Differential roles of RNA binding proteins hnRNP L and LL in hormone production in rat pituitary cells

by

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ABSTRACT

OBJECTIVES: The role of heterogeneous nuclear ribonucleoproteins in controlling hormone production is not well-understood. This study aims to determine the regulatory roles of hnRNP L and LL in hormone production. **RESULTS:** HnRNP LL knockdown dramatically reduced the mRNA level of growth hormone (GH) and prolactin (PRL) in GH_3 pituitary cells, which was efficiently rescued by a Myc-hnRNP LL. In contrast, hnRNP L knockdown caused a 93-nucleotide intron retention with a decrease in the full-length *Prl*, which was rescued by a FLAG-hnRNP L. Double knockdown of hnRNPs indicates the interactions of hnRNPs for particular effects. The essential role of hnRNP LL for the secretion of GH and PRL was verified in primary pituitary cultures. Using RNA sequencing, a group of genes in hormone production that were differentially regulated by hnRNP L or LL were identified. **CONCLUSION:** These findings demonstrate the differential functions of two paralogous RNA processing factors in hormone production.

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DEDICATION

This work is dedicated to my father Jianhui Lei and mother Zhifang Fan.

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ABBREVIATIONS

Aβ	amyloid β-protein
ASF/SF2	alternative splicing factor, pre-mRNA-splicing factor SF2 (alias: Serine/arginine-rich splicing factor 1, SRSF1)
BDNF	brain-derived neurotrophic factor
BK	big potassium
BNP	brain natriuretic peptide
BSA	bovine serum albumin
BTK	Bruton's tyrosine kinase
CA-repeat/CA-rich	cytosine adenine-repeat/cytosine adenine-rich
CACS	Central Animal Care Services
cAMP	Cyclic adenosine 3’,5’-monophosphate
CGRP	calcitonin gene-related peptide
CO_2	carbon dioxide
CREM	cyclic AMP-responsive element modulator gene
CT	calcitonin
ddH_2O	double-distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ER	estrogen receptor

ERK	extracellular signal-regulated kinase
ESE	exonic splicing enhancer
FBS	fetal bovine serum
GDNF	glial cell line-derived neurotrophic factor
GFR	GDNF family receptor
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHRHR	growth-hormone-releasing hormone receptor
GnRH1	gonadotropin-releasing hormone 1
GPCRs	G protein-coupled cell surface receptors
GPI	glycosylphosphatidylinositol
hnRNP	heterogeneous nuclear ribonucleoprotein
hnRNP LL	hnRNP L-like
HS	horse serum
IDE	insulin-degrading enzyme
IGF-I	insulin-like growth factor-I
IGHD	isolated growth hormone deficiency
IMDM	Iscove's Modified Dulbecco's Medium
LHCG	luteinizing hormone/choriogonadotropin
miRNA	microRNA
NMD	nonsense mediated mRNA decay
NGF	nerve growth factor
NPPB	natriuretic peptide precursor B

NRG-1	Neuregulin-1
NT-3	neurotrophins-3
OCT4	octamer-binding transcription factor 4
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG8000	Poly (ethylene glycol), M.W. 8000
PMSF	phenylmethanesulfonyl fluoride
PPII	pyroglutamyl peptidase II
pre-mRNA	precursor messenger RNA
PRL	prolactin
PTB	polypyrimidine tract-binding protein
PTC	premature termination codon
PTH	parathyroid hormone
RBPs	RNA binding proteins
RIPA	radioimmunoprecipitation assay
RNAi	RNA interference
RT-PCR	reverse transcription-polymerase chain reaction
SC35	splicing component, 35 kDa (alias: Serine/arginine-rich splicing factor 2, SRSF2)
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
snRNA	small nuclear RNA

snRNP	small nuclear ribonucleoprotein
SOX	SRY-related HMG-box
SRp20	serine/arginine-rich protein of 20 kDa
	(alias: Serine/arginine-rich splicing factor 3, SRSF3)
STREX	stress axis-regulated exon
T3	triiodothyronine
T4	thyroxine
TBS	Tris-buffered saline
TIA-1	T-cell intracellular antigen 1
TIAR	TIA-1 related protein
TM	transmembrane
TRH	thyrotropin-releasing hormone
UTR	untranslated region
UV	ultraviolet
µg	microgram
µl	microliter
µm	micrometer

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CHAPTER I

Introduction

1. Hormone synthesis, transport, secretion and action

Hormones, a group of extracellular signaling molecules secreted into the bloodstream in organisms, can be biochemically classified as amine, peptide, protein or steroid hormones [1, 2]. Amine hormones comprised of catecholamines and thyroid hormones are derived from tyrosine [2]. Peptide and protein hormones are encoded in genes, ranging from three (thyrotropin-releasing hormone) to more than 200 amino acids (follicle-stimulating hormone) [1, 3, 4]. Last but not least, steroid hormones are derived from cholesterol [2].

Catecholamines are synthesized in the adrenal medulla, including epinephrine (~80% of total catecholamine in adrenal medulla), norepinephrine and dopamine [1, 5]. The three hormones are distinct from each other in their modifications on the side groups of tyrosine [1, 5]. The thyroid hormones (T4 and T3), which are originated from the conjugation of two tyrosines and iodine atoms, are produced by the follicles in the thyroid gland [6]. Thyroglobulin synthesized in follicular cells enters into the colloid and binds iodines with its tyrosine residues, resulting in the formation of mono-iodotyrosine (T1) and di-iodotyrosine (T2) [6]. Combination of two T2 generates a T4 contributing to ~90% of the thyroid hormone, whereas interaction of T1 and T2 forms T3, which is ~4 times more potent than T4 [7, 8].

The synthesis of peptide and protein hormones is initiated from the transcription of the hormone genes into pre-mRNA in the cell nucleus, followed by RNA processing

that includes addition of a 5’ 7-methyl guanosine cap at the 5’ end (5’ capping), addition of a poly-A tail at the 3’ end (Poly-A tailing) and removal of introns along with join of exons (pre-mRNA splicing) [9-12]. Messenger RNA is then transported to the cytoplasm and translated on the ribosomes to preprohormone containing a 15~30 amino acid-long signal peptide at the N-terminal [1]. The preprohormone is directed by interaction of the signal peptide with its docking proteins on the rough endoplasmic reticulum for continuous translation [1]. The removal of the signal peptide by signal peptidases converts the preprohormone to a prohormone, which is translocated to the Golgi apparatus [1]. Post-translational modifications of prohormones in the Golgi apparatus, which including glycosylation, phosphorylation, sulfation and C-Terminal amidation, are critical for the characteristic and specificity of peptide/protein hormones [2, 13-17]. Afterwards the prohormone is packaged in membrane-bound secretory vesicles, where it is cleaved into active hormone and is stored [1, 2].

Steroid hormones are synthesized in the adrenal cortex, the gonads, corpus luteum and placenta through enzymatic reactions from cholesterol [2]. They are characterized by the cyclopentanoperhydrophenanthrene ring (steroid nucleus), excluding some forms of vitamin D [2, 18, 19]. Modifications of cholesterol, including hydroxylation, dehydrogenation/reduction and lyase reaction, endow binding specificity of the steroid hormone to one of the five different types of receptors, which is progestin, glucocorticoid, mineralocorticoid, androgen or estrogen receptor [1, 2, 5].

To maintain biological homeostasis, the secretion of hormones is regulated. Catecholamine hormones and peptide/protein hormones are stored in secretory vesicles in the gland and are secreted via exocytosis into bloodstream upon stimulations [1].

Hormone-containing vesicles traffic, dock and fuse at the porosome by the guidance of soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins (SNAREs) [20-24]. Concurrently, the vesicles swell and create a turgor pressure, facilitating the release of hormones out of the cell [25]. This regulated secretory pathway requires consumption of ATP and calcium ions [26-28].

Upon secretion of thyroid hormones, the thyroglobulin and its iodinated tyrosines stored in the colloid undergo endocytosis into the follicular cells and fuse with a lysosome [29]. Digestases inside the lysosome break down the thyroglobulin backbone, releasing the T4 and T3 [29, 30]. After that, the T4 and T3 are expelled by proteolysis into the bloodstream [2, 5, 31].

Steroid hormones are lipophilic and can freely cross membranes, therefore are not stored in the gland but are released as produced [1].

The binding of hormone with its carrier protein (also called plasma protein) in the bloodstream prolongs the circulating half-life of the hormone by preventing filtration by the kidneys (for amines), preventing destruction (for peptide/protein hormones) or increasing solubility (for steroid and thyroid hormones) [32]. Catecholamine hormones are either unbound or loosely bound to albumin and has a short circulating half-life (a few minutes) [33, 34]. Peptide/protein hormones mostly circulate freely without carrier protein and therefore also have a short half-life (1-30 minutes) [35, 36]. Thyroid hormones that mainly bind to thyroxine binding globulin (TBG) have circulating half-lives of about one day (T3) to six days (T4) [37, 38]. Steroid hormones associated with steroid binding globulins (SBG) have circulating half-lives from 30-300 minutes [39, 40].

Free hormone (unbound form) is biologically active and is ready for interaction with its receptor, inducing conformational changes in the receptor and then activation of second messengers followed by regulation of effector proteins [1, 41]. This process is called signal transduction [1, 41]. Hydrophilic hormones (catecholamines and peptide/protein hormones) cannot freely cross the cell membrane and thus bind to cell surface receptors whereas lipophilic hormones (thyroid hormones and steroid hormones) recognize intracellular receptors [1, 42]. Unlike the cell surface receptors, the intracellular receptors directly interact with the DNA promoter of the target genes upon hormone binding to them [42].

The plasma level of hormones is essential in physiological homeostasis. It is mainly controlled by the rate of hormone secretion, which can be influenced by many factors including direct regulatory inputs (neural or hormone input) and hormone metabolism rates (hormone activation/inactivation/degradation or its binding with plasma proteins) [1, 2, 5].

2. Alternative splicing in endocrine development

The effect of alternative splicing on the regulation of hormones has been studied for decades [43, 44]. Alternative splicing is the selective exon usage of a gene that results in multiple transcript variants, tremendously contributing proteomic diversity (Fig. 1A). A spliceosome that comprises of small nuclear RNAs (snRNAs) and proteins is assembled onto the splice site consensus sequences at the 5’ end and 3’ end of the intron for pre-mRNA splicing (Fig. 1B) [45, 46]. During the assembly, U1 snRNA/snRNP binds to the GU, and U2 recognizes the branch point after U2AF binding to the polypyrimidine

tract and 3’AG [45, 46]. The interactions of U1 and U2 with the pre-mRNA recruit U4, U5 and U6 to form the spliceosome [45, 46]. A sequence, which contains splice site consensus but is not normally used in splicing as an exon, is called cryptic exon; the usage of this exon is called cryptic splicing [47-49]. Alternative splicing happens in almost all RNA Polymerase II transcripts in humans with a wide range of impacts on biological functions. Of great interest to us is its role in the endocrine system.

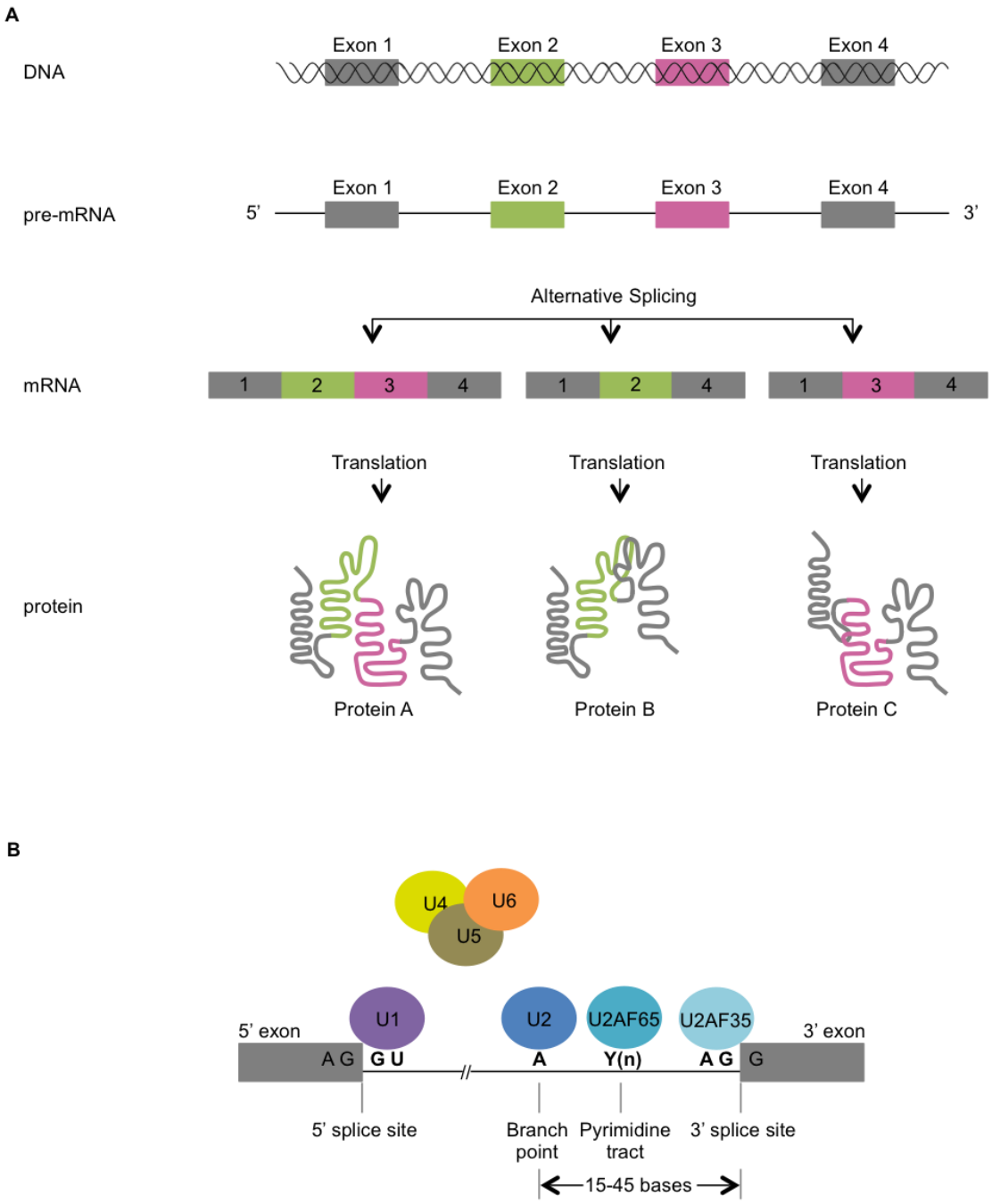


Figure 1. Diagram of alternative splicing and the splice site consensus. (A) The exons from a single gene can be spliced and joined together in different combinations. It is annotated as DNA (black double helix), exon (coloured box), intron (black straight line) and protein (coloured wavy line). **(B)** Within an intron, a 5' splice site, a 3' splice site and a branch point are required for pre-mRNA splicing. The GU and AG at the 5' and 3' end of the intron is highly conserved for most eukaryotic splice sites [50].

Increasing evidence suggests that alternative splicing is important for endocrine system development. For instance, the octamer-binding transcription factor 4 (OCT4) has three isoforms produced by alternative splicing, termed OCT4A, OCT4B and OCT4B1 [51, 52]. The OCT4 is encoded by all five exons of the *OCT4* gene; the OCT4A mRNA also comprises five exons but with a 5′ truncation in the second exon (exon 1b); and the *OCT4B* transcription starts from exon 1b [51]. The variants distribute differently in cells, with higher intensity of OCT4A in the nucleus and strong fluorescent signal of OCT4B in the cytoplasm in human blastocyst detected by microscopic pseudo-coloured imaging [53]. Exogenous overexpression of OCT4A but not OCT4B in embryonic stem cells induces the differentiation of endodermal, which is the primary gem layer in embryo to develop endocrine glands and organs [54]. The functional difference between OCT4A and OCT4B likely comes from their distinct NH_2-termini. The proline-rich region in the NH_2-terminus of OCT4A may form part of the transcriptional activation domain as it does in other transcription factors and thereby gives OCT4A a different functional property from OCT4B. However, this needs to be verified by more evidence.

The Trk family of tyrosine kinase receptors also generates its isoforms by alternative splicing [55-60]. The receptor trkA has two isoforms trkA I and trkA II, which differ by an 18 base-pair insertion in the extracellular domain [55]. The trkA I lacking the insertion is mainly expressed in non-neuronal tissues while the trkA II containing the insertion is abundant in the nervous system [55]. The Trk family binds to neurotrophins, a group of growth factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins-3 (NT-3) and neurotrophins-4/5 (NT4/5) [61]. *In vitro* studies show that NGF/trkA interaction promotes the differentiation of bipotential

precursor cells to the mammotroph phenotype during the anterior pituitary development [62]. Not only pituitary, NGF/trkA interaction also plays a role in pancreatic differentiation and development. Pancreatic islet cells resemble neurons in many functions and ontogenies, yet they are sourced from different gem layers [63-65]. During pancreatic development, signals from the neural crest are sent to the pancreatic epithelium, regulating the size of the β-cell population [66]. Immunocytochemistry detected the expression of trkA in an insulin-secreting β-cell line (RINm5F) that represents an early stage in pancreatic development [67]. This cell line responds to the addition of NGF and thereby extends its neurite-like process, whereas a more mature β-cell line (βTC3) lacking the trkA receptor does not respond to the signal [67].

The insulin-like growth factor-I (IGF-I) provides another example for the impact of alternative splicing on endocrine development. The *IGF-1* gene comprises six exons, of which exons 1 and 2 are mutually exclusive first exons; exons 5 and 6 can be truncated into three alternative terminal exons [68]. Furthermore, the exons 1 and 2 have six and two transcript start sites, respectively, and the exon 6 contains four polyadenylation sites, which adds another layer of complexity to its alternative splicing regulation [69-72]. Based on the selection of the first exon, IGF-I variants can be classified into class 1 transcripts (containing exon 1) and class 2 transcripts (containing exon 2). Researchers believe that class 1 acts in an autocrine/paracrine manner and class 2 is the secreted endocrine form [73, 74]. Although most tissues use class 1 IGF-I, liver has increased protein levels of both class 1 and 2 during development [75, 76]. In the developing liver of rats, RT-PCR identifies that IGF-I class 1 mRNA expresses predominantly (~90%) and reaches its peak at 4 weeks of age followed by a decline at 6 weeks [76]. In contrast,

class 2 mRNA contributes about 70% of total IGF-I mRNA in the growing mouse liver [76]. The species-, tissue- and stage-specific distribution of IGF-I transcript variants implies their roles in the development of liver and other tissues, and this requires further investigation.

GFRa4 belongs to the glycosylphosphatidylinositol (GPI)-linked GDNF family α receptors that bind to the glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors [77]. The gene *Gfra4* encoding GFRa4 consists of seven exons that can be alternatively spliced into eight transcript variants [78]. Based on the usage of two mutually exclusive first exons Ia and Ib, these variants are grouped into two sets: a1-a4 starting with exon Ia, b1-b4 using exon Ib [78]. Semi-quantitative RT-PCR using specific primers detected the expression of the GFRa4 splice variants in a tissue-specific manner. The a1 variant gains a putative GPI linkage at the C-termini through alternative splicing and is preferentially expressed in juvenile (3 weeks-old mice) thyroid C cells and parathyroid gland [78, 79]. Intriguingly, these two glands switch their expression favourite to the a2 variant containing a putative transmembrane (TM) domain at the C-termini upon 6 weeks [78]. Particularly for the developing thyroid, the expression pattern of *Gfra4* transcripts is complex. Variant a1 and a3 peak at the 3 weeks along with the absence of a2 and a4, whereas a2 and a4 are abundant in newborn and 6-week-old mice [78]. As for other tissues, a4 is prominent in the adrenal gland; a2 and a4 are preferred in pituitary; testis and brain only express a4 [78]. Transcript variants b1-b4 are similar to a1-a4 except the relative weak NH_2-terminal signal peptides encoded by exon bI; however their expression pattern and functional difference remain obscure. The binding of GFRa4 with its ligand persephin activates the transmembrane receptor tyrosine kinase

Ret signaling, which has previously been reported to be critical for kidney development and differentiation [80, 81]. Ret-deficient (-/-) mice appear to have a significant decrease in thyroid C cell mass in comparison to their wide-type littermates [78]. These findings suggest the physiological roles for GFRa4 isoforms in the development of related tissues.

Collectively, these observations point to the importance of alternative splicing in endocrine system development. Most of the studies herein concern the developmental distributions of the alternative splicing variants of a gene; however their specific functions are not understood clearly.

3. Alternative splicing in endocrine function

Functional studies in the endocrine system reveal the emerging role of mRNA alternative splicing. The regulation process for hormones is pleiotropic and delicate, including variables from the physical level (e.g. regulatory axis, feedback loops), the cellular level (e.g. secretory pathway, receptor binding, cell signalling) to the molecular level (e.g. DNA transcription, RNA processing, protein modifications). Alternative splicing occurring in hormone, hormone receptor or hormone-related genes involving in any of the processes above could ultimately affect the regulation of endocrine functions [82-118].

3.1 Alternative splicing in hormones-encoding genes

Alternative splicing is one of the key mechanisms that diversify hormone isoforms. Research by far has found that more than one hundred different hormonally active peptides in the gastrointestinal tract are generated by alternative splicing [82].

Natriuretic peptide precursor B (NPPB) is the preprohormone of brain natriuretic peptide (BNP), which is a cardiac hormone [91]. The gene *NPPB* in mammals comprises three exons [84]. Alternative splicing happening at the intron 2 results in a 102 base-pair in-frame retention that encodes a distinct C-terminus, leading to failure in cGMP-dependent signal transduction [86]. In another case, a NPPB isoform generated from exon 2 skipping escaped the nonsense-mediated mRNA decay (NMD) pathway despite the existence of the premature stop codon in the sequence and acts dominant-negatively [85, 90]. It attenuates the expression and secretion of normal NPPB [90].

Alternative splicing can not only produce hormone isoforms but also yield another different hormone from single genes. For example, calcitonin (CT) is a peptide hormone produced by the thyroid gland for reducing calcium in blood and promoting bone formation [92]. Calcitonin gene-related peptide (CGRP) is a regulatory neuropeptide with multi-functions synthesized in neurons [92]. Calcitonin and CGRP are derived from tissue specific splicing of the same gene [83, 88]. The *CT/CGRP* gene contains six exons in its primary mRNA. In neurons, the skipping of exon 4 results in the joining of the rest of the exons with the usage of polyadenylation site at the 3' end of the exon 6 to produce CGRP [83, 87], whilst the inclusion of exon 4 in thyroid cells lead to the removal of the exon 5-6 accompanied by the usage of the polyadenylation site at the 3' end of exon 4 to produce CT [89]. Its alternative splicing involves an intricate regulation by many *cis*-acting elements and *trans*-acting factors. In the neuronal pathway, Fox-1 and Fox-2 proteins repress exon 4 inclusion by binding to two UGCAUG sequences surrounding the 3' splice site of the exon, therefore, spatially blocking U2AF65 recognition of the splice site [106, 107]. In the non-neuronal pathway, an exonic

splicing enhancer (ESE) on exon 4 promotes its inclusion by interacting with Tra2β and SRp55 [103-105]. Meanwhile, an intronic splicing enhancer (ISE) containing a pseudo-5' splice site sequence downstream of the exon 4 binds with PTB, SRp20, U1 snRNP and TIAR to facilitate alternative polyadenylation resulting in the expression of calcitonin [98, 108, 119].

3.2 Alternative splicing in hormone receptors and signalling pathways

The specific affinitive binding of the hormone receptors to the hormones triggers multiple signalling pathways (intracellular signal transduction) [1, 41]. Alternative splicing in hormone receptors altering their properties could lead to changes of their binding specificity, signalling initiation or localization.

Growth hormone-releasing hormone (GHRH) receptor provides an excellent example for alternative splicing events changing receptor affinity and signal transduction. It is a member of the seven-transmembrane, G protein-coupled receptors (GPCRs) superfamily, which is the largest and most diverse group of membrane receptors in eukaryotes [94]. Accumulating evidences indicate the role of alternative splicing in diversifying the activities of G protein-coupled receptors. GHRH receptor binds to growth hormone-resealing hormone, which is a hypothalamic peptide hormone stimulating the synthesis and secretion of growth hormone (GH) [93, 95, 101]. The alternative splicing of GHRH receptor occurring at intron 10 yields distinctive isoforms, leading to different functional consequences. The alternative splicing in rats generates a 123 base-pair intron retention adding a peptide in the third intracellular loop (an important domain to G protein interaction) [96, 100]. This change does not affect the

GHRH binding affinity but invalidates the response to cAMP-medicated signalling [100]. Alternative splicing at the same location in humans results in a 561 base-pair intronic insertion that contains a premature stop codon [97, 102]. This splice variant encodes a truncated protein lacking the sixth and seventh transmembrane domains, which is unable to interact with normal GHRH and transmit signals through the cAMP pathway [97, 99]. It serves as a dominant-negative isoform because of its ability to abolish normal GHRH binding by forming a heteroreceptor complex with the wild-type receptor [99].

Alternatively spliced human luteinizing hormone/choriogonadotropin (LHCG) receptor lacking exon 9 results in the retention of the isoform within the cells instead of at the cell surface [118]. Upon co-transfection of both the isoform and the normal LHCG receptor, they can form a heteroreceptor complex that drives the normal LHCG receptor to the lysosome for degradation and consequentially decreases the expression of normal LHCG receptor at the cell surface [118]. In rats, 3' partial skipping of exon 11 encodes a receptor isoform missing the seven transmembrane domains and the C-terminal intracellular domain [109]. This LHCG receptor isoform is retained in the lumenal side of the endoplasmic reticulum (ER) [109].

Taken together, these observations demonstrate that alternative splicing regulates the function of hormone receptors and their intracellular signaling.

3.3 Alternative splicing in hormone metabolism

Hormone metabolism includes the synthesis, degradation and recycling of hormones and their receptors. As expected, alternative splicing happening in the related regulatory factors, kinases or enzymes may result in functional alteration of the protein and thereby influence the hormone homeostasis.

Insulin-degrading enzyme (IDE) is a zinc metalloendopeptidases served in the degradation of insulin, amyloid β-protein (Aβ) and many other small proteins [111, 114]. Human IDE consists of 25 exons; its exon 3 encodes HXXEH catalytic site (H is histidine, E is glutamate, X is any amino acid) [110]. A recent study identified a novel exon 15b (145 base-pair) that replaces the original exon 15 (145 base-pair) and forms a protein with the same length as the canonical one [120]. Same as the normal IDE, this isoform is predominately located in the cytosol and is marginally expressed in many tissues including liver and brain [120]. Trichloroacetic acid precipitation assays and measurement of insulin and Aβ in conditioned medium reveal that the isoform has inefficient proteolytic activity, which results in the accumulation of insulin and Aβ, and consequently type 2 diabetes mellitus and Alzheimer's disease [115-117, 120].

In another case, an intronic insertion at the 5' end of intron 14 of the pyroglutamyl peptidase II (PPII), a neuropeptide thyrotropin-releasing hormone (TRH) degrading ectoenzyme, encodes a C-terminal truncated PPII, which fails to hydrolyze TRH [112, 113]. The short PPII isoform is able to heterodimerize with the normal form and decreases the activity of PPII [113].

Therefore, alternative splicing also regulates the metabolism of hormones.

4. Alternative splicing in endocrine disorders

The complicated hormone regulation is exquisite, and when disrupted in even the slightest way, can cause disorders [121-161].

Isolated growth hormone deficiency (IGHD) is the most common pituitary hormone deficiency, which can be described into four types: autosomal recessive (type

IA and IB), autosomal dominant (type II) and X-linked recessive (type III) [162]. Known genes involved in the genetic etiology of IGHD include growth hormone gene *GH1* (type I and II), growth-hormone-releasing hormone receptor gene *GHRHR* (type IB), transcript factor gene *SOX3* and Bruton's tyrosine kinase gene *BTK* (type III) [126, 134, 148, 157, 163].

4.1 *GH1* mutations in alternative splicing

GH1 gene contains five exons that encode the 22kDa full-length growth hormone protein with a small portion of alternatively spliced variants (5-10%) under normal physiological conditions [133, 143]. A 20kDa GH (5-10%) is generated by the usage of weak canonical splice site to remove the first 45 nucleotides of exon3; 17.5kDa GH (1-5%) occurs upon exon 3 exclusion [142, 143].

Deletion, frameshift and nonsense mutations of *GH1* have been reported as the causes of IGHD type IA [122, 138, 140]. Mutation at the donor splice site of *GH1* gene intron 4 disrupts the mRNA splicing and causes a bio-inactive form of growth hormone, resulting in IGHD type IB [128]. The dominant negative effect of a 17.5kDa growth hormone isoform is a major cause of type II IGHD [129]. Moreover, mutations in both intronic and exonic elements increase the expression of both exon 3-skipped 17.5kDa and 20kDa isoforms by 35-68% and 20-37%, respectively, accompanied by the decrease of the 22kD isoform. For intronic elements, mutations in the first six nucleotides of intron 3, the intronic splice enhancer motif (ISE) and branching point site in the intron 3 have been found to cause 17.5kDa GH and type II IGHD [135-137, 141, 164]. Exon 3 is also important for the skipping of itself. Four exonic splice enhance motifs (ESE) have been

reported to date to be critical for the integrity of the splicing mechanism, with ESE1 at the 5' of exon 3, ESE2 located 12 nucleotides upstream of the weak canonical splice site, newly discovered pESE1 and pESE2 closed to the 3' of exon 3 [125, 130, 144, 146, 147, 151, 160]. The 17.5kDa fails to form a disulfide bond between the first two helices of growth hormone because of the lack of protein linker domain and cysteine residue (Cys53), which retains the isoform in the endoplasmic reticulum, disrupting secretory pathway and trafficking of growth hormone and even other hormones, for instance prolactin, adrenocorticotropic hormone and luteinizing hormone [145, 150, 152, 153].

4.2 *GHRHR* mutations in alternative splicing

A recent study has identified a compound heterozygous *GHRHR* gene mutation in intron 2 that activates the cryptic donor splice site, resulting in the generation of two aberrant spliced variants and a significant decrease in canonical growth hormone proteins [158]. In fact, over 20 mutations of GHRH receptor have been found to date to cause isolated growth hormone deficiency [124, 131, 139, 154, 156, 158]. Mutations at introns 1, 7 or 13 can result in abnormal splicing of GHRHR transcripts [123, 149, 158, 160]. Mutations at intron 3 or 8 introduce in-frame stop codon in the site and generate abnormal truncated proteins [155, 161]. These mutants all affect the stimulation of growth hormone production and thereby ultimately cause dwarfism of patients.

4.3 *BTK* mutations in alternative splicing

Among other genes that are associated with type III IGHD, mutations of the *BTK* gene have been reported to result in growth hormone insufficiency associated with

abnormal splicing. A single base-pair insertion in exon 6 of *BTK* generates premature stop codon in exon 7, which leads to hypogammaglobulinemia and isolated growth hormone deficiency [121, 159].

Besides isolated growth hormone deficiency, accumulating evidence has pointed to the emerging role of alternative splicing in endocrine diseases with a wider impact on the endocrine system. For instance, inclusion of cryptic exon b2 of androgen receptor gene generates a C terminal truncated protein that is constitutively active and promotes the expression of endogenous AR-dependent genes in a prostate-cancer-therapy-resistance manner [132]. Cyclic AMP-responsive element modulator gene *CREM* has complicated splicing regulation on the fine-tuning between CREM activator and repressor isoforms [127]. The alternative usage of leader exons and the combination of the following exons encode either transcriptional activators or repressors with strong or weak signals, in which deregulation of activators can have male infertility [127]. Nevertheless, the majority of cases in endocrine disorders are idiopathic with no clarified etiology.

Taken together, these reports indicate that alternative splicing plays an important role in the development of the endocrine system, its function, hormone metabolism as well as the development of endocrine diseases. However, in most cases, the role of most of the RNA binding proteins/splicing factors in these processes remain unclear.

5. RNA binding proteins hnRNPs and alternative splicing

Alternative splicing is regulated by *cis*-acting elements within the pre-mRNA and *trans*-acting RNA binding proteins (RBPs), although the majority of the reported

alternative splicing events lacks the identification of the responsible regulatory factors by far. RBPs regulate alternative splicing by interacting with *cis*-acting elements. Two major families of RBPs are the arginine/serine-rich SR protein family and the heterogeneous nuclear ribonucleoprotein (hnRNP) family. HnRNPs consist of one or more RNA recognition motifs (RRM) and at least one auxiliary domain for protein-protein interactions [165]. They are able to shuttle between the nucleus and the cytoplasm and function in post-transcriptional regulation [165]. Of particular interest is hnRNP L and its paralog hnRNP L-like. These two proteins share 68% amino acid sequence homology and the same domain organization of four RRMs [166]. They both favour CA-repeat/CA-rich sequences with distinct specificity, of which the molecular basis is unclear [167-170]. They are ubiquitous and multi-functional: hnRNP L is important for transcription, pre-mRNA processing, mRNA exportation, translation and mRNA stability [171-175]. HnRNP LL is much less known, with only its role in transcription and pre-mRNA processing described by far [170, 176].

Another hnRNP protein that appeals to us is the polypyrimidine tract-binding protein (PTB; also known as hnRNP I). It contains four RRMs and preferentially binds to the polypyrimidine tracts (Py-tracts) of RNA. PTB has been reported to play roles in polyadenylation, RNA splicing, mRNA stability, translation initiation [177-180]. PTB displays 55% amino acid sequence similarity to hnRNP L (32% identity between the RNA recognition motifs) [181, 182]. HnRNP L protein contains a proline-rich region that can bind PTB, whereas hnRNP LL does not have this region [167]. Interaction of hnRNP L with PTB has been reported affecting RNA processing and protein translation in different ways [167, 175, 181-183].

6. Questions leading to the current research of the thesis

Regarding the alternative splicing events happening in the endocrine system, a few RBPs have been identified in some cases. For instance, polypyrimidine tract-binding protein-associated splicing factor (PSF), SF2/ASF and hnRNP H are found to regulate the alternative splicing of the thyroid hormone receptor pre-mRNA [184, 185]. Heterogeneous nuclear ribonucleoprotein (hnRNP) G modulates the splicing of estrogen receptor alpha (ERα) exon 7 [186]. HnRNP E1 is associated with the suppression of a growth hormone receptor pseudo-exon [187]. ASF/SF2 and SC35 control the alternative splicing of growth hormone (GH) [188, 189]. RNA binding factors not only regulate the splicing of targeted genes, but are also involved in other regulatory steps of gene expression. For instance, hnRNP D affects the RNA stability of parathyroid hormone (PTH); hnRNP A/B and hnRNP G control the transcription of gonadotropin-releasing hormone 1 (GnRH1) [190, 191].

Studies in our lab have found that two paralogous RNA binding factors hnRNP L and LL regulate the pre-mRNA splicing of hormone-related genes in GH_3 pituitary cells [192-194]. However, their effects and roles on hormone production (synthesis or secretion) are unknown. More importantly, to our best knowledge, whether the paralogous hnRNPs have similar or distinct roles in hormone gene regulation is not known. Knowledge of the similarity, distinction and cooperation between paralogous hnRNPs in the endocrine system is very limited. In fact, only a handful of studies explored the function of hnRNP L-like mainly the in immune system [167, 169, 170, 176, 195-198]. Investigating the role of paralogous hnRNPs on hormone production can shed

light on the above issues. This study aimed to determine the regulatory roles of hnRNP L and LL in hormone production.

7. Hypothesis

Based on the functions of these two RNA binding factors reported in other researches and the findings in our previous studies, we hypothesize that hnRNP L and LL regulate the production of growth hormone and prolactin by affecting the transcriptional and splicing process of the two hormone genes.

CHAPTER II

Materials and Methods

Materials

Mammalian cell lines

GH_3	rat pituitary tumor cells
HEK293T	human embryonic kidney cells

Materials for cell culture

Cell culture plate	BD FALCON
Disposable serological pipet	FALCON
F-10 Nutrient Mixture (Ham) (1X), liquid	Invitrogen
Fetal bovine serum (FBS)	Invitrogen & Sigma
Gibco® Antibiotic-Antimycotic	Invitrogen
Gibco® Dulbecco's Modified Eagle Medium (D-MEM) powder	Invitrogen
Gibco® Penicillin-Streptomycin, 100X Solution	Invitrogen
Horse serum (HS)	Invitrogen
Iscove's Modified Dulbecco's Medium (IMDM), liquid	Hyclone
L-Glutamine, 200mM Solution	Invitrogen
Polypropylene centrifuge tubes	BD FALCON
Polypropylene tubes with snap cap	VWR
T175 tissue culture flask	FALCON

Trypsin, 2.5% Cellgro

***E.coli* strains**

DH5α A gift from Dr. Peter A Cattini's lab

Antibodies

Name	**Source**
Anti-β-actin (C4)	Santa Cruz
Anti-c-Myc (A-14)	Santa Cruz
Anti-c-Myc (9E10)	Santa Cruz
Anti-FLAG® M2 (F1804)	Sigma-Aldrich
Anti-hnRNP L (4D11)	Santa Cruz
Anti-hnRNP LL (#4783)	Cell signaling
Anti-hnRNP K/J (3C2)	Santa Cruz
Anti-hnRNP F/H (1G11)	Santa Cruz
Anti-rat pituitary growth hormone	NHPP. Harbor-UCLA Medical Centre
Anti-Prolactin (6F11)	Pierce
Goat anti-Mouse IgG (Fc specific)-Peroxidase	Sigma-Aldrich
Goat anti-rabbit IgG-HRP	Santa Cruz

Chemicals and reagents

Name	**Source**
ß-Mercaptoethanol	Sigma-Aldrich
Acrylamide: Bis-Acrylamide 29:1	Fisher
Agarose RA™	AMRESCO
Ammonium Sulphate	Fisher
Ampicillin	Fisher
Bovine serum albumin, RNase free	Fisher
Bromophenol Blue	Fisher
Calcium chloride	Fisher
Coomassie brilliant blue R250	Fisher
Deoxycholic acid, sodium salt	Fisher
Dimethyl Sulfoxide (DMSO)	Fisher
Ethanol	Fisher
Ethidium bromide (EtBr)	Sigma-Aldrich
Ethylenediamine Tetraacetic Acid (EDTA)	Fisher
Gibco® D-Glucose	Sigma-Aldrich

Glycerol	Fisher
Glycine	Fisher
IGEPAL® CA-630	Sigma-Aldrich
Instant skim milk powder	Carnation Milk
Isopropanol	Fisher
Magnesium chloride	Fisher
Methanol	Fisher
$MgCl_2$ 25mM	Promega
N,N,N´,N´-tetramethylenediamine (TEMED)	Fisher
Phenylmethylsulfonyl fluoride (PMSF)	Fisher
Phosphate Buffered Saline (PBS) Tablet	MP Biomedicals
Polyoxyethylene-20-sorbitan Monolaurate (Tween 20)	Fisher
Poly (ethylene glycol), M.W. 8000	Sigma-Aldrich
Ponceau S	Fisher
Potassium chloride (KCl)	Fisher
Sodium chloride (NaCl)	Fisher
Sodium dodecyl sulfate (SDS)	Fisher
Sodium fluoride (NaF)	Sigma-Aldrich
Sodium orthovanadate (Na_3VO_4)	Fisher
Tris base	Fisher (J.T. baker)
Triton X-100	Fisher
TRIzol reagent	Invitrogen

Enzymes and buffers

Product	**Source**
5x First strand buffer	Invitrogen
5x Forward buffer	Invitrogen
Benzonase® Nuclease	Sigma-Aldrich
Collagenase D	Roche Diagnostics
dNTP set	Invitrogen
DpnI	New England Biolabs
Dithioreitol (DTT), 1M	Invitrogen
Mg free buffer M190G	Promega
M-MLV Reverse Transcriptase	Invitrogen
Phusion® High-Fidelity DNA Polymerase	New England Biolabs
Protease inhibitor cocktail	Sigma-Aldrich
SUPERase• In™ RNase Inhibitor	Invitrogen
Taq DNA polymerase	Xie lab
Taq DNA polymerase M186E	Promega

Kits

Kit	Source
GenElute Mammalian Total RNA Miniprep Kit	Sigma-Aldrich
RNeasy® Plus Mini Kit	QIAGEN
QIAquick® Gel Extraction Kit	QIAGEN
QIAprep® Spin Miniprep Kit	QIAGEN

Others

Product	Source
Acrodisc® Syringe Filters (0.45 μm)	PALL
Chromatography paper	Whatman
CL-XPOSURE FILM	Fisher
ECL Western blotting detection reagent	GE Healthcare
Human Neuregulin-1	Cell Signaling
LB Agar	Fisher
LB Broth	Fisher
Lipofectamine® 2000	Invitrogen
Lipofectamine® 3000	Invitrogen
PolyJet™ DNA In Vitro Transfection Reagent	SignaGen
polyvinylidene difluoride membrane	Millipore
Rapid-Flow™ Filter Units (0.22 μm)	NALGENE
Syringe Filters Cellulose Acetate (0.2 μm)	NALGENE
Avanti® J-E, rotor JA-25.50 (for lentiviral production)	BECKMAN COULTER

Buffers and solutions

0.5 M EDTA·2Na

$EDTA \cdot 2Na \cdot 2H_2O$	37.224 g
ddH_2O	150 ml

Adjust pH to 8.0 with NaOH pellets.

Fill up with ddH_2O to 200 ml, autoclave, stored at 4 ˚C.

1 M Tris·HCl

Tris	60.57 g

ddH_2O		400 ml

Adjust pH to 7.5 with 6 N HCl.

Fill up with ddH_2O to 500 ml, autoclave, stored at 4 ˚C.

1 × TE Buffer

10 mM	Tris·HCl	2.0 ml
1 mM	EDTA	0.4 ml

Fill up with ddH_2O to 200 ml, autoclave, stored at 4 ˚C.

4 × Separating Buffer (pH 8.8)

1.5 M	Tris Base	36.342 g
0.4%	SDS	0.8 g
	ddH_2O	150 ml

Adjust pH to 8.8.

Fill up with ddH_2O to 200 ml, through 0.45 μm filter, stored at 4 ˚C.

4 × Stacking Buffer (pH 6.8)

0.5 M	Tris Base	12.114 g
0.4%	SDS	0.8 g
	ddH_2O	150 ml

Adjust pH to 6.8.

Fill up with ddH_2O to 200 ml, through 0.45 μm filter, stored at 4 ˚C.

6 × SDS Loading Dye (50 ml)

300 mM	Tris· HCl (pH 6.8)	15 ml
50%	Glycerol	25 ml
6%	ß-Mercaptoethanol	3 ml

12%	SDS	6 g
0.12%	Bromophenol Blue	6 mg

Mix well, stored at -80 ˚C.

10 × PCR Buffer (40 ml)

200 mM	Tris·HCl (pH 8.3)	8.0 ml
15 mM	$MgCl_2$	6.0 ml
250 mM	KCl	2.5 ml
0.5%	Tween-20	667 μl
1 mg/ml	BSA (Inactivated)	800 μl
	ddH2O	22.033 ml

Stored in aliquots at -20 ˚C.

10 × SDS Running Buffer (pH 8.3)

250 mM	Tris Base	30.285 g
1.92 M	Glycine	144.128 g
1%	SDS	10.0 g

Fill up with ddH_2O to 1 L.

10 × TBS Solution

5 M	NaCl	292.5 g
0.2 M	Tris·HCl (pH 7.5)	200 ml

Fill up with ddH_2O to 1 L, through 0.45 μm filter, stored at room temperature.

20× Transfer Buffer (pH 8.3)

250 mM	Tris Base	30.285 g
1.92 M	Glycine	144.128 g

Fill up with ddH_2O to 1 L.

Blocking Buffer

10 × TBS	20 ml
Instant skim milk powder	10 g
ddH_2O	180 ml

Mix well, stored in aliquots at -20 ˚C.

NaF (Sodium Fluoride) 200 mM

NaF	0.4199 g
ddH2O	50 ml

Mix well, stored at 4 ˚C.

NP-40 Buffer (Nonidet-P40)

150 mM	NaCl	4 M
10 mM	Tris· HCl (pH 7.5)	1 M
1 mM	EDTA·2Na	0.5 M
0.325%	Igepal CA630	20%

Fill up with ddH_2O to 200 ml, autoclave, stored at 4 ˚C.

PCR Mix I (21.64 ml)

10 × PCR buffer	2.5 ml
dNTP (100 mM) 2.5 µl each	50 × 4 µl
MgCl2	125 µl
ddH2O	18.815 ml

Final concentration of Mg^{++} in PCR reaction is 2mM.

Stored in aliquots at -20 ˚C.

PMSF (Phenylmethylsulfonyl Fluoride) 100 mM

PMSF	0.34 g
100% ETOH	20 ml

Stored at 4 ˚C.

RIPA Buffer (RadioImmunoPrecipitation Assay)

150 mM	NaCl	4M
50 mM	Tris· HCl (pH 7.5)	1M
1%	Triton X-100	20%
0.5%	Deoxycholic Acid	1.0 g
1%	SDS	0.2 g

Fill up with ddH_2O to 200 ml, autoclave, stored at 4 ˚C.

RT Mix I

5 × First strand buffer	400 μl
dNTP (100 mM) 2.5 μl each	2.5 × 4 μl
DTT (0.1 M)	200 μl
ddH2O	90 μl

Mix well, stored at -20 ˚C.

Sodium Vanadate (Na_3VO_4) 100 mM (Need be activated)

Na_3VO_4	0.9196 g
ddH_2O	50 ml

Adjust pH to 10.0 with 1 N HCl, boil it until colorless, cool down to room temperature.

Re-adjust pH to 10.0, boil it again until colorless.

Repeat the steps until the solution remains at pH 10.0 after boiling and cooling.

Fill up with ddH_2O to 200 ml, stored in aliquots at -20 ˚C.

Stripping Buffer

62.5 mM	Tris· HCl	12.5 ml
2%	SDS	40 ml
0.7%	ß-mercaptoethanol	1.6 ml
	ddH_2O	100 ml

Adjust pH to 6.8. Fill up with ddH_2O to 200 ml.

Stored at room temperature.

Methods

Plasmid Construction

The lentiviral plasmid pFG12-shL (shL), which targets the 3' untranslated region of hnRNP L, was lab-designed as described previously [194]. The pLKO.1-shLL (shLL) against the open reading frame of hnRNP LL was purchased from Open Biosystems (clone ID TRCN0000075101) [193].

The hnRNP L-FLAG expression plasmid was provided by Drs. Peter Stoilov and Stefan Stamm as in our previous studies [192-194]. Human hnRNP LL cDNA was purchased from Open Biosystems and was cloned into pCMV-Myc [193]. These two plasmids hnRNPL-FLAG and Myc-hnRNP LL were then subcloned into the lentiviral vector cppt2E as described [193]. To avoid being targeted by the shLL, a silent mutant of cppt2E-Myc-hnRNP LL was generated by site-directed mutagenesis polymerase chain

reaction (PCR). It contains three mutation sites (below) to ensure sufficient insensitivity to the shLL.

By referring to the sequence of human hnRNP LL isoform 1:

Site 1: T-342-A, Ala-114-Ala

Site 2: G-348-T, Val-116-Val

Site 3: A-351-G, Glu-117-Glu

The primers used for the mutagenesis PCR are shown below.

Forward strand (shLL target region bolded, mutated sites underlined):

5'-CATTTAAA**CGACAGGCACTAGTTGAGTTT**GAAAACATAGATAGTGC-3'

Reverse strand:

5'-CTATCTATGTTTTC**AAACTCAACTAGTGCCTGTCG**TTTAAATGGCATC-3'

A 25 μl mutagenesis PCR reaction was carried out with 50ng cppt2E-Myc-hnRNP LL plasmid template, primers as mentioned above, 2 units of Pfu DNA polymerase and PCR Mix I for 18 cycles (denaturation at 95 °C for 60 s, annealing at 55 °C for 60 s, elongation at 68 °C for 20 minutes (2 minutes per kb of plasmid)). PCR product was digested with 10 units of DpnI at 37 °C for 1 hour. The nicks of the digested dsDNA were repaired by DH5α upon transformation. The cppt2E-Myc-hnRNP LL (silent mutant) was confirmed by sequencing.

Cell Culture

Rat GH_3 pituitary cells spontaneously produce and secrete growth hormone and prolactin. This tumor cell line is a well-established *in vitro* model widely used in hormone studies. Previous studies in our lab have shown that hnRNP L and LL regulate

the alternative splicing of three hormone-related genes, which are including the big potassium (BK) channel gene *Slo1*, the Neuregulin-1 gene *Nrg-1* and the phosphatidylinositol transfer protein beta gene *Pitpnb* [192, 193, 199]. Therefore, the same cell line was chosen as an experimental model in this study. GH_3 cells were maintained in Ham's F10 nutrient mixture with 10% of horse serum (HS), 2.5% of fetal bovine serum (FBS) and 1% of penicillin-streptomycin-glutamine solution (PSG).

HEK293T human embryonic kidney cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplied with 10% FBS and 1% PSG. For lentivirus production, HEK293T cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS and 1% PSG at about 30 min prior to transfection.

For primary pituitary cell culture, Sprague Dawley rats were originally from the University of Manitoba Genetic Modeling of Disease Centre (GMC) and bred at the Central Animal Care Services (CACS) (Abbreviated Protocol for Minimal Animal Involvement, doc08087920140829142937). Adult (5-6 month) rats underwent euthanasia via carbon dioxide inhalation. Following decapitation the craniums were immediately opened and the whole pituitaries were dissected free of the pituitary capsule *in situ*. The glands were then harvested and washed in calcium-free phosphate buffered saline (PBS) with 0.1% bovine serum albumin, 1% of penicillin-streptomycin and 1% Antibiotic-Antimycotic (0.1% BSA/PSA-PBS). After several washes, the glands were minced into 1-3 mm pieces and incubated with 0.1% BSA/PSA-PBS containing 2 mg/ml Collagenase D, 2.5 mM $CaCl_2$ and 250 unites/ml Nuclease at 37 °C for 10 min. The dispersed cells were collected in FBS with 5 mM ethylenediamine tetraacetic acid (EDTA). Fresh digestion solution was added to the residual tissue fragments for further disaggregation,

and steps were repeated until all the fragments were dissociated. Cells were pelleted and resuspended in Ham's F10 nutrient mixture supplemented with 10% HS, 10% FBS and 1% PSG, followed by 72 h incubation at 37 °C, 5% carbon dioxide.

Transfection

Overnight cultures of HEK293T cells were transfected with Lipofectamine 2000 or 3000 according to the manufacturer's protocols. For the HEK293T cells in 12-well plates at about 90% confluence, 1 μg of cppt2E-Myc-hnRNP LL or cppt2E-Myc-hnRNP LL (silent mutant) were used. Transfected cells were analyzed after 72 h incubation.

Lentivirus Production

Lentiviruses were generated by the cotransfection of HEK293T cells with the packaging plasmid pCMV-dR8.2 dvpr, the envelope plasmid pCMV-VSV-G and expressing plasmid pFG12-empty vector, pLKO.1-EGFP or cppt2E-GFP for vector control, pFG12-shL or pLKO.1-shLL for gene knockdown, cppt2E-hnRNPL-FLAG or cppt2E-Myc-hnRNPLL (silent mutant) for gene overexpression using PolyJet^{TM} DNA *in vitro* transfection reagent according to the instructions of the producer.

HEK293T cells in T175 flasks at about 90% confluence were transfected with 11.25 μg of pCMV-dR8.2 dvpr, 3.75 μg of pCMV-VSV-G, and 15 μg of the expressing plasmids. Culture medium was changed at 12 h after transfection. At 24 h, 48 h and 72 h post-transfection, culture supernatant was collected and filtered through a 0.22 μm filter.

Precipitation and concentration (about 100 times) of viral supernatant was performed with 8% PEG8000 and 250 mM NaCl, and centrifuged at 17,000 rpm for 30 min. Virus pellets were resuspended in culture medium.

Table 1. Plasmids for lentivirus production

RELEVANT CHARACTERISTICS	PLASMID
Packaging plasmid	pCMV-dR8.2 dvpr
Envelope plasmid	pCMV-VSV-G
Vector control plasmid	pFG12-empty vector pLKO.1-EGFP cppt2E-GFP
Gene knockdown plasmid	pFG12-shL pLKO.1-shLL
Gene overexpression plasmid	cppt2E-hnRNPL-FLAG cppt2E-Myc-hnRNPLL (silent mutant)

Lentiviral Transduction

Culture medium was refreshed 1 day before transduction. For gene knockdown or overexpression in GH_3 cells, lentivirus dilution containing 100 μl lentivirus stock, 250 μl F10 culture medium and 0.8 ng/μl Polybrene was prepared on the next day. About 2 x 10^5 cells was incubated with the lentivirus dilution at 37 °C, 5% CO_2 for 3 h before the lentivirus dilution was replaced by fresh medium. Culture medium was changed on the 3rd and 5th day post transduction. On day 7 (the 6th day post transduction), the cells and conditioned medium were harvested for protein and RNA analysis. 2 mM PMSF, 2 mM Na_3VO_4, 2 mM NaF and Protease Inhibitor Cocktail were added to conditioned medium.

Method for concurrent rescue of RNAi targeted genes was almost identical to that of gene knockdown, except that 100 μl shRNA lentivirus stock, 100 μl RNAi-resistant gene expression lentivirus stock, 150 μl F10 culture medium and 0.8 ng/μl Polybrene were contained in the lentivirus dilution.

For gene knockdown and rescue in rat primary pituitary cells, small alterations to transduction protocol were made. Briefly, cells were placed in 6-well plates, followed by 24 h incubation in 2 ml lentivirus dilution per well. For gene silencing, each lentivirus dilution comprises of 200 ul lentivirus stock and 0.8 ng/ul Polybrene; for rescue, 200 ul shRNA lentivirus stock, 200 ul RNAi-resistant gene expression lentivirus stock and 0.8 ng/ul Polybrene were included.

Western Blot Analysis

Western blot was performed according to [200] with slight modifications. For cell lysates, approximately 10 μg of total protein or 5 μg of nuclear protein was subjected to 10% SDS-PAGE. For detecting secreted hormones, 7.5 μl of conditioned medium (12-24 hours) was subjected to 12% SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membranes (PVDF) at 100 mA for overnight at 4 °C, followed by blocking in blocking buffer for 1 h at room temperature. Primary antibody was incubated for overnight at 4 °C. Secondary antibody was incubated for 1 h at room temperature with a dilution of 1:3000. All incubations and washes were in blocking buffer or TBS, respectively. Proteins were detected by applying ECL Western blotting detection reagent and exposed to X-ray films.

For reprobing, the blots were incubated in stripping buffer for 15-30 min at 62 °C, followed by sufficient wash in TBS, and then proceeded with blocking and antibody incubations as mentioned above.

Regarding the loading control of conditioned medium, firstly Ponceau S staining after protein transfer confirmed the similar loading. Secondly, the protein level of hnRNP

F/H was used as the loading control for the equal amount of total proteins of cells that secreted hormones detected in the same volume of conditioned medium.

RNA Sequencing (RNA-Seq)

The knockdown efficiency of GH_3 cells with shL or shLL was confirmed by western blot. Approximately 3 μg of total RNA from Mock (non-transduced, treated with 0.8 ng/μl Polybrene), L knockdown (shL) and LL knockdown (shLL) samples (each in triplicate) were used as templates for cDNA library construction and Illumina Hi-Seq sequencing. RNA quality prior to sequencing was analyzed on Nano Chip Bioanalyzer and Nanodrop to ensure RIN ≥ 8.

RNA quality control, library preparation, Illumina Hi-Seq 2000/2500 paired-end 100bp sequencing and reference mapping to the *Rattus norvegicus* genome (rn5) were conducted by the McGill University and Génome Québec Innovation Centre (Montréal, Québec, Canada). An average of 129±0.22 million purity filtered reads were acquired with 91±0.20 million of uniquely mapped pairs and 15169±0.02 mapped genes for each samples. The average mapping rate and the unique mapping rate were 100%±0.00 and 71%±0.03, respectively.

Bioinformatics Analyses of RNA-Seq Read Data

The differential gene expression and aberrant splicing were calculated in the Bioconductor package edgeR [201, 202]. Alternative exon usage was calculated in the package DEXSeq [203]. Difference at adjusted p values < 0.05 were considered significant.

Visualization of sequencing data was presented in Integrative Genomics Viewer (IGV) (developed at the Broad Institute of MIT and Harvard, available for download from http://www.broadinstitute.org/igv)[204].

Functional annotation and classification of significant genes was done with a Database for Annotation, Visualization, and Integrated Discovery (DAVID) (developed at the U.S. National Institute of Allergy and Infectious Diseases, available on the Internet at http://david.abcc.ncifcrf.gov)[205] and Gene Ontology Consortium (GOC) (a web tool that is accessible at http://geneontology.org/)[206, 207].

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cytoplasmic RNA was fractionated and extracted according to our previous procedures [194, 208]. About 400 ng of cytoplasmic RNA was used for 10 μl of reverse transcription reaction. PCR reaction was performed for 28 cycles for *Actb* and *Gh1*, 30 cycles for *Prl*. PCR products were resolved in 1.5-3.5% agarose gels containing ethidium bromide (EtBr) and visualized with a digital camera under ultraviolet (UV).

GH_3 total RNA for RNA-Seq was extracted using RNeasy® Plus Mini Kit (QIAGEN) following its protocol. Similar RT-PCR procedures were followed for validation, except about 500 ng of total RNA was used for 10 μl of reverse transcription reaction. The number of PCR cycles was 28-39 for different genes.

Rat pancreas total RNA was extracted using TRIzol reagent according to the manufacturer's directions.

Table 2. Primers for RT-PCR analysis

Sequence Amplified	Primers (5'->3')	Exon Location	PCR Conditions	Fragment Size (bp)
Gh1 exon 1-5	F: CAGGTCCTGTGGACAGATCAC R: TGGCAGTTGCCAGAGTACAG	1 5	2 mM $MgCl_2$ 60 °C	741
Prl exon 1-5 /3-5	F: CAGTGGTCATCACCATGAACAG F: GCTACTCCTGAAGACAAGGAACAAG R: TGCTGAAAGTTGTAATGCAAATAGAAC	1 3 5	2 mM $MgCl_2$ 60 °C	778/494
Cdh1 exon 6-9	F: AAGGCGCTCTTCCAGGAAC R: GCACTTGACCCTGGTACGTG	6/7 8/9	2 mM $MgCl_2$ 60 °C	336
Cdh2 exon 4-6	F: GCAAGACTGGATTTCCTGAAG R: GATGACCCAGTCTCTCTTCTGC	4 6	2 mM $MgCl_2$ 60 °C	395
Actb exon 3-4	F: TGGGACGATATGGAGAAGATTTG R: CCATCACAATGCCAGTGGTAC	3 4	2 mM $MgCl_2$ 60 °C	226
Gapdh exon 3-4	F: CTTCATTGACCTCAACTACATGGTT R: GCTCCTGGAAGATGGTGATG	3 3/4	2 mM $MgCl_2$ 60 °C	131
Adm exon 5-7	F: GTGCAATGCTTGTTATACAGCC R: CTTCATCCACAGGCGGTAAT	5 7	2 mM $MgCl_2$ 60 °C	288
Atp2b2 exon 25	F: GAGTCCTTTGTCACATTTTTCATC R: TGTACTACAGTGAGTTCTGACCAACC	25 25	2 mM $MgCl_2$ 60 °C	332
Car9 exon 9-11	F: CATGTGAATTCCTGCCTCTCT R: CACTTGCTTAGCAAGCTCGAG	9 11	2 mM $MgCl_2$ 60 °C	329
Chaf1b exon 13-14	F: GAAGAGGTCCAGCCAGAGGT R: CGCTCTAATATCACCATCACCAG	13 14	2 mM $MgCl_2$ 60 °C	200
Chgb Exon 4-5	F: CTGACCAGAGAGTTCTGACCG R: AAAGACAGTAACACGGTCATTGTC	4 5	2 mM $MgCl_2$ 60 °C	320
Filip1 exon 6-7	F: TTTGCTGAATGTTCTGTTGAAGAT R: CCCTGTGATAGCTCTGAAAGTATTC	6 7	2 mM $MgCl_2$ 60 °C	324
Fsd2 exon 12	F: AACTGCAGTTTGTGCTCTGTAAAG R: AGCACTCGTCACAGGAGCAG	12 12	2 mM $MgCl_2$ 60 °C	353
Fstl3 exon 2-4	F: CAATAAAATCAGCCTGCTAGGATTC R: CCGAACTCCAATGGAACG	2 4	2 mM $MgCl_2$ 60 °C	480
Ghrl exon 1-4	F: ATGGTGTCTTCAGCGACTATCTG R: GCAGAAGCTGGATGTGAGTTC	1 4	2 mM $MgCl_2$ 60 °C	414
Hmox1 exon 5	F: TCCTGTGTCTTCCTTTGTCTCTC R: AAACAAGACAGAAACACAAGACAGAA	5 5	2 mM $MgCl_2$ 60 °C	272
Ins2 exon 3	F: TTCAGACCTTGGCACTGGAG R: CAGAGGGGTGGACAGGGTAG	3 3	2 mM $MgCl_2$ 61 °C	132
Insl6 exon 1-2	F: AGCAGCTGTGCTGTTCTTGTC R: ACCAGTGATGTTTGTTTAATCGTTAAG	1 2	2 mM $MgCl_2$ 60 °C	591
Mr1 exon 6	F: CTTTGGCTCCAGAAGAACTGTC R: AAGAGCCAAGTCTGTGACATTTTAG	6 6	2 mM $MgCl_2$ 60 °C	176
Nrg1 exon 2-5	F: GGGACCAGCCATCTCATAAAG R: CCACCAGCAGGGCGATAC	2 5	2 mM $MgCl_2$ 60 °C	243, 258, 312
Olr321 exon 1	F: GTCACTACCCTCTGGTTTATCTTCC R: AGAGTGTAGATCAATGGGTTCAATAG	1 1	2 mM $MgCl_2$ 60 °C	250
Olr324 exon 1	F: ATCACGACACTGTGGTTTGTTC R: TCAGGACAGGGACAATTATATTTAGG	1 1	2 mM $MgCl_2$ 60 °C	231
Prima1 exon 5	F: AGGTGTTTCTACAAGCCTTCTAGAAG R: GAAGAGGCTGCTGAGTCTTGAC	5 5	2 mM $MgCl_2$ 60 °C	364
Prlhr exon 2-3	F: TCATGGCCAGAATATGACCG R: GCTCTTCTACTCCAAAGCCTATCTAAC	2 3	2 mM $MgCl_2$ 60 °C	212
Rnase4 exon 2	F: GCAGCTCACAACGGTCTGTATC R: GGGTAAACCAATCTTAGTGATGTTCTAC	2 2	2 mM $MgCl_2$ 60 °C	214
Rplp2 exon 1-4	F: CCGACATGCGCTACGTTG R: ACTGGCCAGCTTGCCAAC	1/2 4	2 mM $MgCl_2$ 60 °C	196

Tep1 exon 54-55	F: TGCTGGTGACTGCTTCAGAG R: AGAATATAGACACTGGTGTCTTCAGG	54 55	2 mM $MgCl_2$ 60 °C	254
Tmprss11d exon 8-10	F: CCTGATTCCGTCGCATATG R: GCTGTCACTCGCGTATACACTC	8 10	2 mM $MgCl_2$ 60 °C	316
Ube2c	F: TCTGCACTGTATGATGTCAGGAC R: CGGAGAAAGGACAGACATCTAAG	4 5	2 mM $MgCl_2$ 60 °C	233
Wdr76	F: CGTCTTCGTAGGCAACTTTCC R: TTGCTGAGCGAAAACGGGTAGAG		2 mM $MgCl_2$ 60 °C	196, 211

Image Data Analysis

TIFF images were quantitated with Image J software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/)[209].

Statistical Analysis

Data were presented as means ± S.D. for independent experiments repeated at least three times. Statistical analysis was done with a two-tailed unpaired Student's *t*-test. *P* values < 0.05 were considered to be statistically significant.

CHAPTER III

Results and Discussion

1. Differential effects of hnRNP L and L-like proteins on hormone production in GH_3 rat pituitary tumor cells

Studies in our lab have shown the effect of membrane depolarization on the alternative splicing of the STREX exon of *Slo1* gene and the regulatory factors hnRNP L/LL in GH_3 rat pituitary cells [193, 194]. Since both depolarization and the *Slo1* gene are components of the electrical firing system that controls the quantal release of hormones, namely growth hormones and prolactin, from GH_3 cells, we examined the effect of hnRNP L, LL as well as a homologous hnRNP PTB on hormone production in GH_3 cells using short hairpin RNA (shRNA)-mediated gene silencing. Cells treated with Polybrene (transduction reagent) or transduced with empty lentiviral vector were used as controls (Fig. 2A, lanes 1-2). The knockdown efficiency was confirmed to be ~80% reduction of hnRNPs by immunoblotting with specific antibodies (Fig. 2A, lanes 3-6). The hnRNP L level in the shLL-expressing cells appeared to be lower but this was only observed in this particular set of experiments and not in the rest of the thesis work; therefore this variation in hnRNP L level is not associated with the observed changes (see Further considerations).

To allow for comparison between the hormone levels upon different conditions, we set the level of each hormone in non-treated cells (NT) and mock-transduced (Mock) as 100% (Fig. 2B). The levels of the hormones under other conditions were scaled relative to this level.

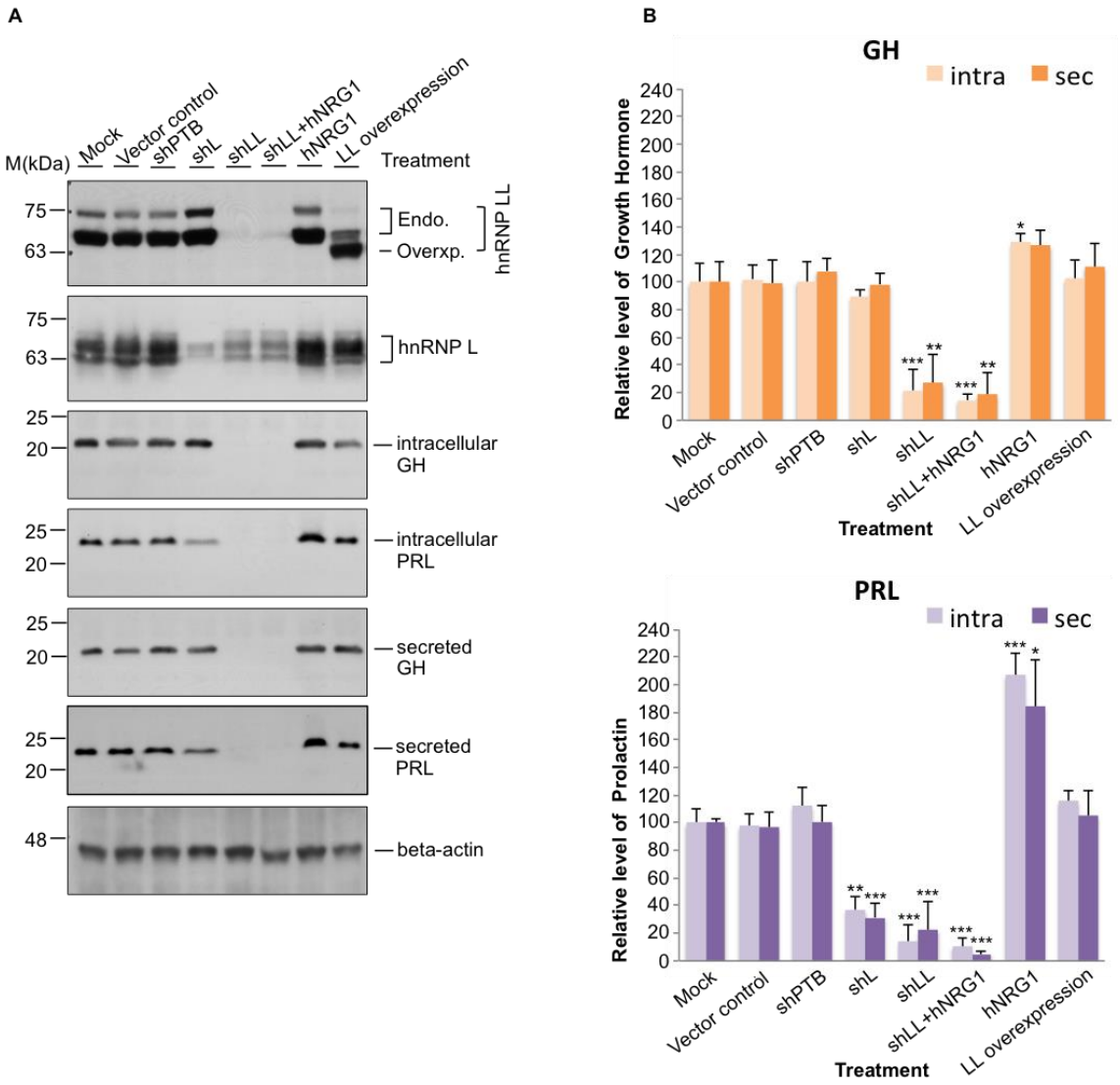


Figure 2. Effect of hnRNP L and L-like on the production of growth hormone and prolactin in GH_3 cells. (A) Representative western blots demonstrating the efficiency and specificity of RNAi against the hnRNP L (shL) or LL (shLL) using lentiviral vector-expressed gene-specific shRNAs. Intracellular and secreted levels of both growth hormone (GH) and prolactin (PRL) were determined in comparison with beta-actin (protein loading control). Note that the overexpressed hnRNP LL protein (~60kDa) is from human. **(B)** Bar graphs summarizing the protein levels of intracellular or secreted GH and PRL relative to that of hnRNP F/H (protein loading control) upon different treatments (mean ± S.D., n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs. Mock, in two tailed Student's t-test.

Upon hnRNP L knockdown, the protein levels of intracellular and secreted PRL were decreased by about 60%; yet those of GH did not change significantly (Fig. 2B).

Upon hnRNP LL knockdown, both the intracellular and secreted protein levels of GH and PRL were reduced by approximately 80%. PTB knockdown did not seem to have an effect on hormone production compared to control samples. Thus, it is possible that hnRNP L/LL are specifically and differentially required for maintaining the protein levels of GH or PRL.

The transcription and secretion of PRL can be upregulated by Neuregulin-1 (NRG-1) through activating AKT and extracellular signal-regulated kinase (ERK) signaling pathways [210, 211]. Interestingly, the exon 18 of *Nrg1* is repressed by hnRNP L and LL upon depolarization in our previous study, and CA-repeat elements are found in the adjacent introns [199]. Knockdown of hnRNP L or LL increases the exon 18 usage resulting in protein truncation and intracellular retention of neuregulin. Therefore, we investigated whether the reduction of extracellular NRG-1 is responsible for the decrease of PRL upon hnRNP LL knockdown by adding NRG1 peptide to the culture media. Knockdown and untransduced cells were treated with human Neuregulin-1 (hNRG-1) at a concentration of 6nmol/L for 48 hours [210] (Fig. 2A, lane 6-7). Addition of hNRG-1 was not able to restore the hormone levels from the hnRNP LL-knockdown cells, Consistent with the reported observation [199], hNRG-1 increased the synthesis and secretion of PRL twice as much as the controls and slightly raised the intercellular level of GH (~110%) in untransduced cells. These observations suggest that (1) hnRNP LL is required for the NRG1-induced increase of prolactin and growth hormones, and (2) there are more defects than just disrupted NRG1 exon 18 splicing that caused the defective hormone production in the hnRNP LL-knockdown cells. Taking this together with the

NRG-1 promotion of prolactin gene transcription [210, 211], it is likely that loss of hnRNP LL interrupted prolactin gene expression.

Since the knockdown of hnRNP LL decreased the protein levels of both GH and PRL, we then wanted to examine if overexpressing hnRNP LL would promote the production of these two hormones. Lentiviral overexpression of Myc-tagged hnRNP LL was performed in GH_3 cells. Interestingly, the expression of exogenous Myc-hnRNP LL consistently reduced the protein level of endogenous hnRNP LL (Fig. 2A, lane 8). Comparison of the band intensities indicated that the hnRNP LL total expression between control and overexpression samples were similar (approximately 1-1.2 fold), implicating the possible existence of an hnRNP LL negative autoregulatory feedback loop. The intracellular and secreted levels of both GH and PRL did not have significant changes upon hnRNP LL overexpression compared with those of controls. This was likely due to that hnRNP LL was not in excess in cells. HnRNP L but not LL, has been shown to be autoregulated through NMD triggered by its own excess protein [168]. It might be interesting to investigate the mechanism of hnRNP LL homeostasis, which contributes to the balanced functions of RNA binding proteins [168, 172, 212-214].

2. HnRNP LL is required for the mRNA expression of *Gh1* and *Prl*

To determine whether the protein decrease of the two hormones were due to changes at the mRNA level in GH_3 cells, we designed specific PCR primers covering all the five exons of rat *Gh1* (Fig. 3A) or *Prl* (Fig. 4A), respectively. They encode the growth hormone and prolactin. RT-PCR results demonstrated that the *Gh1* mRNA level was decreased to approximately 40% compared with controls (Fig. 3B). This transcript

reduction rate is lower than that observed in protein level, which could be due to the difference of sensitivity between western blot and RT-PCR with its correlated protein reduction. Nevertheless, this suggests that RNA change is likely the primary cause for the hormone downregulation in the hnRNP LL knockdown cells. No statistical significance of the transcript changes was found in the hnRNP L or PTB knockdown samples (Fig. 3C). Thus, hnRNP LL is specifically required for maintaining the mRNA level of *Gh1*.

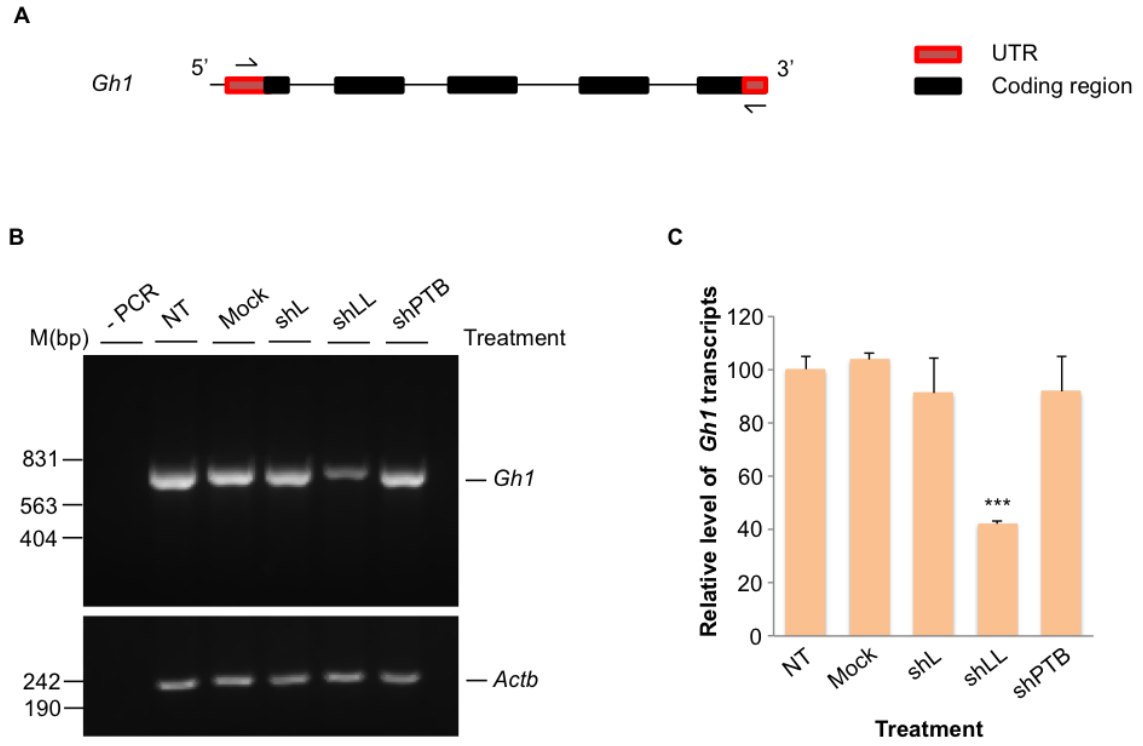


Figure 3. Effect of hnRNP L and LL on growth hormone (*Gh1*) gene expression. (A) Diagram of the rat growth hormone gene. It is annotated as UTR (red), exon (black box), intron (black line) and primer locations (black arrows). **(B)** Agarose gels of RT-PCR products of *Gh1* transcript in GH_3 cells with knockdown of the splicing factors, and **(C)** a bar graph of its changes relative to that of *Actb*. Mean ± S.D., n=3, ***: $p<0.001$ vs. NT. -PCR: PCR negative control. NT: Non-treated GH_3 cells were used as a control. *Actb*: RNA loading control.

RT-PCR revealed that the *Prl* gene expression was also dramatically repressed upon hnRNP LL knockdown (Fig. 4B). Only about 20% remaining *Prl* mRNA was

detected in these cells (Fig. 4D), which was consistent with those of its protein changes, suggesting that hnRNP LL regulates prolactin synthesis mainly through controlling its mRNA transcripts.

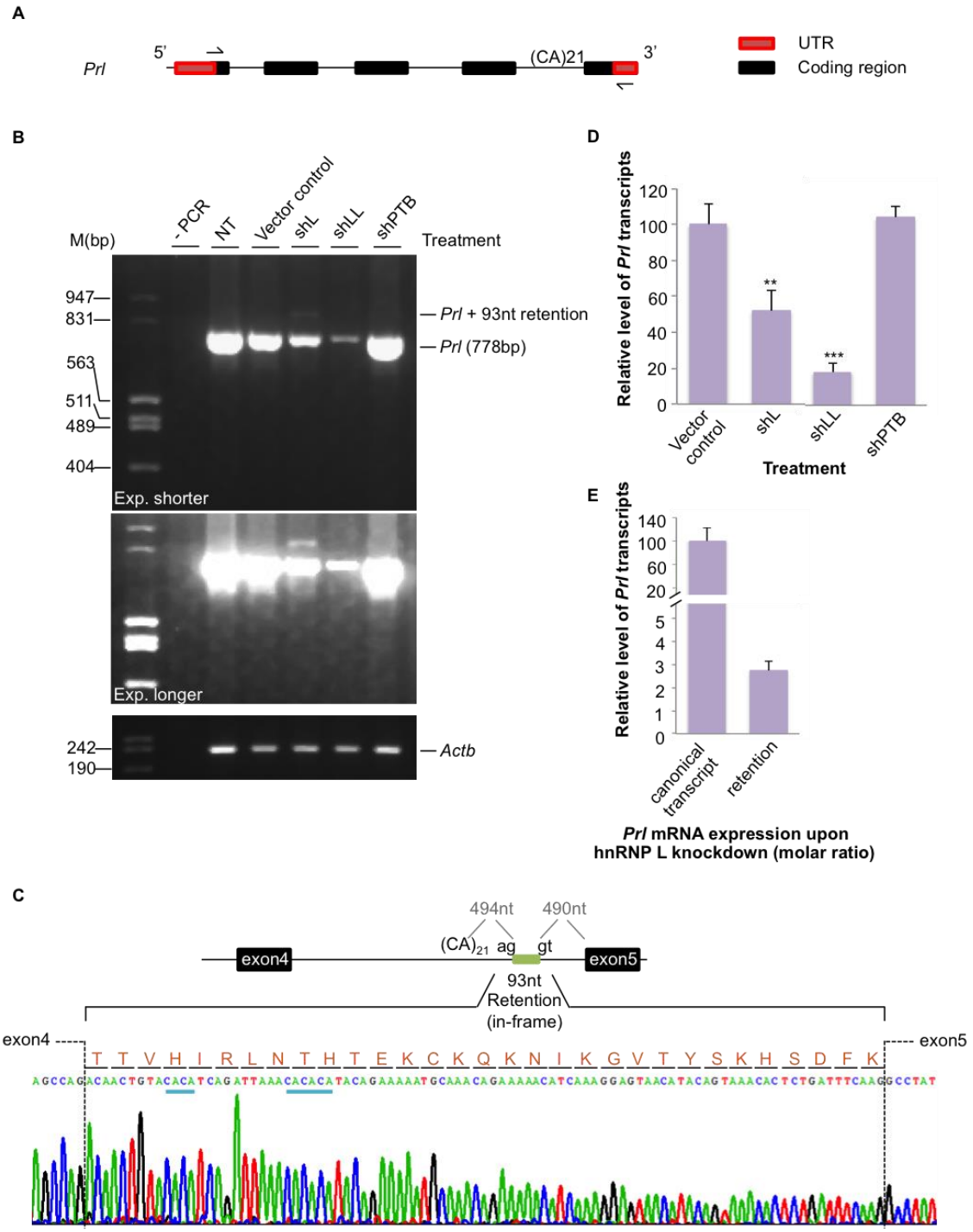


Figure 4. Differential regulations of prolactin (*Prl*) gene expression by hnRNP L and LL. (A) Diagram of the rat prolactin gene. It is annotated as UTR (red), exon (black box), intron (black line) and primer locations (black arrows). A sequence with 21 CA-repeat was located in intron 4 as marked. **(B)** Shown are agarose gels of RT-PCR products of *Prl* transcript in GH_3 cells upon knockdown of hnRNP L, LL or PTB. To the left are DNA size markers (bp). -PCR: PCR negative control. NT: Non-treated GH_3 cells were used as a control. **(C)** Sequencing chromatogram of the RT-PCR product of *Prl* 93nt retention (green box), which has two CA-repeat regions (blue underlines) and can be translated into 31 amino acids (orange capital letters). **(D)** A bar graph elucidating the relative percentage of *Prl* transcript upon different treatments, and a bar graph **(E)** comparing the relative level of *Prl* 93nt retention to that of the conventional transcript (set as 100%). Mean ± S.D., n=3, **: $p<0.01$, ***: $p<0.001$.

3. HnRNP L downregulation generates a splice variant of prolactin with 93-nucleotide in-frame retention of intron 4

The level of prolactin but not growth hormone decreased upon hnRNP L knockdown according to the above data. RT-PCR results indicated that downregulation of hnRNP L resulted in an about 50% decrease of *Prl* mRNA expression (Fig. 4B and D); this decrease was similar to changes at the protein level. A weak band above the major 778bp band of *Prl* was observed (Fig. 4B). Sequencing result of this band indicated an insertion of 93-nucleotide from intron 4 into the *Prl* mRNA (Fig. 4C). The 93-nt is flanked by canonical consensus sequences of splice sites: 3' AG in its upstream and 5' GU downstream, as for most eukaryotic splice sites [50]. It is not found in any known rat transcripts in the UCSC Genome Browser or in the NCBI database. The exon is also not conserved in human or mouse by sequence alignment. It is a CA-rich sequence (16 CA dinucleotides in total) containing one $(CA)_2$ repeat and one $(CA)_3$ repeat. Presumably hnRNP L suppresses the splicing of this cryptic exon by interaction with these CA-dinucleotide repeats, as in other cases [215, 216]. The suppression of a cryptic exon is a novel function of hnRNP L that has not been observed previously.

The *Prl* 93-nt retention of the intron fragment was approximately 3% of the major *Prl* transcript (Fig. 4E). Using NCBI Open Reading Frame Finder (available on the Internet at http://www.ncbi.nlm.nih.gov/gorf/gorf.html), we confirm that it is an in-frame insertion. However, we did not observe a corresponding protein band in western blot by using the prolactin antibody, which recognizes the carboxyl-terminal disulfide loop that is conserved among prolactins from several species. The lack of a western blot signal is probably due to the low abundance of the cryptic transcript.

4. Predicting the functional impact of the prolactin variant with the 93-nt in-frame retention

Since the *Prl* 93-nt retention induced by hnRNP L knockdown is an in-frame retention (contains no premature stop codons and can be translated into protein theoretically), we evaluated the functional impact of this protein isoform by comparing its structure with the known ones. The rat prolactin consists of 197 amino acids, which forms an antiparallel four-alpha-helix bundle [217] (Fig. 5A). We used SWISS-MODEL (available on the Internet at http://swissmodel.expasy.org [218]) to predict the three-dimensional (3D) molecular structure of the canonical form of rat prolactin as well as this isoform (Fig. 5B). According to the prediction, the canonical PRL folded into a structure consistent with its known crystal structure [219]. This 93-nt insertion translated into 31 amino acids that are high charged and forms an extra alpha-helix in vertical orientation to all the other four helices. It also forms an extended linker immediately downstream of the 3rd alpha-helix (Fig. 5C). By analyzing the structural changes by the 31aa insertion, it is

possible to deduce its potential impact on post-transcriptional modifications of PRL and its binding activity with the prolactin receptor (PRLR).

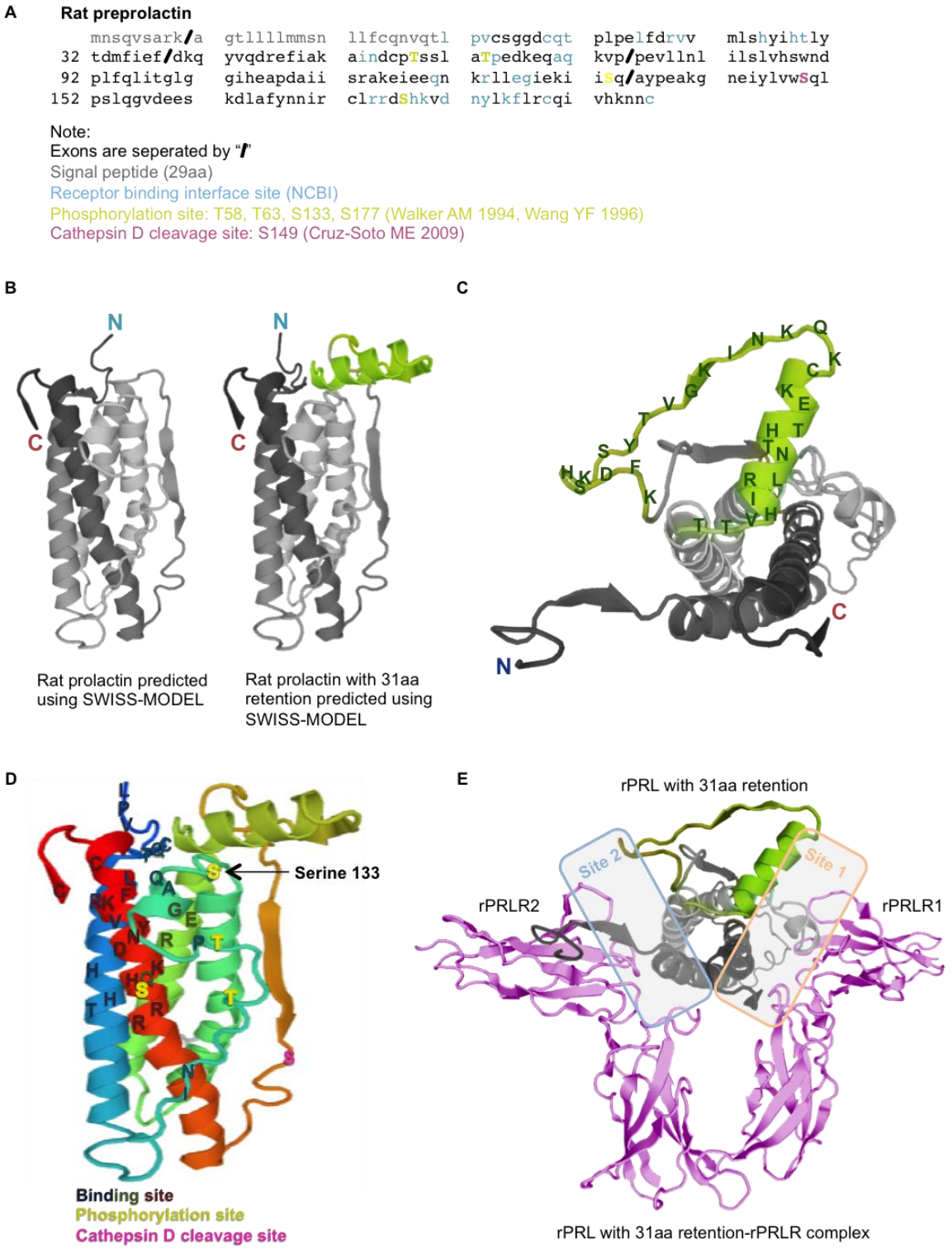


Figure 5. Function prediction of the prolactin variant with the 93-nt retention. (A) Amino acid sequence of rat prolactin (from NCBI database) contains a signal peptide of 29 amino acids (grey), receptor binding interface sites (blue), phosphorylation sites (yellow) and a cathepsin D cleavage site (pink). Regions encoded by different exons are separated by "/". **(B** and **C)** Predicted three dimensional solution structures of rat prolactin (B left), prolactin with the 31aa insertion (neon green, B right) and its aerial view (C) with mapped amino acids. **(D)** Receptor binding sites (coloured based on their located alpha-helix), phosphorylation sites (orange) and the cathepsin D cleavage site (pink) are mapped to the predicted 3D structure of rat prolactin with 31aa insertion. **(E)** Binding simulation of the 31aa inserted prolactin by mapping to the crystal structure of the PRL-$PRLR_2$ complex [220, 221]. This picture shows the two binding sites (orange: site 1 and blue: site 2) of the 31aa (green) inserted PRL (gray) to its receptor (purple).

Prolactin undergoes various types of protein modifications including proteolytic cleavage, glycosylation and phosphorylation, which affect its stability, half-life, polymerization, receptor binding and bioactivity [217, 222-224]. At least four phosphorylation sites (Tyrosine 58, Tyrosine 63, Serine 133 and Serine 177) have been reported in rat prolactin and the Serine 177 is considered as a major site [223, 225-227]. Of particular interest to us is the Serine 133 located one amino acid upstream of the 31aa insertion. This phosphorylation site is exposed to the external surface in the canonical protein structure but is blocked by the insertion in the predict model (Fig. 5D). This spatial blockage could interrupt the interaction between this substrate with its kinases, result in phosphorylation failure and consequently physiological alterations. The function of the Serine 133 remains to be identified.

It is also possible that the 31aa insertion in the 288aa prolactin might affect other modifications, such as proteolytic cleavage or glycosylation. The change of protein modifications not only can alter microheterogeneity, but also may bring about a dominant negative effect on other prolactin isoforms. All these possibilities require further exanimations.

Another property of PRL likely to be impacted by the 31aa insertion is polymerization. High molecular weight forms of prolactin have been found in the pituitary [228-230]. Dimerized prolactin consists of two prolactin monomers that one's N-terminal vertically joined to the other one's. The even bigger form – macroprolactin – comes from association between monomeric PRL and immunoglobulin G (IgG) [231]. Based on our 3D structure prediction, the 31aa insertion, which is spatially close to both N- and C-terminals of the protein, is very likely to disrupt the formation of these complexes. The dimerized prolactin and macroprolactin molecular forms of PRL are involved in storage, transformation and secretion [123], yet their biological significance is not fully understood.

To evaluate the potential effect of the 31aa insertion on the binding activity with prolactin receptors, we aligned the receptor binding interface sites on the 228aa PRL using NCBI database and mapped the 3D structure to the crystal structure of the hPRL-rPRLR2 complex [220, 221]. According to this alignment, the extra alpha-helix formed by the insertion is pointing toward the binding site 1, and the extended linker towards the binding site 2 (Fig. 5E). This spatial rearrangement of the protein likely affects its binding affinity with the prolactin receptors.

5. Knockdown of hnRNP LL does not induce cryptic splicing variants

The fact that knockdown of hnRNP L induced a 93-nt in-framed retention in *Prl* intron 4 promoted us to ask if knockdown of hnRNP LL also causes splice variants from the two hormone genes. However, the potential variants may not be visible due to the low transcript levels of *Gh1* and *Prl* in the knockdown cells. To clarify whether hnRNP LL

regulates their RNA splicing, RT-PCR was carried out followed by concentration of the PCR products using the gel extraction column from QIAGEN. PCR product of hnRNP LL knockdown sample was concentrated from one to eight times and the intensity of its major band was enhanced proportional to the concentrations (Fig. 6A and B). However, no potential transcript variants were detected on both genes upon hnRNP LL downregulation. Therefore, the cryptic splicing is specific for the hnRNP L knockdown samples.

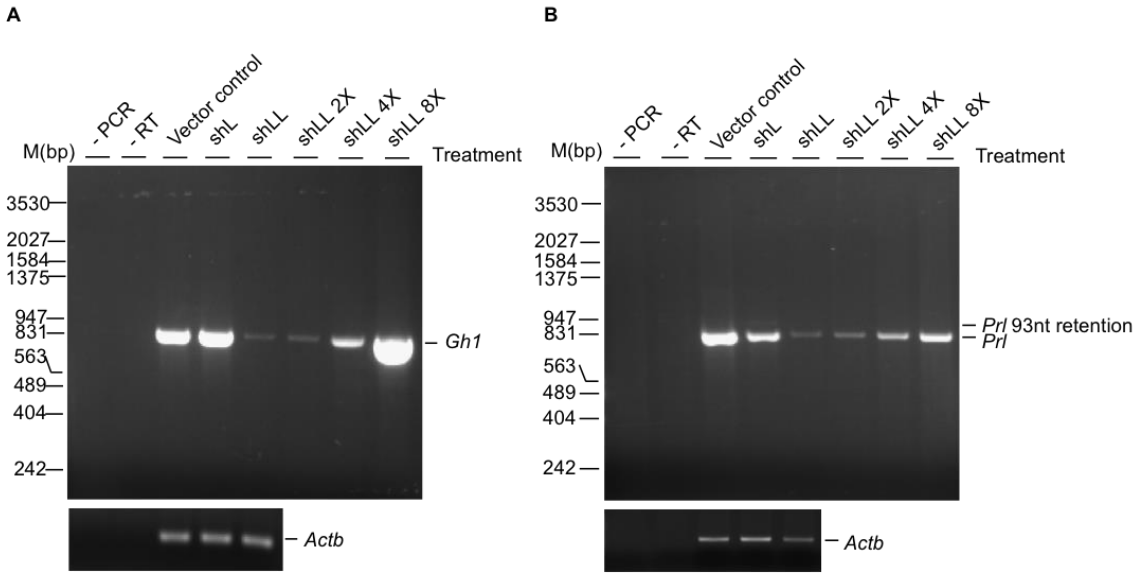


Figure 6. No potential cryptic transcript variant of *Gh1* and *Prl* was detected upon hnRNPLL knockdown. Agarose gels of RT-PCR products of *Gh1* **(A)** or *Prl* transcripts **(B)** in GH_3 cells upon hnRNP L or LL knockdown. PCR products in shLL are concentrated into 2 times (2X), 4 times (4X) or 8 times (8X). -PCR: PCR negative control, -RT: RT negative control. *Actb*: RNA loading control.

6. Expression of exogenous hnRNP LL rescues the hormone production in hnRNP LL knockdown cells

To examine the specificity of shLL on the changes of GH and PRL, we carried out RNA interference/rescue with lentiviral expression of an RNAi-resistant Myc-hnRNP

LL. Western blotting confirmed the knockdown efficiency of hnRNP LL by a ~80% reduction (Fig. 7B). The hormone expression in knockdown cells and their medium were decreased sharply - by about 60% for growth hormone and 70% for prolactin.

Since our shLL RNA is targeting the open reading frame of hnRNP LL at a region conserved in human, mouse and rat, site-directed mutagenesis PCR was used to mutate three nucleotides in the corresponding region of the Myc-hnRNP LL and generated three silent mutations (Fig. 7A). Two of the mutation sites (G-348-T, A-351-G) are located in the siRNA seed region [232] and the other site (T-342-A) is at the 3' end. They are made together to ensure sufficient insensitivity of the expression transcript to the shLL.

Myc-hnRNP LL was expressed at ~90% of the controls. The expressed hnRNP LL efficiently restored the protein levels of both intracellular and secreted growth hormone and prolactin, to the same level as controls.

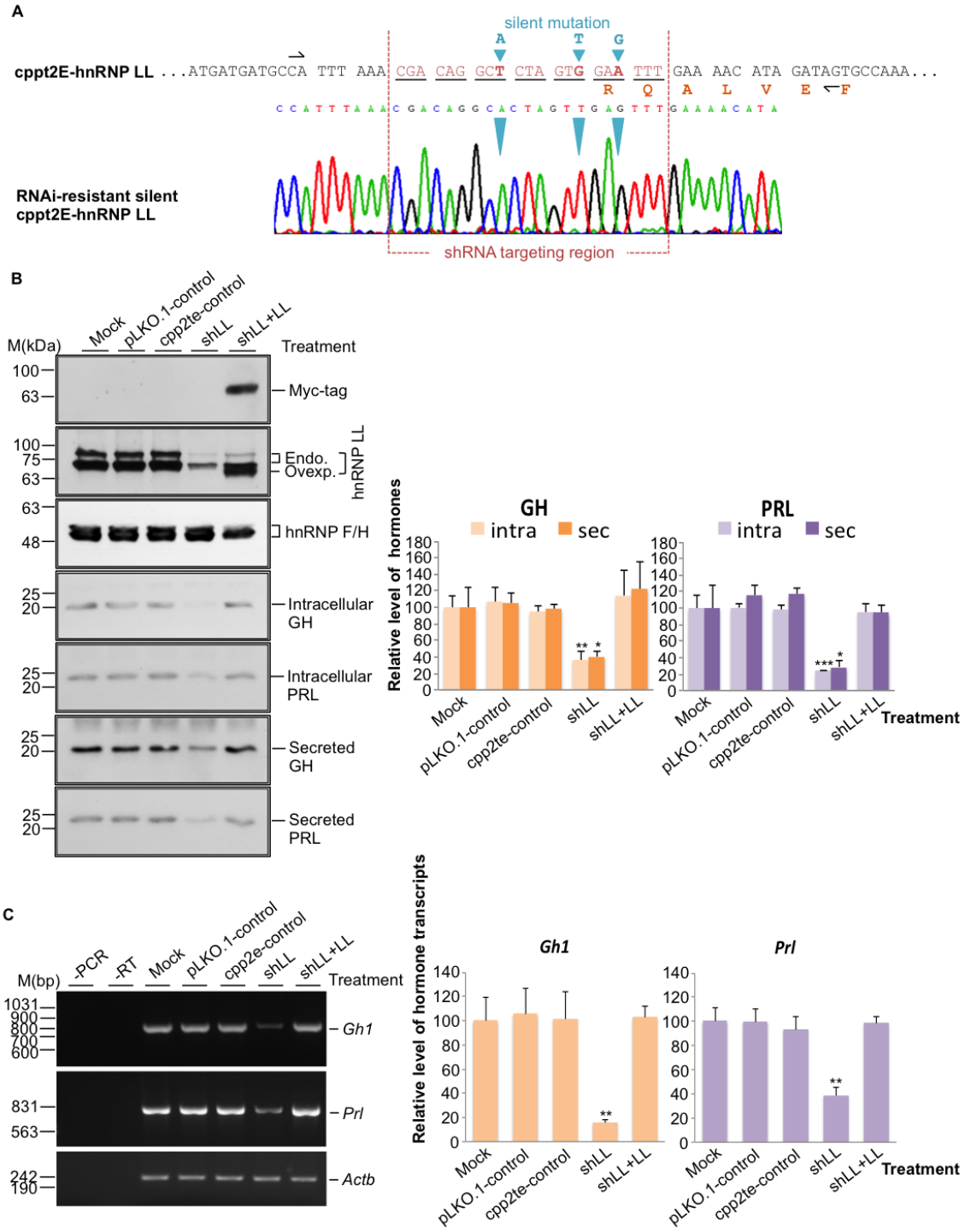

A
silent mutation
cppt2E-hnRNP LL ...ATGATGATGCCA TTT AAA CGA CAG GCT CTA GTG GAA TTT GAA AAC ATA GATAGTGCCAAA...
R Q A L V E F
RNAi-resistant silent cppt2E-hnRNP LL
shRNA targeting region
B
M(kDa)
Mock
pLKO.1-control
cpp2te-control
shLL
shLL+LL
Treatment
Myc-tag
Endo.
Ovexp.
hnRNP LL
hnRNP F/H
Intracellular GH
Intracellular PRL
Secreted GH
Secreted PRL
GH
PRL
intra
sec
Relative level of hormones
Treatment
C
M(bp)
-PCR
-RT
Gh1
Prl
Actb
Relative level of hormone transcripts

Figure 7. Essential role of hnRNP LL in hormone production in GH_3 cells. (A) Construction of the RNAi-resistant silent mutation of hnRNP LL overexpression plasmid using site-directed mutagenesis PCR. Diagram demonstrating the shRNA-targeting region in the plasmid cppt2E-hnRNP LL and the sequencing chromatogram of the mutated plasmid. It is annotated as primer locations (black arrows), mutation sites (aqua blue letters and triangles) and coding amino acids (orange). **(B)** Representative western blots of hnRNP LL and the hormone levels from GH_3 cells expressing Myc-hnRNP LL or shRNA against endogenous hnRNP LL. **(C)** Agarose gels of the RT-PCR products of *Gh1* and *Prl* transcripts in these samples. -PCR: PCR negative control, -RT: RT negative control, Mock: mock-transduced cells as a control. Right panel are bar graphs of the protein levels (upper) and of the transcript level (lower) of the hormones relative to that of hnRNP F/H (protein loading control) or that of *Actb* (RNA loading control). Mean ± S.D., n=3, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs. Mock.

RT-PCR analysis of the two hormone genes also indicated that the reduction of their transcript levels caused by hnRNP LL knockdown was brought back after expression of the exogenous hnRNP LL (Fig. 7C). Thus, hnRNP LL is likely the specific factor lost in shLL samples for maintaining the transcript level of the hormones.

7. Exogenous hnRNP L expression in its knockdown cells confirms the novel role of hnRNP L in preventing aberrant splicing of *Prl* in two directions

To verify that the reduction of *Prl* mRNA and the 93-nt insertion are indeed due to the knockdown of hnRNP L, lentiviral knockdown/rescue assay was again performed similarly as for the hnRNP LL. Prolactin expression was indeed partially (~60%) brought back upon the expression of exogenous hnRNP L (Fig. 8A). Moreover, the 93-nt insertion was eliminated as well (Fig. 8B). Moreover interestingly, transcripts resulted from *Prl* exon 2 skipping or *Prl* exons 2-3 skipping were also generated according to the sequencing result (Fig. 8C). They each comprised about 1.5% of the *Prl* transcripts in hnRNP L rescued samples (Fig. 8D). The average prolactin mRNA level appeared

slightly less in hnRNP L rescued cells than that in controls (~80%) but with no statistical significance.

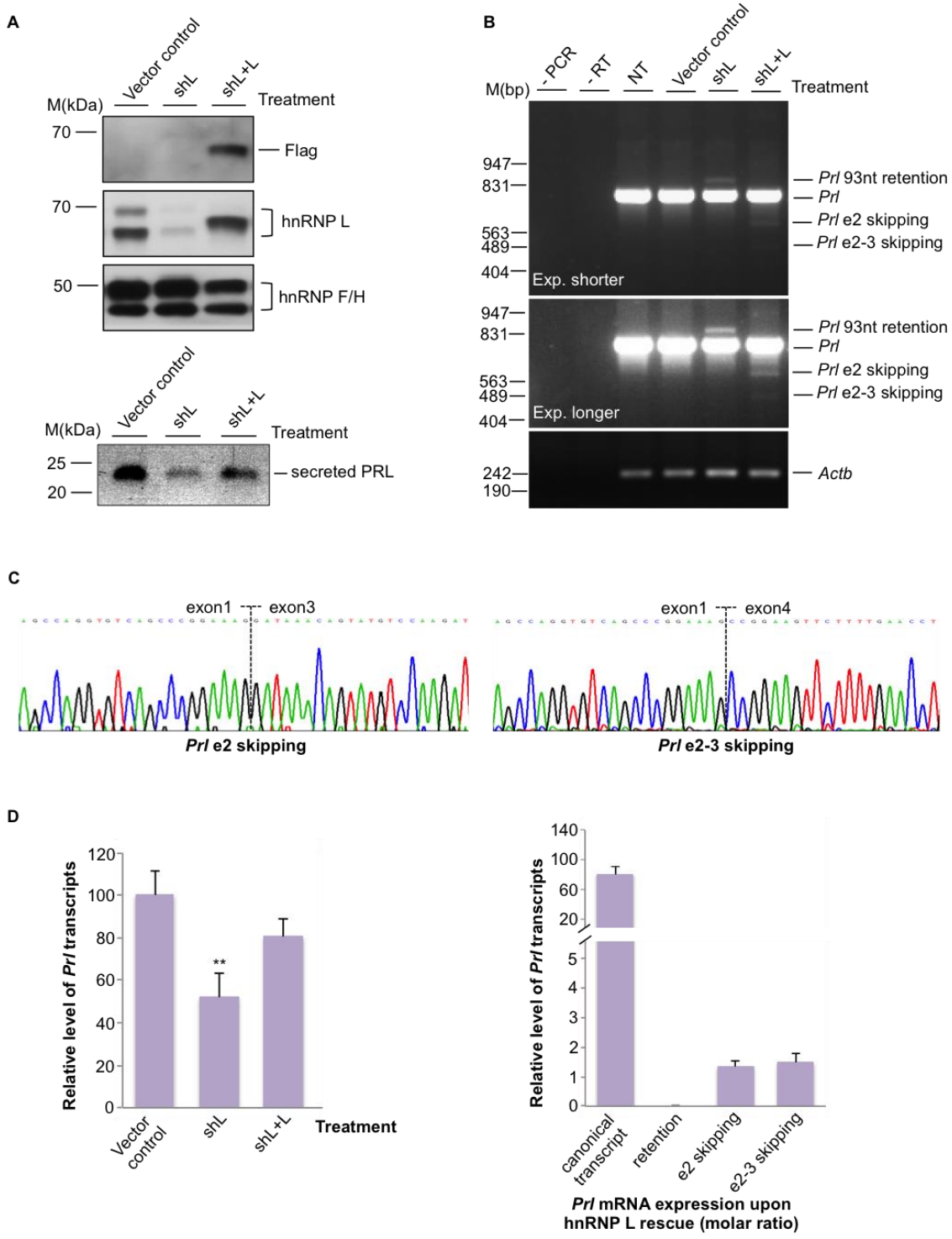


Figure 8. HnRNP L overexpression in shL-expressing cells prevented the 93nt intron retention but also induced two PTC-containing transcripts resulted from exon skipping of *Prl*. (A) Western blots of hnRNP L from GH_3 cells expressing FLAG–hnRNP L or shRNA against endogenous hnRNP L. This work is done by Guodong Liu [199]. Below is a western blot showing the secreted prolactin from conditioned medium upon hnRNP L knockdown/rescue. HnRNP F/H: protein loading control. **(B)** Agarose gels of the RT-PCR products of *Prl* in hnRNP L knocked down/rescued GH_3 cells. *Actb*: RNA loading control. -PCR: PCR negative control, -RT: RT negative control, NT: non-treated. **(C)** Sequencing chromatograms of the RT-PCR products of *Prl* exon skipping. **(D)** Bar graphs of the levels of *Prl* transcripts relative to that of *Actb* (RNA loading control). Mean ± S.D., n=3, **: $p<0.01$.

The expression of hnRNP L in rescued cells was about 2~4 times of that of control (Fig. 8A). The higher level of hnRNP L was likely responsible for the appearance of these exon exclusions. The up and down stream introns of exon 2 and 3 contain CA-repeats as well, which presumably are bound by hnRNP L for modulating splicing events of their adjacent exons.

The skipping of *Prl* exon 2 or exon 2-3 generated premature termination codons (PTCs) >50nt away from the last exon-exon junction [233, 234], therefore probably resulting in nonsense-mediated mRNA decay (NMD) of the transcripts [235] (Fig. 9A). The total level of the two transcript variants was not equal to the shortage of expression in rescue samples, perhaps due to that a portion of the variants were degraded by NMD.

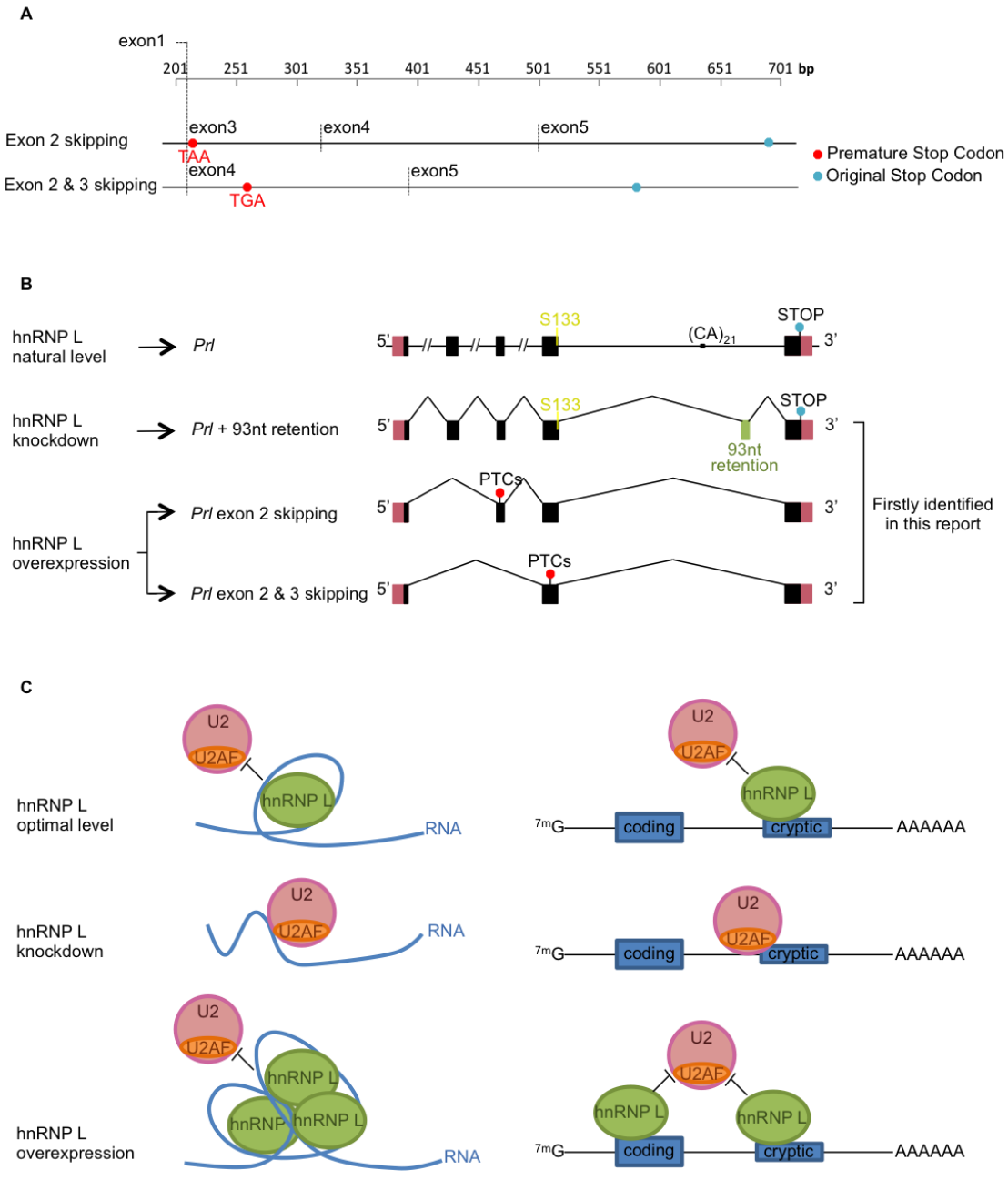


Figure 9. The expression level of hnRNP L is essential for *Prl* proper splicing. (A) Locations of premature stop codons (red) and original stop codons (blue) in *Prl* exon-skipping transcripts induced by hnRNP L overexpression. **(B)** Diagram of the four *Prl* variants detected in GH_3 cells upon different expression level of hnRNP L, based on the *Prl* information from the UCSC Genome Browser and our sequencing results. **(C)** A schematic diagram showing the proposed relationship between hnRNP L level and alternative/cryptic splicing.

Taken together, hnRNP L regulates the splicing and expression of *Prl* in three directions: 1) downregulation of hnRNP L induces a cryptic exon along with a decrease in full-length prolactin mRNA; 2) overexpression of hnRNP L leads to exon skipping; 3) only the optimal endogenous level of hnRNP L yields efficient proper splicing of *Prl* (Fig. 9B). Thus, hnRNP L controls the expression of *Prl* gene and its proper expression level is critical for preventing aberrant splicing of *Prl* in cells. To our knowledge by far, this is the first identification for the *trans*-acting factor in prolactin pre-mRNA alternative splicing. Moreover this is the first case of hnRNPs regulating splicing of an endogenous gene in two directions. Here we proposed a model based on our data (Fig. 9C), which might happen to other RNA binding factors as well.

8. Regulation of the production of growth hormone and prolactin by the interaction of hnRNP L/LL with other hnRNPs

HnRNP L protein contains a proline-rich region that can bind PTB, whereas hnRNP LL does not have this region [167]. Interaction of hnRNP L with PTB has been reported affecting RNA processing and protein translation in different ways [167, 175, 181-183]. Furthermore PTB displays 55% similarity to hnRNP L (32% identity between the RNA recognition motifs) [181, 236]. To further investigate the role of the interaction of these hnRNP proteins on hormone production in GH_3 cells, double knockdown of hnRNP L with LL or hnRNP L with PTB was performed respectively. Knockdown efficiencies of target genes were at least 90% (Fig. 10A). A tremendous decrease was obtained in secreted and intracellular levels of growth hormone and prolactin upon hnRNP L and LL double knockdown.

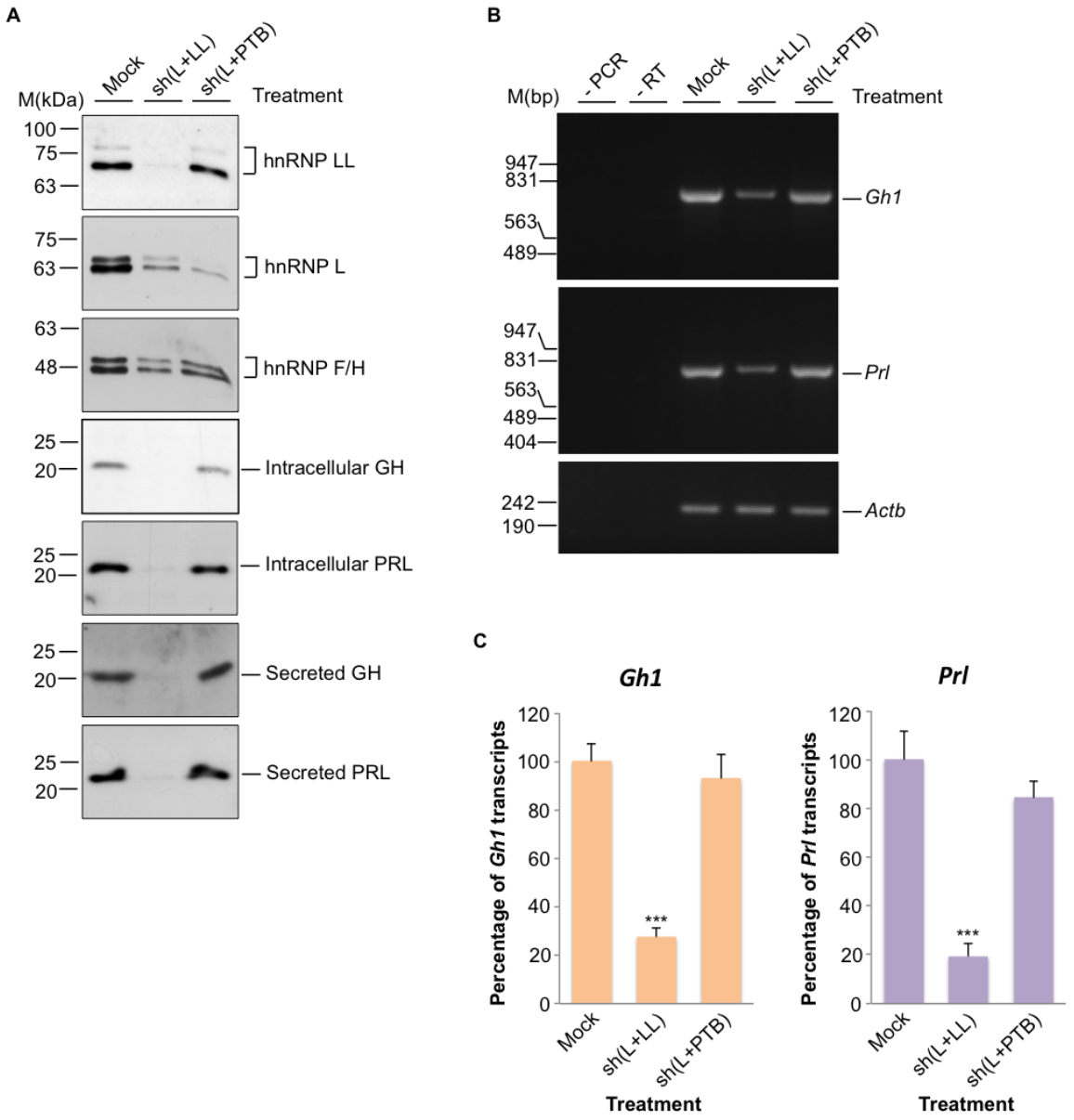


Figure 10. Effect of hnRNP proteins double knockdown on hormone production. (A and **B)** Western blots of hormone levels (A) and agarose gels of the RT-PCR of *Gh1* and *Prl* transcripts (B) in GH_3 cells with double knockdown of selected splicing factors. HnRNP F/H: protein loading control. -PCR: PCR negative control, -RT: RT negative control, Mock: mock-transduced. (C) The transcript levels of *Gh1* and *Prl* relative to that of *Actb* (RNA loading control). Mean ± S.D., n=3, ***: $p<0.001$ vs. Mock.

RT-PCR detected about 70% and 80% reductions in *Gh1* and *Prl* in hnRNP L and LL double knockdown cells, respectively (Fig. 10B-C). These reduction extents were

consistent with the corresponding data in hnRNP LL knockdown cells, which means that double knockdown of hnRNP L and LL did not further decrease the *Prl* transcript level. Also the *Prl* 93-nt retention was not detected in this group. These observations (Figs. 3B, 4D, 7C and 10) suggest that the two proteins likely regulate the prolactin gene through the same pathway and the effect of hnRNP L on *Prl* requires the presence of hnRNP LL.

Equally interesting, double knockdown of hnRNP L with PTB did not significantly reduce the average level of *Prl* expression (Fig. 10C), nor did it cause the appearance of the cryptic exon. This is in contrast to their effect on *Prl* transcripts upon single hnRNP L or PTB knockdown (Fig. 4D). The knockdown of PTB abolished the effect of hnRNP L knockdown on *Prl* mRNA. Based on the observation that PTB interacts with the RNA 3' UTR to destabilize the transcript [237], the reduction in *Prl* mRNA upon hnRNP L knockdown could be resulted from PTB binding to the 3'UTR. In fact, a predicted PTB binding site "tctcttctt" was found about 21 nucleotides upstream the poly(A) in the *Prl* 3'UTR.

9. The regulatory role of hnRNP LL on hormone secretion is confirmed in primary cultures of rat pituitary

To confirm the functional effects of hnRNP L and LL on hormone production in the pituitary, primary cultures of rat pituitary tissues were examined. Pituitary tissues dissected from adult females were digested into single cells and cultured for 72 hours before lentiviral transduction. Cells were handled in a similar way as done in GH_3 cells. The cellular morphology of each treatment group was stable (Fig. 11A). Culture medium was relatively cloudy in transduced samples, which was likely due to some components

in the homemade lentivirus stocks. Importantly, these cells produce growth hormone and prolactin from day 3 up to 16 days in culture as confirmed in western blots (Fig. 11B)

The knockdown efficiency of ~85% reduction of hnRNP LL and the LL protein expression of ~65% in concurrent rescue samples were verified by Western blots (Fig. 11C). The secreted growth hormone detected from the medium was reduced by about half upon hnRNP LL knockdown and recovered to ~100% of control after rescue (Fig. 11D). The intracellular level of growth hormone remained statistically unchanged among all treatment groups. Both intracellular and secreted prolactin decreased, by approximately 20% and 80%, respectively. Their levels were restored to ~115% and ~80% of control by rescuing hnRNP LL. Thus, hnRNP LL appears to affect the secretion of GH and PRL from primary pituitary cells but has limited impact on hormone protein synthesis inside the cells.

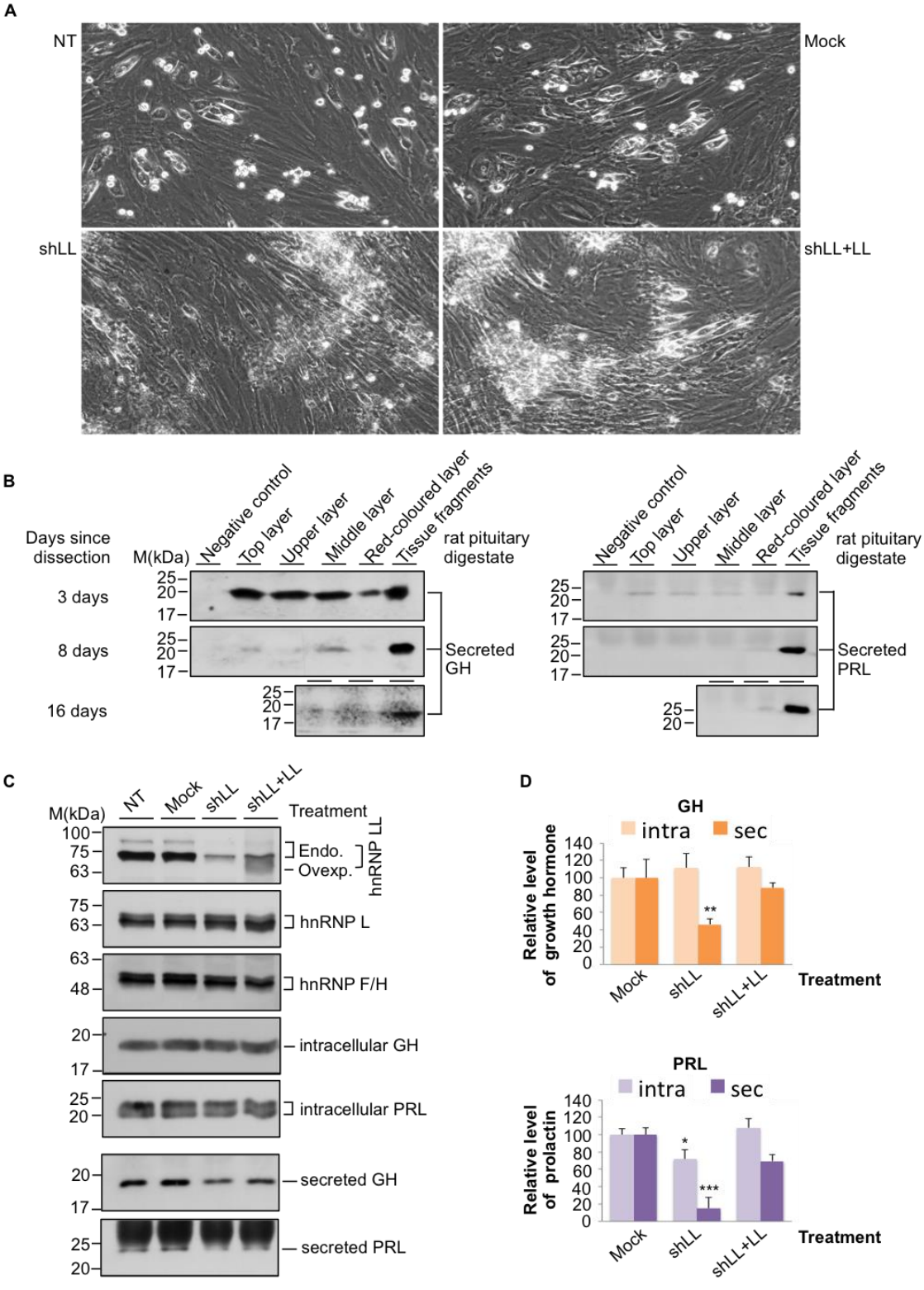

A
NT
Mock
shLL
shLL+LL
B
Days since dissection
M(kDa)
Negative control
Top layer
Upper layer
Middle layer
Red-coloured layer
Tissue fragments
rat pituitary digestate
3 days
8 days
16 days
Secreted GH
Secreted PRL
C
M(kDa)
NT
Mock
shLL
shLL+LL
Treatment
hnRNP LL
Endo.
Ovexp.
hnRNP L
hnRNP F/H
intracellular GH
intracellular PRL
secreted GH
secreted PRL
D
GH
intra
sec
Relative level of growth hormone
Mock
shLL
shLL+LL
Treatment
PRL
Relative level of prolactin

Figure 11. Effect of hnRNP L and LL on hormone production in rat primary pituitary culture. (A) Microscopic images of primary rat pituitary cell culture on the third day post-transduction. **(B)** Western blots of secreted GH or PRL from different layers of rat pituitary digests at different days. Medium contains no cells is used as negative control. **(C** and **D)** Representative western blots (C) showing the efficiency of knockdown/rescue of hnRNP LL and the hormones from primary rat pituitary cells upon different treatments, and (D) bar graphs of the levels of hormones relative to that of hnRNP F/H (protein loading control). NT: non-treated, Mock: mock-transduced. Mean ± S.D., n=3, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs. Mock.

10. Deep sequencing of total RNA reveals differential regulation of hormone genes by hnRNP L and LL, as well as a novel function of hnRNP L on preventing aberrant splicing in the transcriptome

GH_3 cells with specific knockdown of hnRNP L or hnRNP LL, as well as control (non-transfected) were sent for RNA Hi-Seq after verification of gene-silencing efficiency (Fig. 12A). Sequencing data was analyzed to identify gene expressions affected by the knockdown of hnRNP L or LL. By using the Bioconductor package edgeR, the reads of 8413 and 9465 genes were significantly changed by shL and shLL, respectively (Table 3A). The reads of 10301 genes exhibited significant differences between the hnRNP L and LL knockdown samples. More interestingly, with a similar number of changed genes between hnRNP L and RNP LL, hnRNP LL seems to have a stronger effect in changing the total level of gene expression (~2-3 times than that by L in general). A three-way comparison showed that the reads of 7239 genes were changed significantly among the three treatment groups (Table 3B).

Table 3A. Summary of two-way comparison of the number of genes whose reads were changed by the knockdown of hnRNP L or LL proteins using edgeR analysis

edgeR	Total Detected Genes	Significantly changed genes (P<0.05)	Genes with fold change <0.5 or >2	Fold change <0.5	Fold change >2
Mock vs. shL	26689	8413	445	121	324
Mock vs. shLL	26689	9465	988	291	697
shL vs. shLL	26689	10301	1677	817	860

Table 3B. Summary of three-way comparison of the total number of genes whose reads were changed by the knockdown of hnRNP L or LL proteins using edgeR analysis

edgeR	Significantly changed genes (P<0.05)	Genes with fold change <0.5 or >2	Increased in both shL & shLL	Decreased in both shL & shLL	Different effects in shL & shLL
Mock vs. shL vs. shLL (3-ways comparison)	7239	105	54	27	24

Twenty eight (28) genes were selected from the group of significantly changed genes (P-value < 0.05) in the three-way comparison for validation. Validation of gene expression by semi-quantitative RT-PCR successfully confirmed the trend of changes in 25 genes, which gave a success rate at ~89.3% out of 28 genes successfully amplified from the samples.

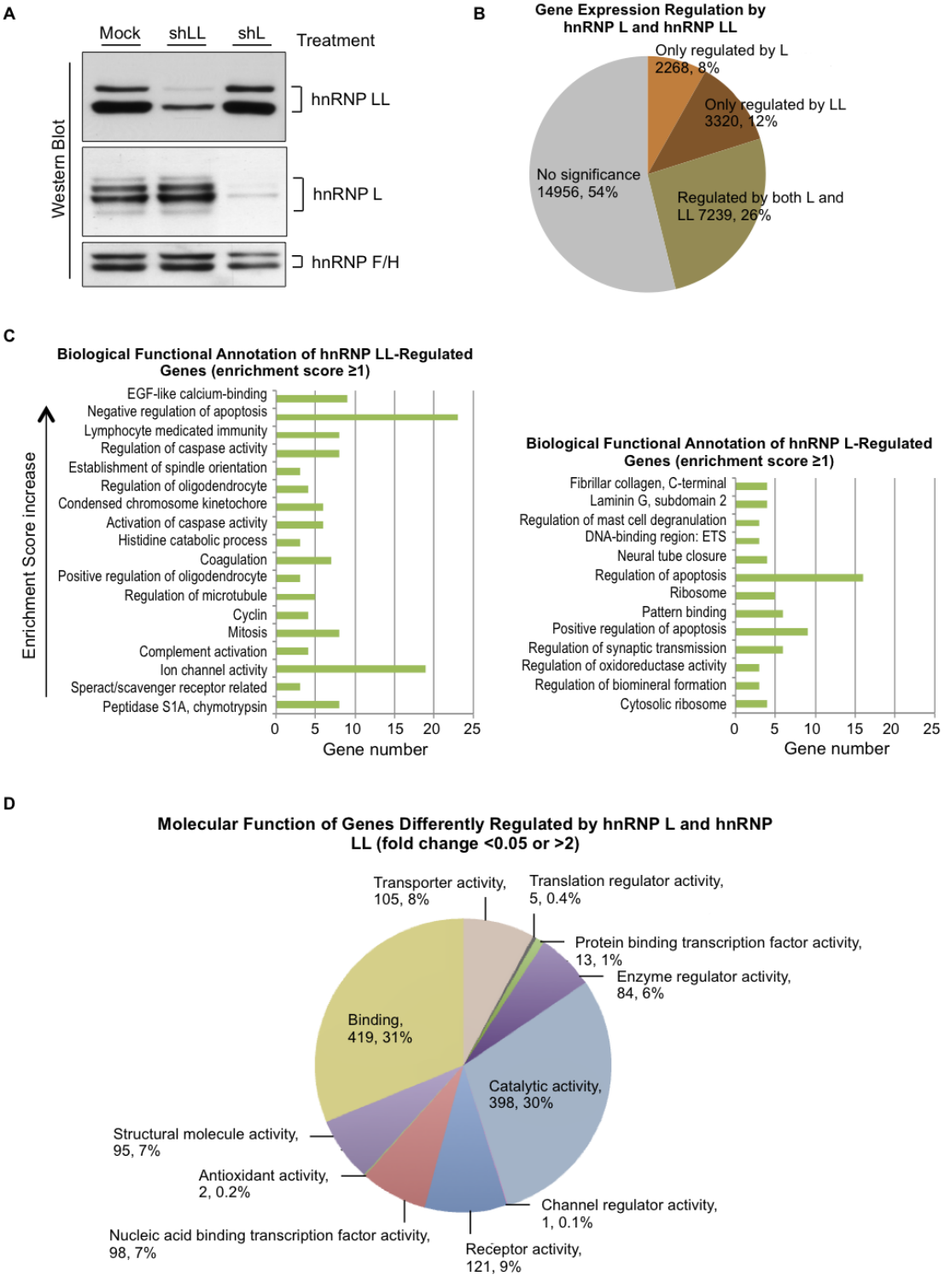

A
Mock
shLL
shL
Treatment
Western Blot
hnRNP LL
hnRNP L
hnRNP F/H
B
Gene Expression Regulation by hnRNP L and hnRNP LL
Only regulated by L 2268, 8%
Only regulated by LL 3320, 12%
No significance 14956, 54%
Regulated by both L and LL 7239, 26%
C
Biological Functional Annotation of hnRNP LL-Regulated Genes (enrichment score ≥1)
Enrichment Score increase
EGF-like calcium-binding
Negative regulation of apoptosis
Lymphocyte medicated immunity
Regulation of caspase activity
Establishment of spindle orientation
Regulation of oligodendrocyte
Condensed chromosome kinetochore
Activation of caspase activity
Histidine catabolic process
Coagulation
Positive regulation of oligodendrocyte
Regulation of microtubule
Cyclin
Mitosis
Complement activation
Ion channel activity
Speract/scavenger receptor related
Peptidase S1A, chymotrypsin
0 5 10 15 20 25
Gene number
Biological Functional Annotation of hnRNP L-Regulated Genes (enrichment score ≥1)
Fibrillar collagen, C-terminal
Laminin G, subdomain 2
Regulation of mast cell degranulation
DNA-binding region: ETS
Neural tube closure
Regulation of apoptosis
Ribosome
Pattern binding
Positive regulation of apoptosis
Regulation of synaptic transmission
Regulation of oxidoreductase activity
Regulation of biomineral formation
Cytosolic ribosome
0 5 10 15 20 25
Gene number
D
Molecular Function of Genes Differently Regulated by hnRNP L and hnRNP LL (fold change <0.05 or >2)
Transporter activity, 105, 8%
Translation regulator activity, 5, 0.4%
Protein binding transcription factor activity, 13, 1%
Enzyme regulator activity, 84, 6%
Binding, 419, 31%
Catalytic activity, 398, 30%
Structural molecule activity, 95, 7%
Antioxidant activity, 2, 0.2%
Channel regulator activity, 1, 0.1%
Nucleic acid binding transcription factor activity, 98, 7%
Receptor activity, 121, 9%

Figure 12. Functional annotations of hnRNP L and LL targeted genes. (A) Representative western blots demonstrating the efficiency and specificity of RNAi against the hnRNP L or LL. HnRNP F/H: protein loading control. **(B)** Pie chart showing gene expression regulation by hnRNP L, hnRNP LL and by both according to RNA-Seq analysis. **(C)** DAVID biological functional clustering of genes with hnRNP L- or hnRNP LL-regulated expressional changes identified by RNA-Seq analysis (fold change <0.5 or >2; enrichment score ≥1). **(D)** Molecular Function of Genes that differently regulated by hnRNP L and LL using Gene Ontology (fold change <0.5 or >2). Pie chart is labeled as "category name, gene number, percentage of total".

Although hnRNP L and LL share high similarities in their amino acid sequences, RNA-recognition motifs (RRMs) and binding preferences, they have shown distinctive expression patterns and functional consequences [167, 169, 195]. RNA-Seq data analysis showed that these two regulatory factors have different preference on biological systems (Fig. 12B-C). Based on the highly successful prediction rate of the edgeR analysis, we examined the functional clusters of these genes. Four hundred and forty five (445) or 988 genes with hnRNP L- or hnRNP LL-related expression changes (fold change > 2 or < 0.5) identified by RNA-Seq were collected and analyzed using Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics web tool. In total, 53 and 92 functional clusters were identified in hnRNP L- or hnRNP LL-related genes, respectively, suggesting hnRNP LL might play roles in a wider range of biological system in the cells. Among these genes, those that the expressions are differentially regulated by the two factors were analyzed for the molecular function by Gene Ontology Consortium (GOC) (Fig. 12D). It is interesting to see that the two major molecular functions of these genes are binding activity (~31%) and catalytic activity (~30%) along with other important functions including receptor activity (~9%), transporter activity (~8%), transcription factor activity (~7%) and structural molecule activity (~7%). (Source of these genes can not be clearly traced back to the original categories.)

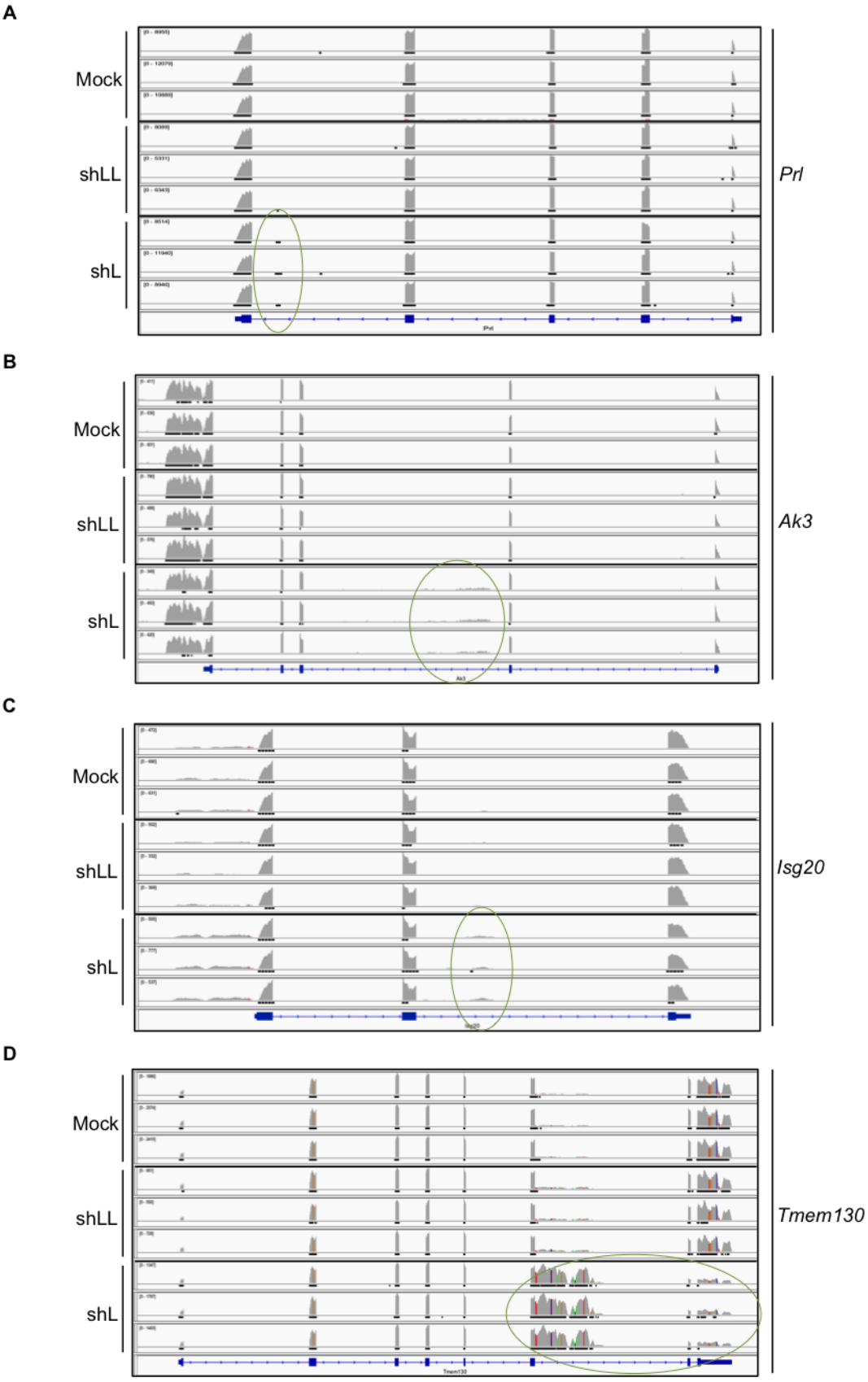
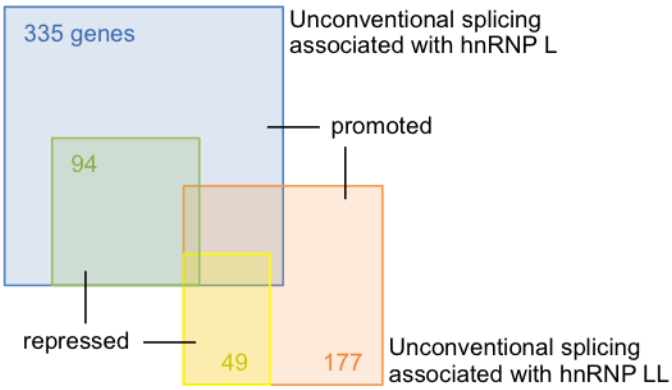

A
Mock
shLL
shL
Prl
B
Mock
shLL
shL
Ak3
C
Mock
shLL
shL
Isg20
D
Mock
shLL
shL
Tmem130

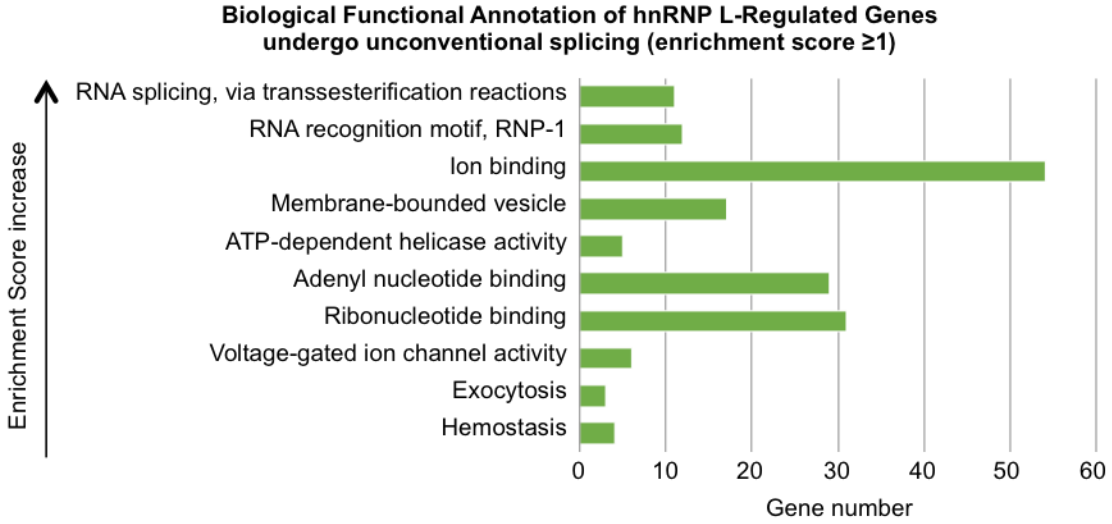
Figure 13. HnRNP L prevents aberrant splicing events. (A-D) IGV images visualizing RNA-Seq reads mapped on selected genes with cryptic splicing in hnRNP L knockdown GH_3 cells. Reads located in cryptic splicing regions are circled by green lines. Each row represents one sample, each treatment group (triplicated) is separated by black thick lines. Reads are accumulated into gray areas, the more reads, the higher peak in the region. To the left of each row is a number (within brackets) representing the read scale of that row. Reference genes (blue) are shown at the bottom of the view (blue big box: exons, blue small box: UTR, blue line: introns, arrows: transcription direction).

The RNA-Seq data was visualized using Integrative Genomics Viewer (IGV). Very interestingly, cases of intron retention and 3' end retention were observed in hnRNP L knockdown samples (Figure 13A-D), implying a role of hnRNP L in preventing the usage of cryptic splicing sites and alternative polyadenylation sites. To explore further about this important observation, RNA-Seq read counts located in non-coding regions were mapped and calculated by edgeR for their expressional significance among the three treatment groups. Those with fold changes > 2 or < 0.5 were subjected to further filtration using IGV sequence analysis to eliminate false positive (background, inconsistent variance, gene overlapping/ imbedding) and only genes with distinct consistent changes in introns were counted and sorted. Unconventional splicing events, including intron retention/cryptic exons/cryptic transcription initiation sites/cryptic polyadenylation sites, occurred in 335 (94 repressed) and 177 (49 repressed) genes upon hnRNP L and LL knockdown, separately (Fig. 14A). The changes of 46 (2 repressed) genes were associated with both hnRNP L and LL knockdown. By comparing the locations of these cryptic splicing events, it was found that 38% of hnRNP LL-related happened in the first intron, which was much more than that of hnRNP L-related (17%). This suggests that hnRNP LL likely prefers binding to the first intron of a gene for regulating its splicing or alternative start sites.

A


335 genes
Unconventional splicing
associated with hnRNP L
promoted
94
repressed
49
177
Unconventional splicing
associated with hnRNP LL

B


Biological Functional Annotation of hnRNP L-Regulated Genes
undergo unconventional splicing (enrichment score ≥1)
Enrichment Score increase
RNA splicing, via transsesterification reactions
RNA recognition motif, RNP-1
Ion binding
Membrane-bounded vesicle
ATP-dependent helicase activity
Adenyl nucleotide binding
Ribonucleotide binding
Voltage-gated ion channel activity
Exocytosis
Hemostasis
0
10
20
30
40
50
60
Gene number

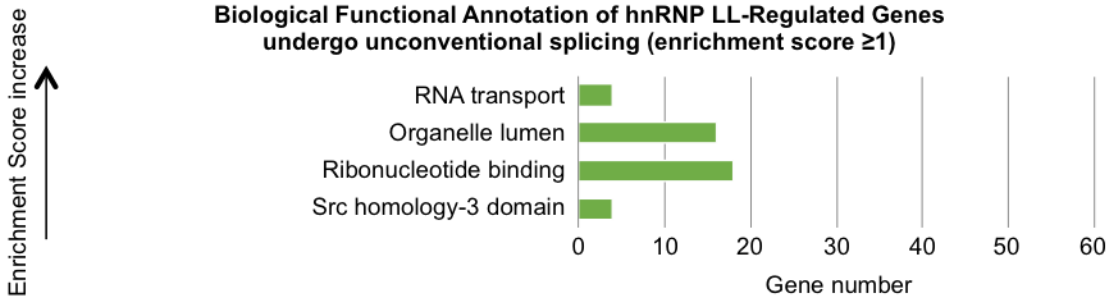

Biological Functional Annotation of hnRNP LL-Regulated Genes
undergo unconventional splicing (enrichment score ≥1)
Enrichment Score increase
RNA transport
Organelle lumen
Ribonucleotide binding
Src homology-3 domain
0
10
20
30
40
50
60
Gene number

Figure 14. Gene ontology of genes that undergo unconventional splicing associated with hnRNP L and LL. (A) Distribution of genes with hnRNP L- or hnRNP LL-related unconventional splicing. Unconventional splicing in 335 genes is hnRNP L-related (blue box) with 94 of them are repressed (green box). HnRNP LL-related (orange box) is comprised of 177 genes with 49 of them are repressed (yellow box). Overlapping regions indicating the amount of genes with common attributes. (B) DAVID biological functional clustering of genes underwent hnRNP L- or hnRNP LL-preferred unconventional splicing events identified by RNA-Seq analysis (enrichment score ≥1).

Functional annotation of these genes was carried out by using DAVID. Of the 53 functional groups of hnRNP L, the top three with highest enrichment scores are RNA splicing, RNA recognition motif RNP-1 and ion binding (Fig. 14B). The top three with largest gene numbers are ion binding, ribonucleotide binding and adenyl nucleotide binding. HnRNP LL has relatively less width of impact as it causes fewer cryptic splicing events in genes. The top three out of 25 related functions with highest enrichment scores are RNA transport, organelle lumen and ribonucleotide binding. The top three with largest gene numbers are ion binding, ribonucleotide binding and organelle lumen. Particularly, the "transcription initiation" function is only in the hnRNP LL knockdown group, which is consistent with the previous observation. To analyze in detail, we found that hnRNP L is involved in a group of function-specific protein families. They are the A-kinase anchor protein family (*Akap6, Akap8, Akap8l*), the ATPase family (*Atp11a, Atp6v0a1, Atp6v1d, Atp6v1g3*), the Centrosomal Protein family (*Cep19, Cep44, Cep57l1*), the hnRNP protein family (*Hnrnpa3, Hnrnpab, Hnrnpc, Hnrnph1, Hnrnpl*), the Solute Carrier Family (*Slc12a4, Slc25a26, Slc35e4, Slc6a8*), the zinc finger protein family (*Zfp167, Zfp385a, Zfp386*). More interestingly, the intronic cryptic splicing in the transmembrane protein family (*Tmem120a, Tmem128, Tmem130, Tmem184c, Tmem186, Tmem209, Tmem38a Tmem5*) was all repressed by hnRNP L, whereas that of the

Serine/Arginine-Rich Splicing Factor (SRSF) family (*Srsf2, Srsf3, Srsf5, Srsf6, Srsf7, Srsf10, Srsf11*) was promoted by the presence of hnRNP L. As for hnRNP LL, the DNAJ/HSP40 protein family (*Dnajb5, Dnajc6, Dnajc8*), the Solute Carrier Family (*Slc12a1, Slc12a6, Slc25a26, Slc37a4, Slc39a3*) and the zinc finger protein family (*Zfp295, Zfp385a, Zfp451, Zfp532, Zfp780b, Zfp787*) are related with either enhancement or repression for intronic sequence by the presence of hnRNP LL. However, the functional annotation is based on bioinformatics, which needs to be confirmed and consolidated by further experiments.

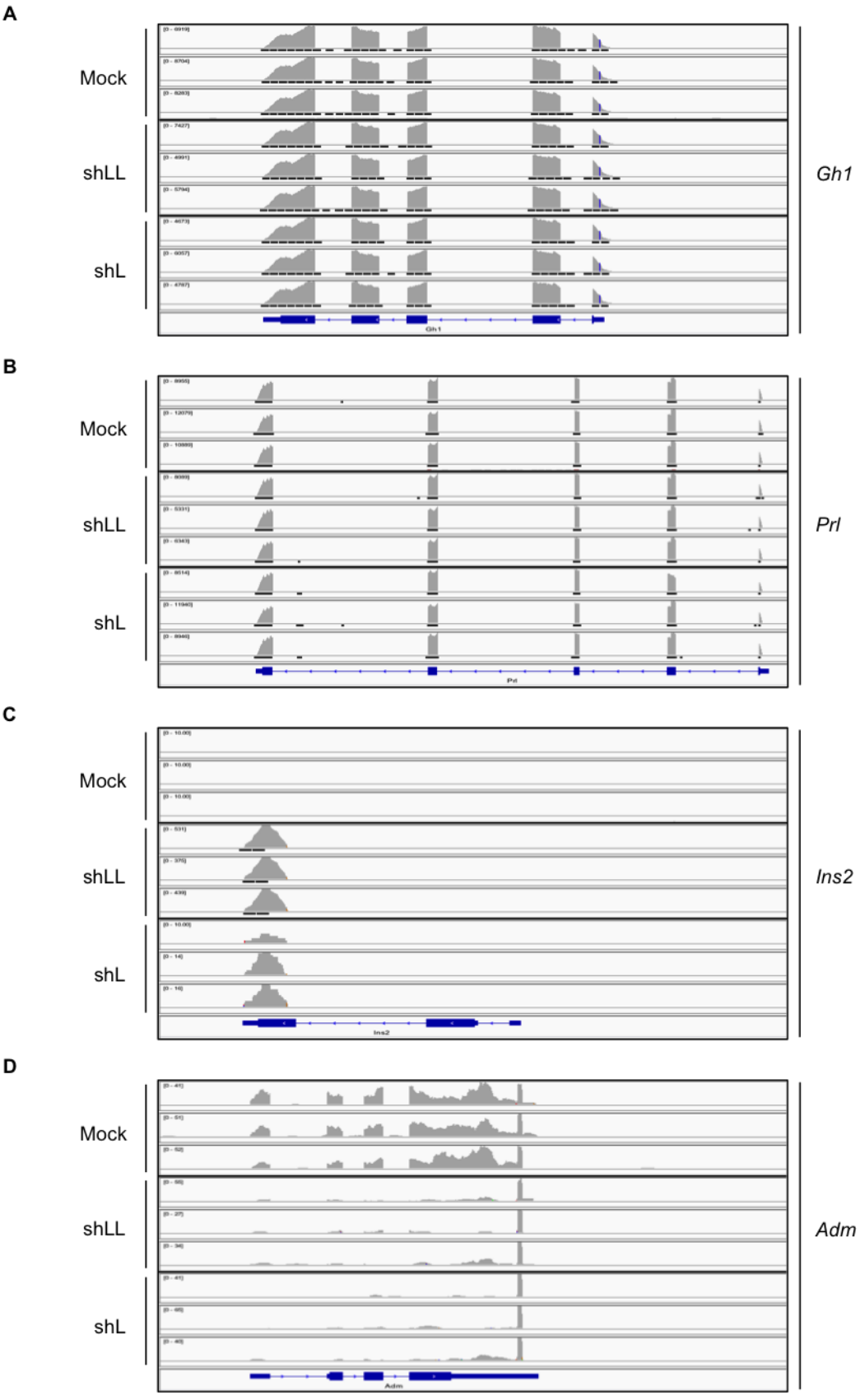

A
Mock
shLL
shL
Gh1
B
Mock
shLL
shL
Prl
C
Mock
shLL
shL
Ins2
D
Mock
shLL
shL
Adm

Figure 15. HnRNP L and LL regulate a network of hormone-related genes. (A-D) IGV images visualizing RNA-Seq reads mapped on selected hormone genes. Each row represents one sample, each treatment group (triplicated) is separated by black thick lines. Reads are accumulated into gray areas, the more reads, the higher peak in the region. To the left of each row is a number (within brackets) representing the read scale of that row. Reference genes (blue) are shown at the bottom of the view (blue big box: exons, blue small box: UTR, blue line: introns, arrows: transcription direction).

11. RNA-Seq data identifies a network of hnRNP L/LL-regulated genes in hormone production and secretion

Of great interest from the RNA-Seq analysis was the identification of a category of hormone-related genes. Among the significantly changed genes (P-value < 0.05) in the three-way comparison, 13 hormone-related genes were validated and confirmed. Besides the growth hormone and prolactin that were studied, additional four hormones were shown to change their gene expression significantly upon hnRNP L or hnRNP LL downregulation. They are adrenomedullin, ghrelin/obestatin prepropeptide, insulin2 and insulin-like 6 (Fig. 15A-D). Insulin2 gives a very interesting case here because of its independent expression of exon3. *Ins2*, which encodes insulin2, is one of the two non-allelic insulin genes in rats, of which both expressed nearly equally in pancreas [238-240]. Although the expression of insulin has been reported in rat brain (especially in hypothalamus) and tilapia pituitary [241, 242], no study so far discovered its synthesis in rat pituitary. Consistent with the literatures, *Ins2* expression was not detected in the control group (0 read counts in triplicate), but was detected in knockdown samples and dramatically promoted by hnRNP LL. Only its exon 3 was detected but not the other exons using an exon x-specific primer, implicating an alternative initiation site located in the region, whose usage is inhibited by the presence of hnRNP L/LL under normal conditions. To our best knowledge by far, the solo expression of *Ins2* exon3 has not been

reported in human, mouse or rat. The molecular mechanism and biological meaning of its existence remains to be pursued. However, this provides another example for the prevention of cryptic expression of hormone by hnRNP LL and L.

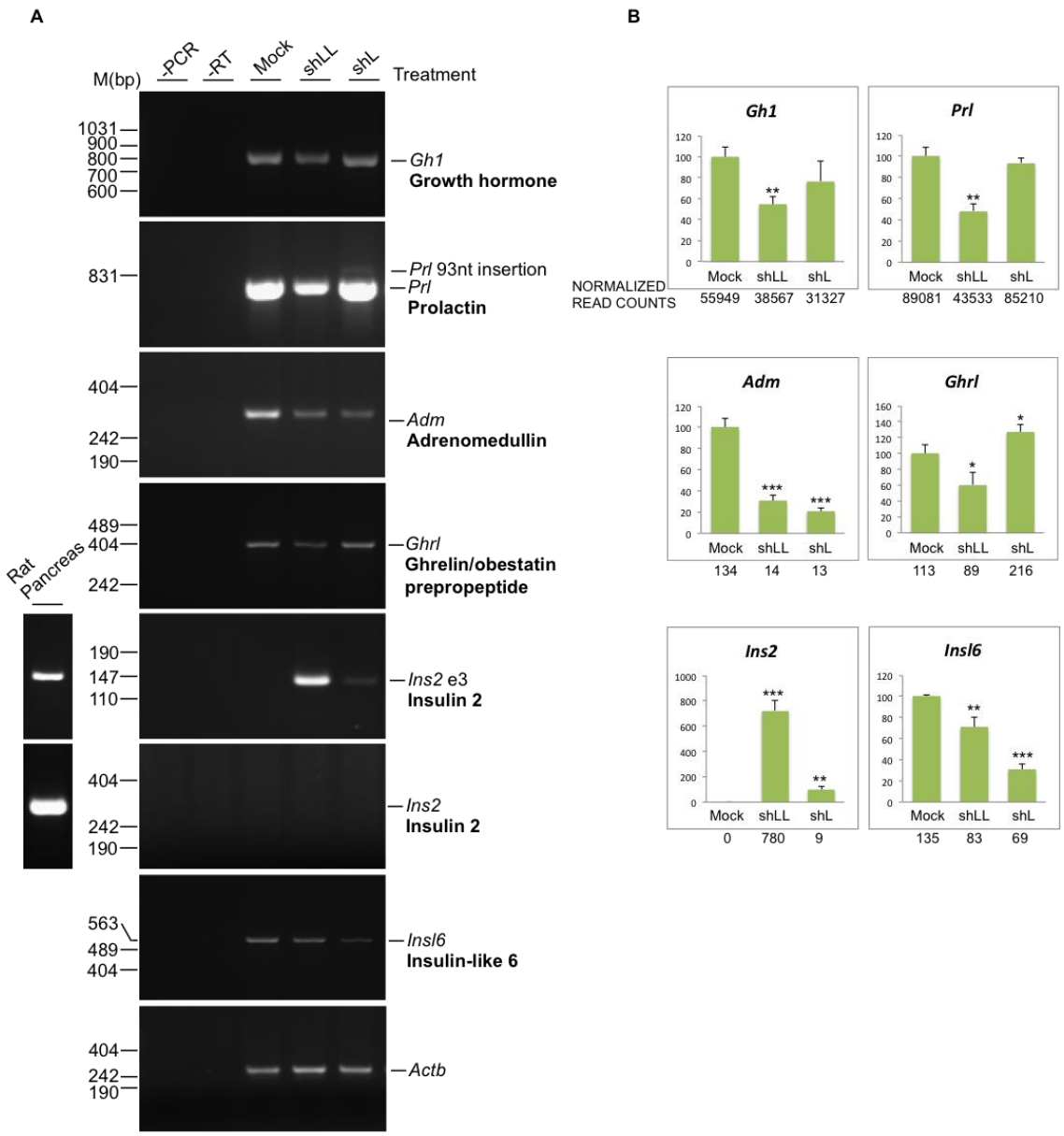


Figure 16. Validation of RNA-Seq data I. (A) Agarose gels of hormone genes, which identified by RNA-Seq analysis, preferentially regulated by hnRNP L or LL in GH_3 cells. -PCR: PCR negative control, -RT: RT negative control, Mock: mock-transduced. Name of protein encoded by each gene is bolded under the gene name. **(B)** Bar graphs summarizing the level of transcripts relative to that of *Actb* (RNA loading control). Normalized read counts from RNA-Seq analysis are marked under corresponding bars. Mean ± S.D., n=3, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs. Mock.

RT-PCR validation confirmed the trend of expression significance of these hormone genes (Fig. 16A-B). To confirm the solo expression of *Ins2* exon 3, two pairs of primers were used to detect the full length and the exon 3 of the gene. Compared to rat pancreas RNA as a positive control, no insulin 2 full-length transcripts were detected in three treatments while its exon 3 was highly expressed in hnRNP LL knockdown cells and some in hnRNP L knockdown ones.

Besides hormone genes, seven hormone-related genes with abundant counts and significant changes were also validated and confirmed of their trend of expression difference (Fig. 17A). More hormone-related genes showed to be differentially targeted by hnRNP L and/or L-like proteins in our RNA-Seq data (not yet confirmed by RT-PCR), for instance *Lep*, *Pdyn*, *Syt1*, *Syt4*, *etc*. These genes are involve in a full spectrum of hormone production processes including synthesis, modification, storage, release, cell signaling and receptor activity, suggesting a wider roles of hnRNP L and LL in the differential control of hormone production.

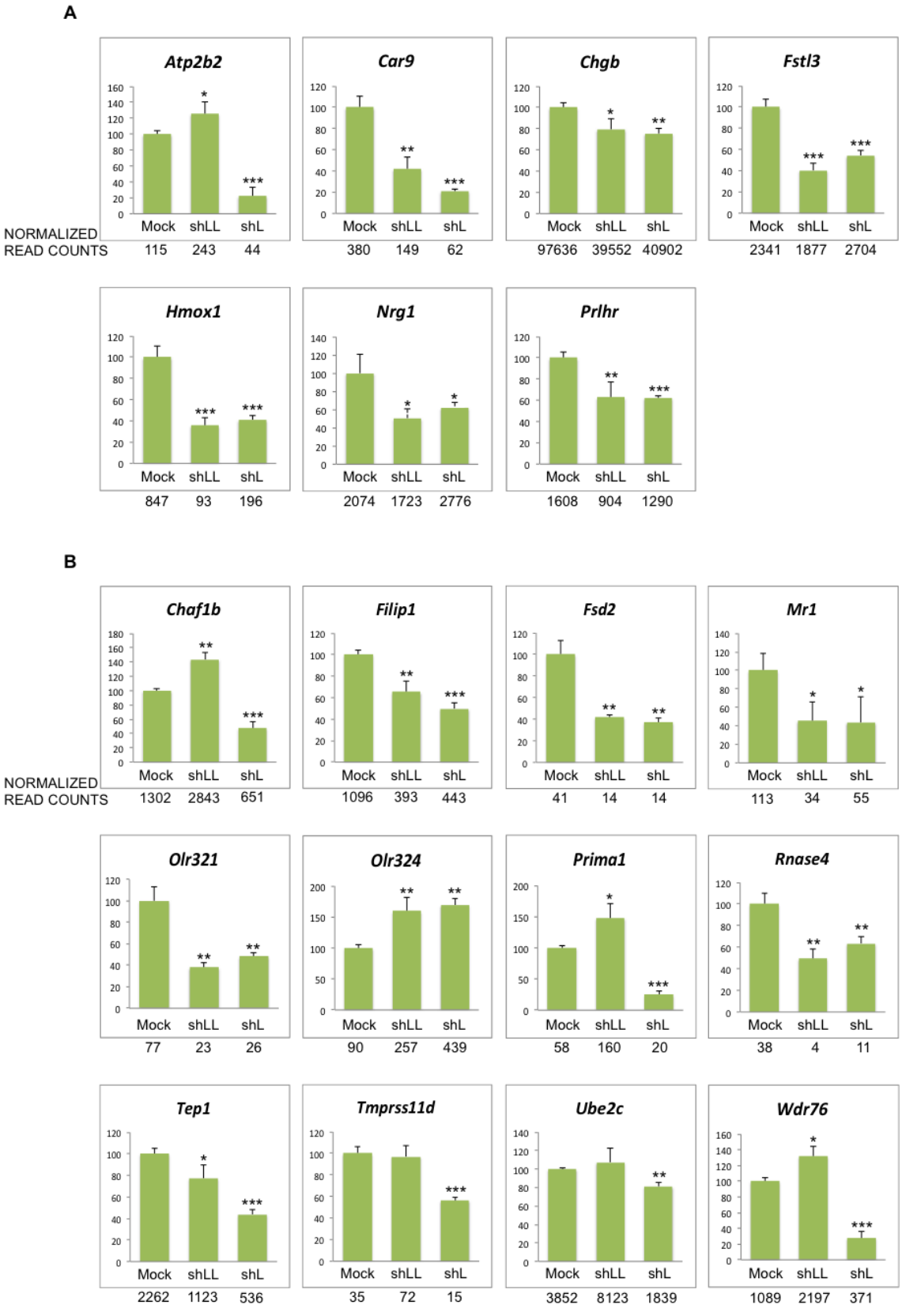

A
Atp2b2
Car9
Chgb
Fstl3
Hmox1
Nrg1
Prlhr
Mock shLL shL
NORMALIZED READ COUNTS
115 243 44
380 149 62
97636 39552 40902
2341 1877 2704
847 93 196
2074 1723 2776
1608 904 1290
B
Chaf1b
Filip1
Fsd2
Mr1
Olr321
Olr324
Prima1
Rnase4
Tep1
Tmprss11d
Ube2c
Wdr76
1302 2843 651
1096 393 443
41 14 14
113 34 55
77 23 26
90 257 439
58 160 20
38 4 11
2262 1123 536
35 72 15
3852 8123 1839
1089 2197 371

Figure 17. Validation of RNA-Seq data II. (A and **B)** Hormone related genes (A) and other genes (B) preferentially targeted by hnRNP L or LL in GH_3 cells. Normalized read counts from RNA-Seq analysis are marked under corresponding bars. Mean ± S.D., n=3, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$ vs. Mock.

Summary

This study identified a network of hormone-related genes differentially regulated by hnRNP L, LL and PTB, particularly *Gh1* and *Prl*. This points to the common, distinctive, as well as cooperative properties of paralogous or homologous RNA processing factors on hormone production, particularly the role of hnRNP L in preventing aberrant splicing of endogenous genes.

CHAPTER IV

Conclusions

1. The production of growth hormone and prolactin is differentially controlled by hnRNP L and LL at both the level of gene expression and splicing.

2. Specifically, the endogeneous level of hnRNP L is crucial for the proper splicing of prolactin transcripts; and that of LL is required for the expression of both hormone genes.

3. There are cooperative effects among the hnRNP proteins L, LL and PTB in the regulation of the expression and splicing of the hormone genes.

4. The role of hnRNP LL in the secretion of both hormones is also confirmed in the primary culture of pituitary cells.

5. RNA-Seq analyses of the transcriptomes identified a larger group of hormone or hormone-related genes whose expression and splicing are also differentially controlled by hnRNP L and LL.

6. This suggests that the larger family of hnRNP proteins may play critically differential roles in controlling particular functions of differentiated cells.

CHAPTER V

Further Considerations and Future Directions

1. The common, distinct roles of and cooperation among hnRNPs

Knowledge of their common, distinct or cooperative properties, particularly between paralogous ones, is essential for understanding their specific regulatory roles in gene expression. Studies have been carried out for the PTB and nPTB or hnRNP L and LL during cell differentiation [170, 195, 243]. This study provides another example demonstrating these properties but in differentiated cells. Firstly, both hnRNP L and LL regulate a category of hormone-related genes (Figs. 16-17). Secondly, these two factors can have different targets or different effects on a target (Figs. 16-17). It is particularly exemplified in their differential roles in the regulation of growth hormone and prolactin genes (Figs. 2 & 4). Thirdly, the effect of hnRNP L on prolactin transcript is likely resulted from the cooperation with hnRNP LL and/or the competition with PTB (Fig. 10). These observations support their different but cooperative roles in gene regulation and hormone production.

2. Direct or indirect effect of hnRNP L and LL

Lentiviral transduction usually takes 48-72 hours for efficient expression depending on the cell types. After that, transduced cells are harvested for analysis [244]. In this study, transduced GH_3 cells or primary pituitary cells were harvested on day 7 (the 6th day post transduction) since the maximum transduction efficiency is needed. This protocol has been optimized and followed in our lab for lentiviral transduction in GH_3

cells. Therefore, based on the time taken for the changes to occur, indirect effect could have also occurred during the changes in the growth hormone and prolactin gene expression. However, since potential hnRNP L/LL binding sites (CA repeats) have been found in the regulated target genes, it is likely that the effects of hnRNP L/LL knockdown is from direct interaction of the proteins with the target mRNAs. Work is currently ongoing in the lab to confirm this effect.

3. Knockdown of hnRNP L and LL

As shown in Fig. 2A, the protein levels of hnRNP L were ~15% in shL, ~43% in shLL and ~60% in shLL+hNRG1 treatment groups in comparison to that of Mock (Fig. 2A). The hnRNP L expression was decreased upon hnRNP LL knockdown. However, this phenomenon happened in the set of experiment was not consistent to other experiments in my study, for instance Figs. 7, 11 and 12. The cause of it is not clear. It may be resulted from the very low expression level of hnRNP LL since the knockdown efficiency of hnRNP LL (~95%) was the highest among all the experiments. However, this variation is unlikely to severely affect the result of hormone detection because of two reasons. Firstly, the decrease in GH and PRL brought by hnRNP LL knockdown in the experiment was consistent to all the other experiments that had showed no change in hnRNP L expression upon shLL expression (Figs. 7, 11 and 12). Secondly, as discussed above, double knockdown of hnRNP L and LL showed similar effect on the expression of hormones as compare to that of hnRNP LL knockdown, suggesting the dominant effect of hnRNP LL in controlling the expression of GH and PRL.

4. Possible competition between hnRNP L/LL and microRNAs for the binding sites on the 3'UTR of hormone genes

Competition between hnRNP L and microRNAs has been reported previously [245]. According to the search-results in microRNA.org database (developed at Memorial Sloan-Kettering Cancer Center, available on the Internet at http://www.microrna.org/microrna/home.do [246]), CA-rich element-binding microRNAs targeting sites are found at the 3'UTR of the prolactin gene. Human *PRL* 3'UTR is targeted by miR-299, miR-203 and miR-223 on the CA-rich elements. Rat *Prl* 3'UTR is bound by miR-382 on the CA-rich elements. Therefore it is possible that the CA-rich element-binding microRNA competes with hnRNP L/LL and binds to the prolactin mRNA for RNA degradation upon knockdown of hnRNP L/LL.

As for the growth hormone gene, CA-rich element-binding miR-138 is found at the 3'UTR region in humans. MiR-590 recognizes a non-CA-rich sequence at the 3'UTR in human, mouse and rats. Other CA-rich element-binding microRNAs include miR-297, miR-567 and miR-609. Whether microRNA are involved in the effect of hnRNP L/LL on hormone production remains to be examined.

5. Remaining questions on hormone analysis in primary cultures

Regarding the RNAi/rescue assay in primary rat pituitary cultures, it remains to be determined as a number of differences in growth hormone and prolactin production were found between GH_3 cells and primary rat pituitary cultures. Firstly, alterations of gene regulation between tumor cell lines and primary culture exist [109]. Secondly, primary pituitary cultures are comprised of various types of pituitary cells. In fact, there

are at least three different types of pituitary cells producing growth hormone and/or prolactin (acidophils) - somatotrophs (constitute 40-50% of anterior pituitary cells, produce GH), lactotrophs (10-25%, PRL) and somatomammotrophs (15-25%, both GH and PRL) [111]. Whether hnRNP LL acts in the same way in all these cells as in GH_3 cells remains a question. Thirdly, the abundance of intracellular hormone protein could have reduced the visual differences in Western blots. We noticed that the experiment condition used for GH_3 cells might not be suitable for primary cultures. For instance, the film exposure for detecting GH_3 growth hormone by Western blot is minimum 30 minutes whereas only 5 minutes is enough for pituitary samples; exposure time for detecting hnRNP LL in primary culture is 10-20 minutes more than that of GH_3. Therefore, optimization of experiment techniques and conditions needs to be done in the future. Last but not least, since there was no protein accumulation in these cells while as the secreted product is reduced, it is likely that protein production is affected as well. All these possibilities and questions need to be explained before we could consolidate any conclusion from the primary culture experiment.

6. HnRNP regulation of different groups of genes

There are at least 20 major hnRNPs named through hnRNP A1 to U [172]. This small number of factors regulates the processing of numerous RNA transcripts. How do they divide their tasks? What is the specific and unique role for each hnRNP? These questions are still not answered. A recent study has found that different hnRNPs, even the closely related ones, regulate distinct groups of targets with little overlap [247]. Consistent with this, our data showed that 1) for gene expression regulation, about 24%

of hnRNP L-targeted genes and 32% of hnRNP LL-targeted genes do not overlap with those targeted by the other protein; even for the overlapped ones, hnRNP L and LL can have either similar or opposite effects; 2) for unconventional splicing regulation, about 86% of hnRNP L-targeted genes and 74% of hnRNP LL-targeted genes do not overlap; most of these genes cluster in different biological functions.

Besides the difference in target selection, hnRNP L and LL proteins also seem to have different behavior patterns in the regulation of gene expression and unconventional splicing. Our data indicates that hnRNP LL has ~11% more target genes regarding its function in gene expression regulation; it also has stronger effect in terms of the changes on the expression level; while hnRNP L affects the unconventional splicing of about twice as many genes (these results are based on the effect of hnRNP LL or L at their knockdown efficiencies of ~76% and ~92%, respectively). These findings point out that paralogous hnRNP proteins are likely to have preferences on different clusters of genes with distinct behavior patterns in their different functions.

7. Important genes with less regulatory factors

Growth hormone and prolactin are encoded by the genes that evolved from a common ancestral gene happened about 400 million years ago [248, 249]. These two proteins are both peptide hormones that have similar gene architectures and share about 40% amino acid sequence homology [250, 251]. In this study, hnRNP L targets the transcripts of prolactin while hnRNP LL targets both growth hormone and prolactin, suggesting that hnRNP LL interacts a common site in both hormones but hnRNP L does not.

These two hormones function differently despite their sequential and structural similarities. Studies on the growth hormone receptor knockout mice (GHR/BP -/-) revealed that they appeared small in size, defected organism development, delayed sexual maturation, retarded, low insulin and undetectable IGF-I [252-254]. Prolactin knockout mice (-/-) and prolactin receptor knockout mice (-/-) showed no effect on body size, body composition and metabolic phenotype in either sex, but reduced bone formation in young and completely infertile in female due to multiple reproductive abnormalities [255-257]. These studies indicate that growth hormone has more important global effects in species than prolactin does. In combination with our study, it provides a point for understanding the physiological meaning of why prolactin can be regulated by both paralogous RNA binding factors but growth hormone is only subject to the regulation by one of them. By being subjected to less regulatory hnRNPs, presumably a gene would have less chance to be disrupted by disturbance of the hnRNPs, especially for those important genes. Therefore, individual could be exposed to less life-threatening situations that are caused by mutation or regulatory defect/impairment.

8. Potential application in therapy for curing hormone deficiency/hyperproduction

Abnormal growth hormone/prolactin secretion includes hormone deficiency and hormone hyperproduction. In the case of growth hormone deficiency, the causes are largely unidentified [163]. Our study identified the critical role of hnRNP LL on the control of hormone production, which should be included in clinical diagnosis as a

disease cause or associated factor. It is possible that a type of hormone deficiency in patients is resulted from hnRNP LL defect.

Regarding the situation of hormone hyperproduction, it is commonly caused by tumor [1]. The regulatory function of hnRNP LL on hormones can be applied in therapy by selectively knocking out this RNA binding factor in the hormone-secreting tumor to abolish the excess hormone production. Moreover, the gene functional cluster of our RNA-Seq data showed that hnRNP LL-targeted genes involve in the negative regulation of apoptosis (cell survival) (Fig. 12C). Therefore, its knockout in tumor cells promotes cell death, which will be an additional benefit for treating tumors.

9. Further Directions

Identification of the differential regulation of hnRNP L and LL on growth hormone and prolactin mRNA raises the question of what is the mechanism of hnRNP L-regulated splicing of the prolactin pre-mRNA. Actually it is very interesting that a region with $(CA)_{21}$ repeat is found at 494 base pair upstream of the *Prl* retained intron sequence (Fig. 4C). Similarly, prolonged CA-repeat sequences are also seen in the last intron of *Prl* in human, rhesus, rabbit, alpaca, horse, cat and opossum. Meanwhile, CA-repeats are also found in the retained sequence. Based on the known position-dependent regulation mechanism of hnRNP L that splicing repression tend to happen when it bind to intronic regions upstream of the exon or inside the exon [193, 258], it is possible that hnRNP L interacts with these CA-repeats and subsequently represses the splicing of this 93-nt cryptic exon under normal physiological conditions. This is being investigated in the lab.

Another question is that how hnRNP L controls the gene expression of the two hormones. CA-repeats are found in the coding region and the 3' UTR of both *Gh1* and *Prl* genes. It is known that hnRNP L can enhance RNA stability by binding to the CA-repeats in 3'UTR [174]. Similar regulatory behaviour may exist for hnRNP LL, which means it is possible that loss of hnRNP LL binding with *Gh1* or *Prl* at their 3'UTR results in RNA degradation. This could be a direction for investigating the mechanism of hnRNP LL on hormone production. Another possibility for the hormone mRNA reduction would be transcription inhibition based on reported experiment results [176].

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