLRP-Six6 in LβT2 cells as well as GT1-7 cells. Using these stable cell lines, we should be able to have a higher amount of biotinylated target proteins that could be pulled down to be used for protein-DNA or protein-protein analyses. We are currently in the process of testing the stable cell line for protein expression and activity using the same methods as was done in this thesis.

Ultimately, we seek to identify the interacting partners through high-throughput sequencing (ChIP-seq) as well as mass spectrophotometry. We want to use ChIP-Seq analysis to identify the binding locations of SF-1 in the chromatin of LβT2 cells and Six6 in LβT2 cells and GT1-7 cells. Previously, it was difficult to do this assay for SF-1 and Six6 in the pituitary and hypothalamus due to the lack of specific antibodies. However, this biotinylation system should allow us to use ChIP for this purpose. At the same time, we would like to apply mass spectrometry techniques to identify and quantify protein interactions of SF-1 in LβT2 cells or Six6 in LβT2 and GT1-7 cells. If this BLRP-biotinylation system works well, we would commence with *in vivo* assays.

In the future, we would like to do *in vivo* experiments with transgenic mice that express the bacterial biotin ligase and a specific BLRP-tagged protein to confirm the results of our *in vitro* experiments. Briefly, the transgenic mouse that expresses the tagged transcription factor driven by a promoter that is only specific to a certain cell type will be crossed with a transgenic mouse that expresses BirA in all cells. Currently, there exists a transgenic mouse from Dr. Dies Meijer's lab from Erasmus Medical Center that expresses the biotin ligase BirA gene from the ROSA26 locus [1]. Since this locus is active in all cells, the resulting mouse will express the BirA gene, allowing for large-scale *in vivo* biotinylation of target proteins in the mouse. Similar to the *in vitro* experiments that were done in this thesis, the target protein and its interacting partners will be purified using streptavidin beads and identified through ChIP, ChIP-seq, as well as mass spectrometry techniques. Similar experiments have been done with these types of transgenic mice and have shown that *in vivo* biotinylation of the tagged protein is possible [1, 65].

In conclusion, the efficient *in vitro* BLRP-biotinylation of our target proteins in hypothalamic and pituitary cells demonstrated in this thesis raises the possibility of applying this technology in the purification and characterization of protein complexes in the hypothalamic-pituitary-gonadal (HPG) axis. This thesis focused on two transcription factors, SF-1 and Six6, which are critical for the transcriptional regulation of the HPG axis. SF-1 knockout mice are characterized by an abnormal development of the VMH and the lack of gonadotrope markers such as LH and FSH. Six6 knockout mice are characterized by a reduced number of GnRH neurons and reduced fertility. Thus, the determination of complex protein interactions in the mouse model would allow us to understand the biological processes in which genes interact to regulate the expression of specific gene targets in the HPG axis at a cellular and molecular level. We would then be able to apply these findings to disorders that are common in the human body such as IHH, hypogonadism, and other forms of infertility. Therapeutic intervention for treating disorders of the HPG axis will be a possibility in the future.

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