Title

In Vitro Validation of Putative Patient Mutations Implicate ZIC1 as Causative Gene for Isolated GnRH Deficiency

Author

Sung, Kaitlin Emily

Publication Date

2021

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA SAN DIEGO

In Vitro Validation of Putative Patient Mutations Implicate ZIC1 as Causative Gene for Isolated GnRH Deficiency

A thesis submitted in partial satisfaction of the requirements of the degree Master of Science

in

Biology

by

Kaitlin Emily Sung

Committee in charge:

Professor Pamela L. Mellon, Chair
Professor Susan Golden, Co-chair
Professor Maho Niwa Rosen

2021

The thesis of Kaitlin Emily Sung is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

I would like to dedicate this thesis to:

My parents, who have supported me with my goals through thick and thin throughout my life.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ARC	Arcuate nucleus of the hypothalamus
AVPV	Anteroventral periventricular nucleus of the hypothalamus
βGal	Beta-Galactosidase
E299K	ZIC1 substitution at amino acid position 299 of a glutamate to a lysine
GnRH	Gonadotropin-releasing hormone
GnRH (e/p)-Luc	Gonadotropin-releasing hormone enhancer/promoter Luciferase
H134Rfs*21	ZIC1 frameshift mutation at amino acid 134 and stops after 21 bps
hKiss-Luc	Human Kisspeptin Luciferase
HPG	Hypothalamic-Pituitary-Gonadal
IGD	Isolated GnRH Deficiency
IHC	Immunohistochemistry
S413Y	ZIC1 mutation at amino acid position 413 from a serine to a tyrosine
VB bb	Vector Builder Backbone
Y286X	ZIC1 premature stop codon at amino acid position 286
ZIC1	Zinc finger of the cerebellum family member 1

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to acknowledge my Principal Investigator and committee chair, Dr. Pamela Mellon, for allowing me to join the lab first and foremost.

I would also like to acknowledge my mentor, Dr. Jessica Cassin, for her excellent teaching and incessant guidance during the entirety of my time in lab as well as everyone I have interacted with in our lab for helping me with any part of this project.

ABSTRACT OF THE THESIS

In Vitro Validation of Putative Patient Mutations Implicate ZIC1 as Causative Gene for Isolated GnRH Deficiency

by

Kaitlin Emily Sung

Master of Science in Biology

University of California San Diego, 2021

Professor Pamela L. Mellon, Chair

Professor Susan Golden, Co-Chair

Isolated Gonadotropin-Releasing Hormone Deficiency (IGD) is a condition characteristically marked by the absence or delay of puberty. Previous work suggests that there is a disconnect between the hypothalamus and pituitary in the hypothalamic-pituitary gonadal

(HPG) axis that may be a cause for this condition. In particular, the expression of Kisspeptin, as well as GnRH, in the hypothalamus which are vital aspects of the HPG axis for maintaining fertility may be disrupted. Whole exome sequencing performed on IGD patients revealed mutations in a gene called ZIC1. There has been little to no previous work done on the role of ZIC1 in the reproductive system. We developed a patient mutation model in the immortalized KTaV and KTaR cell lines to test the implications of ZIC1 on the expression of Kisspeptin. We also use the GT1-7 cell line to test ZIC1 on expression of GnRH. In our study, we find that novel patient mutations in ZIC1 reverse ZIC1 effects on Kisspeptin luciferase and GnRH luciferase expression. Select mutations also produce truncated proteins and display a dispersion of localization in the cell. Our results provide preliminary mechanisms that support further study of the etiology of IGD as well as identifying a novel gene that regulates the HPG axis.

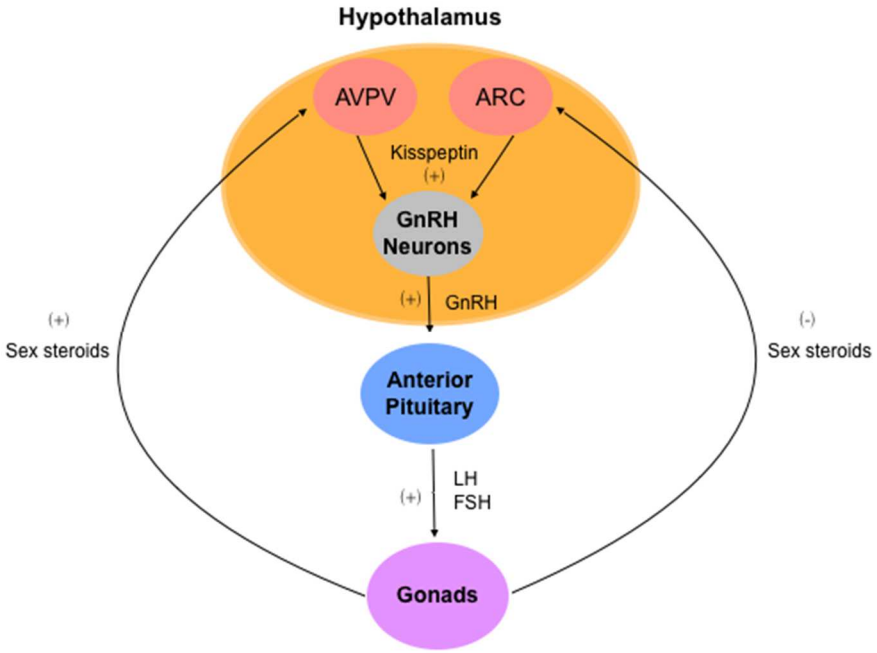
INTRODUCTION

Fertility and the HPG Axis

Fertility is a major component of biological evolution and reproductive health that is relevant to every living organism with a reproductive system. There are many conditions and underlying reasons that may involve disruptions in normal hormone secretion that may result in reproductive failure and infertility. As of 2013, about 15% of the population in the United States are affected by infertility [1]. An important feedback loop of the neuroendocrine system that regulates and maintains fertility is called the hypothalamic-pituitary-gonadal (HPG) axis.

The HPG axis is a critical feedback loop mechanism of the mammalian reproductive system (Fig. 1). In the hypothalamus, Kisspeptin neurons secrete Kisspeptin neuropeptide expressed by the *KISS1* gene to trigger the pulsatile release of downstream GnRH, an important hormone that plays a key role in maintaining this axis [2, 3]. GnRH neurons originate in the olfactory placode in the nose and migrate to their final destination in the preoptic area of the hypothalamus during embryonic development [4]. In the hypothalamus, GnRH neurons project their axons to the median eminence to release GnRH in a pulsatile manner [2]. GnRH then docks at receptors in the anterior pituitary, triggering the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [3, 5]. Two regions in the hypothalamus called the arcuate (ARC) nucleus and the anteroventral periventricular (AVPV) nucleus release Kisspeptin under different regulatory mechanisms. Prior studies indicate that the ARC Kiss neurons are important for pulsatile GnRH, while the AVPV neurons are responsible for the LH surge that initiates ovulation [6]. LH and FSH subsequently travel to the gonads and trigger the release of sex hormones, including estradiol and progesterone in females, and testosterone in males, thus completing the cycle [7]. These sex steroids feedback to the ARC and AVPV Kiss neurons in the

hypothalamus to control the rate of secretion of GnRH [7]. Defects at any site in this axis can disrupt the cycle and cause symptoms of infertility.



Abbreviations:

ARC: Arcuate Nucleus
AVPV: Anteroventral periventricular nucleus
GnRH: Gonadotropin-releasing hormone
FSH: Follicle-stimulating hormone
LH: Luteinizing hormone

Figure 1. The Hypothalamic-Pituitary-Gonadal (HPG) Axis. In the hypothalamus, Kisspeptin neurons from two different regions, the ARC nucleus and AVPV nucleus, secrete Kisspeptin hormone to GnRH neurons. GnRH neurons are stimulated by Kisspeptin to secrete GnRH that then migrates to the anterior pituitary, which secretes LH and FSH. These pituitary hormones then stimulate the gonads and cause the release of sex steroids. To maintain a homeostatic level of sex steroids circulating, these sex steroids signal to the hypothalamus to alter Kiss secretion rate. Kisspeptin secretion from Kisspeptin neurons in the ARC is inhibited by low to mid-level concentrations of sex steroids. Kisspeptin secretion from Kisspeptin neurons in the AVPV is stimulated at exclusively high concentrations of sex steroids.

Isolated GnRH Deficiency

There exist various diseases and conditions that result in infertility in affected individuals. Of note, a genetic condition called Isolated GnRH Deficiency (IGD) occurs when GnRH is not sufficient and does not properly reach the anterior pituitary in the HPG axis. IGD is marked by abnormally low levels of LH and FSH and circulating sex steroids in both males and females, causing delayed or absent puberty [8]. There are currently approximately 50 identified causative genes of the disorder, yet 50% of people who are diagnosed with IGD do not have a known genetic origin for their condition [9]. Although the causes of IGD in those 50% of cases is not known, it is defined as being due to defects in the HPG axis and studies to identify additional causative genes are ongoing [10].

Because of the nature of the disease, most affected individuals with IGD have rare and unique mutation in their genes, most of which are *de novo* [9]. Two thirds of individuals diagnosed with IGD are anosmic, or unable to smell, which is a telltale symptom of one major subtype of IGD called Kallman's Syndrome (KS) [11]. As mentioned, GnRH neurons migrate from the medial olfactory placode to the septal preoptic area and hypothalamus, and a non-migration of these neurons may be the origin for the observed GnRH deficiency presented in Kallman's Syndrome [12]. A common treatment for IGD patients is gonadotropin therapy or pulsatile GnRH therapy, during which serum gonadotropins and circulating sex steroids are restored to normal levels [10]. This suggests that there may be an abnormality within the hypothalamus because from the pituitary downwards, the organs and mechanisms remain intact and function normally [10].

GnRH and Kisspeptin Signaling

GnRH is a vital regulator of the HPG axis and the maintenance of fertility as well as onset of puberty. GnRH is made by the *GNRH1* gene, which expresses a preprohormone that is processed to produce GnRH in the HPG axis [4]. Previous studies have shown evidence that a de novo deleterious mutation in *GNRH1* in a mouse caused its IGD, displaying the telltale symptoms of inappropriately low circulating gonadotropins and sex steroid levels as well as inhibited sexual development [13]. When given gene therapy to restore *GNRH1*, normal reproductive functions and development were rescued [14]. On a study done in several IGD patients, frameshift and missense mutations in *GNRH1* were discovered that were suggested to cause their phenotypes [15]. Evidence suggests that the mutations had loss of function and misfunction effects on GnRH, thus explaining IGD symptoms [15]. This study highlights the importance of proper GnRH function in the HPG axis to maintain fertility and illustrates that defects in GnRH release cause IGD.

The GnRH neurons in the HPG axis are stimulated to release GnRH by Kisspeptin, which is released by Kisspeptin neurons that reside in the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV) of the hypothalamus [6]. For GnRH to be released into the HPG axis, Kisspeptin peptides, which are encoded by the *KISS1* gene and produced from specific Kiss neurons in these two regions of the hypothalamus must first stimulate GnRH neurons [16]. Cases of hypogonadotropic hypogonadism occur when this stimulation is halted or disrupted, causing insufficient release of gonadotropins [17].

ZIC1 Gene Importance

Our lab's collaborators at Harvard University Massachusetts General Hospital have performed whole exome sequencing (WES) on IGD patients. They performed bioinformatics analysis on the patients' genomes to screen for mutations that were novel, highly constrained, and predicted to be deleterious. They provided us with a resultant list of genes in which novel, heterozygous mutations were found in these patients that were candidates for causing IGD. Of those genes, one is called zinc finger of the cerebellum family member 1 (ZIC1). The mutations found in ZIC1 are the following: H134Rfs*21, Y286X, E299K, and S413Y. In situ hybridization data displayed on the Allen Brain Atlas site show that ZIC1 is highly localized in the ARC region in the hypothalamus, one of the regions of Kisspeptin neurons important in the HPG axis [18]. However, its implication in the reproductive system and fertility is largely unstudied and yet to be elucidated.

ZIC1 is a C2H2 zinc finger transcription factor found in the nucleus of a cell that is important for many important developmental processes such as neurogenesis [19]. Figure 2 shows the C2H2 Zinc finger binding domains of ZIC1. ZIC1 contributes to the formation of the medial forebrain, and deletion of ZIC1 leads to a brain defect called hypoplasia [20]. Diseases that have been suggested to be associated with ZIC1 include Dandy-Walker Syndrome malformation of the cerebellum and craniosynostosis, both of which involve underdevelopment or abnormalities of the brain and can cause cognitive defects [15]. Additionally, ZIC1 is able to regulate sonic hedgehog, PI3K, and MAPK signaling pathways, and it can be further studied to investigate similar functions and involvement in similar mechanistic pathways as part of the HPG axis [21].

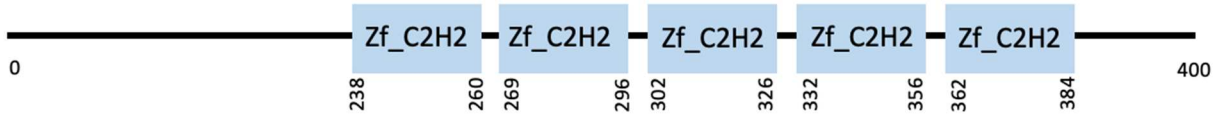


Figure 2. C2H2 Zinc Finger Protein Domains of ZIC1. ZIC1 is a known transcription factor that contains five highly conserved protein domains that allow it to interact with DNA and other proteins. These five C2H2 zinc finger protein domains on ZIC1 are shown. The numbers represent the position in ZIC1 of the beginning and end of each domain.

Goals and Aims of this Project

The main goal of this project is to determine if the H134Rfs*21, Y286X, E299K, and S413Y mutations found by our collaborators contribute to IGD phenotypes and if ZIC1 is a gene that is important for the function of the HPG axis. We studied wildtype ZIC1 as well as the putative patient mutations *in vitro* in cell culture using KTaR-3 and KTaV-1 murine cell lines that mimic ARC and AVPV Kisspeptin neurons, respectively [22]. Additionally, we used the GT1-7 immortalized cell line that mimics GnRH neurons in the hypothalamus [23]. Using luciferase assays, we tested the ZIC1 mutations' effects on Kisspeptin and GnRH luciferase expression. We also verified protein size and synthesis and total protein content through Western Blotting as well as localization of wildtype ZIC1 and ZIC1 mutations in the cell through immunostaining. Lastly, we utilized DNA precipitation assays to determine if ZIC1 binds onto the human Kisspeptin promoter, thus elucidating a possible mechanism by which it affects Kisspeptin expression.

MATERIALS AND METHODS

Cell Culture

The KTaV-1 and KTaR-3 cell lines were provided kindly by Dr. Patrick Chappell [24]. The GT1-7 cell line is an immortalized cell line derived from neurons in mice and was created in the Mellon lab [23]. Each of the KTaV, KTaR, and GT1-7 cell lines were cultured in complete media consisting of DMEM (MediaTech), 10% fetal bovine serum (Omega Scientific, Inc.), and 1% penicillin-streptomycin (Hyclone Laboratories) at 37°C in a 5% CO2 humidified incubator. All cells that were utilized in experiments were between passages 3 to 28.

Site-directed Mutagenesis and Plasmid Construction

The ZIC1 expression plasmid was subcloned into a pRP backbone with a GFP tag and CMV promoter by Vector Builder. An EFS promoter was cloned upstream for constitutive mammalian expression. Using the ZIC1 expression plasmid, the mutations ZIC1 H134Rfs*21, ZIC1 Y286X, and ZIC1 E299K were created using Q5 Mutagenesis (New England Biolabs). The ZIC1 S413Y mutation was created using Quikchange II Mutagenesis (Agilent). Additionally, the second endogenous intron of ZIC1 was put into each of the plasmids by Vector Builder. A 3x HA-tag was added to the N-terminus end of the ZIC1 expression plasmid to allow visualizations in western blots and immunohistochemistry using Q5 Mutagenesis (New England Biolabs). The plasmid also contains a GFP sequence under the control of a CMV promoter. Sequences were sent to EtonBio for verification of successful input of the mutations and HA-tag.

For all transfection assays, the empty Vector Builder backbone (VB bb), which contains the complete background structure without the ZIC1 construct, was used as an empty vector control. To normalize for transfection efficiency, a β-galactosidase expression vector driven by

thymidine kinase promoter (TKβGal) was used and we calculated the value of luciferase divided by the value of β-galactosidase in each condition. -1313/+27 human Kisspeptin Luciferase (hKiss-luc) was put into a PGL2 backbone [24]. KTaR and KTaV cells were transfected with 400 ng per well of ZIC1 or one of the ZIC1 mutations with 400 ng of hKiss-luc or 200ng/200ng of PGL2/pcDNA3.1+ (pcDNA3.1+ was included to normalize for the difference in plasmid size) and 200 ng of TKβGal. Transfection of 400 ng VB bb, 200ng/200ng PGL2 and pcDNA3.1+, and 200 TKβGal served as the all vehicle control to normalize the other values for analysis of fold-change.

In our GnRH enhancer/promoter (e/p) luciferase (GnRH e/p-luc) assays, the GnRH (e/p) luciferase plasmids include rat GnRH enhancers (position -4199 to -3895 and position -3135 to -2980 relative to the transcription start site), rat GnRH enhancer (position -1863 to -1571), and GnRH minimal promoter (position -173 to +1) that were put upstream of the luciferase reporter gene in a PGL3 backbone (Promega) [24, 25]. GT1-7 cells were transfected with 400 ng per well of ZIC1 or any one of the ZIC1 mutations, 400 ng of GnRH e/p-luc or 315 ng/85 ng of PGL3/pcDNA3.1+, and 200 ng of TKβGal. The condition of 400 ng VB bb, 315 ng/85 ng of PGL3/pcDNA3.1+, and 200 TKβGal served as the all vehicle control to normalize the other values for fold-change analysis.

Luciferase Assays

KTaV and KTaR cells were seeded onto 12-well plates (Nunc) at 20,000 cells per well. GT1-7 cells were seeded onto 12-well plates (Nunc) at 125,000 cells per well. All cells were transfected with the appropriate plasmids, Polyjet (SigmaGen), and serum-free DMEM (MediaTech) 24 hours after seeding. 24 hours after transfection, the media was replaced with

fresh complete media (DMEM, 10% FBS, 1% Penicillin-Streptomycin). 24 hours after the media change, the transfected cells were washed using Phosphate Buffered Saline (PBS) and harvested in 60 μL of lysis buffer (100 mM potassium phosphate, pH 7.8, and 0.2% Triton X-100). Luciferase and β-Galactosidase expression was measured using 25 μL of lysed cells each onto 96 well, flat bottom assay plates (Corning Inc.). Activity of luciferase was first measured through the injection of 100 μL of sample luciferase buffer (65 μM D-luciferin, 10 mM ATP, 25 mM Tris pH 7.4, 15 mM MgSO4), and luminescence was measured and quantified 1 second after injection. Following luciferase analysis, β-galactosidase activity was measured using 100 μL of Tropix II Galacto-light β-galactosidase assay buffer (Applied Biosystems) input into each sample and the luminescence from it was measured and quantified 1 second after injection. The luciferase assay activity measurements were performed using the Veritas Microplate Luminometer (Turner Biosystems).

Protein Collection

The KTaV and KTaR cells were seeded onto 10 cm plates on Day 1 at 200,000 cells per plate in 10 mL of complete media. They were transfected once they were at 70% confluency with 1.5 μg each of ZIC1 wildtype and each of the four ZIC1 mutations with opti-MEM (MediaTech) and Lipofectamine 3000 (SigmaGen) 24 hours after seeding. The cells were lysed and collected in Pierce RIPA buffer (Thermo Fisher Scientific) added with 1X Protease Inhibitor Cocktail (Sigma Aldrich) 48 hours after transfection. The protein concentrations were analyzed in triplicate using a standard bicinchoninic acid (BCA) protein assay.

Western Blotting

The collected proteins were allowed to denature in loading buffer (1X Laemmli buffer, 20 nM DTT). We measured 30 μg of each protein sample and adjusted with purified PCR water for each sample to yield consistent volumes. They were boiled at 95-100℃ for 10 minutes to fully denature and then separated in a 4-20% acrylamide SDS-PAGE gel (Bio-Rad, CAT #4561095). After being fully run through the gel, the proteins were transferred onto polyvinylidene fluoride membranes (Millipore) for 1 hour in 4℃ at 100V. The membranes were washed in 1X TBS and blocked in SuperBlock T20 (TBS) blocking buffer (Thermo Scientific, LOT #VJ315797). They were then incubated with primary antibodies diluted into TBS-T (SuperBlock T20 TBS, 0.4% tween) at 4℃ overnight with gentle shaking. Each blot was stripped with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, LOT #VJ308530) in between incubation with the different primary antibodies. Rabbit anti-HA (Cell Signaling Technologies, CAT #C29F4, LOT #10) was diluted in TBS-T (SuperBlock T20 TBS, 0.4% tween) and stored at 4℃ overnight with gentle shaking. Mouse anti-GFP (1:10000, Invitrogen, CAT #GF28R, LOT# UA277185) was diluted in TBS-T (SuperBlock T20 TBS, 0.4% tween) and stored at 4℃ overnight with gentle shaking. Anti-actin-HRP (1:30000, Abcam, CAT #ab49900, LOT #GR276781-11) was diluted in TBS-T (SuperBlock T20 TBS, 0.4% tween) and stored at 4℃ overnight with gentle shaking. Goat anti-rabbit (1:10000, Santa Cruz Biotechnology, CAT #sc-2004, LOT #G2111) and goat anti-mouse (1:10000, Cell Signaling Technologies, CAT #7076S, LOT #36) horseradish peroxidase (HRP)-conjugated secondary antibodies were diluted in TBS-T (SuperBlock T20 TBS, 0.4% tween) and incubated at room temperature for 1 hour with gentle shaking. SuperSignal West Dura Extended Duration Substrate

(Thermo Fisher Scientific) was used to detect antibody staining for all three primary antibodies. GeneSys was used to image all blots.

Immunohistochemistry

The KTaV and KTaR cell lines were seeded onto glass coverslips (Nunc, 8-well) at 7,500 cells per well. 24 hours after seeding, they were transfected with 200 ng of wildtype ZIC1 and each of the ZIC1 mutation plasmids with serum-free DMEM (MediaTech) and Polyjet (SigmaGen). 48 hours after transfection, the cells were washed with cold 1X PBS and fixed with 4% paraformaldehyde diluted in 1X PBS for 10 minutes at room temperature. They were blocked in PBS-T (1X PBS, 0.4% triton, 5% Normal Goat Serum) for 30 minutes at room temperature with gentle shaking.

The primary antibodies rabbit anti-HA (1:1000, Cell Signaling Technologies, LOT #C29F4) and mouse anti-GFP (1:1000, Invitrogen, LOT #UA277185) were diluted in PBS-T (1X PBS, 0.4% triton, 5% Normal Goat Serum). In another replicate, we used rabbit anti-HA (1:1000, Cell Signaling Technologies, LOT #C29F4) and GFP-conjugated Alexa Fluor 488 rabbit polyclonal antibody (1:1000, Invitrogen, LOT #2207528). They were added to the fixed cells and allowed to incubate overnight at 4℃ with gentle shaking. Secondary antibody Alexa Fluor Plus 555 goat anti-rabbit IgG (1:1000, Invitrogen, LOT #TE266006) was diluted in PBS-T (1X PBS, 0.4% triton, 5% Normal Goat Serum). The cells were incubated with secondary antibodies at room temperature for 1 hour with gentle shaking. The fixed cells were covered with ProLong Gold antifade reagent with DAPI (Invitrogen, LOT #2217039) and imaged using ImageJ.

Brains were extracted from ethically sacrificed wildtype mice and fixed in 4% paraformaldehyde at 4℃ overnight with gentle shaking. The fixed brains were treated in 30% sucrose solution and incubated at 4℃ overnight with gentle shaking. After the sucrose treatment, they were mounted and frozen in OCT. They were sliced coronally in 40 micron sections and stored in 1X PBS. The brain sections were washed in boiled Concentrated 10X Antigen Retrieval Citra Plus Solution (Biogenex, LOT #HK0800320) diluted to 1X and additional 1X PBS. They were blocked in PBS-T (1X PBS, 0.4% triton, 5% Normal Goat Serum) for 30 minutes at room temperature with gentle shaking. Primary antibody rabbit anti-ZIC1 (1:500, Invitrogen, LOT #WC3231268A) was diluted in PBS-T (1X PBS, 0.4% triton, 5% Normal Goat Serum) and incubated at 4℃ overnight with gentle shaking. Secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (1:1000, Abcam, CAT #A11008, LOT #2284594) was diluted in PBS-T (1X PBS, 0.4% triton, 5% Normal Goat Serum) and incubated at room temperature for 1 hour with gentle shaking. The sections were coverslipped with ProLong Gold antifade reagent with DAPI (Invitrogen, LOT #2217039).

DNA Precipitation

The oligonucleotides representing the human Kisspeptin promoter were created using an online transcription factor binding profile database called JASPAR [26]. We found that the consensus binding sequence of ZIC1 is CCYRYNG, where Y represents a C or T, R represents A or G, and N represents any base. We used this consensus sequence to find sections of the human Kisspeptin promoter that ZIC1 could potentially bind to. Table 1 lists the primers to create oligonucleotides that replicate sequences of sections on the human Kisspeptin promoter (Integrated DNA Technologies). Biotin tags were added onto each of the forward primers. The

consensus multimer acts as a positive control since it matches the binding sequence of ZIC1. Each of the forward and reverse primers were annealed to each other to create the oligonucleotides.

KTaV and KTaR cells were seeded onto 10 cm plates at 200,000 cells per plate in complete media. 24 hours after seeding, they were transfected with 1.5 μg of ZIC1 wildtype and each of the ZIC1 mutants in opti-MEM (Thermo Fisher Scientific) and Lipofectamine 3000 (Thermo Fisher Scientific, LOT #2274248). 48 hours after transfection, the cells were collected in a FLAG lysis buffer (300 mM NaCl, 20 nM Tris pH 7.5, 1% Triton, 1mM PMSF, 1X protease inhibitor). Streptavidin MagnoSphere Paramagnetic Particles (Promega, LOT #454152) were washed in 2X B&W buffer (10 mM Tris-Cl pH 8, 1 mM EDTA, 2 M NaCl) and were annealed to 100 ng of each of the annealed oligonucleotides in 1X binding buffer for 15 minutes at room temperature with gentle shaking. 1X binding buffer was diluted in purified PCR water with 3X binding buffer (15% glycerol, 60 mM Tris pH 7.5, 3 mM EDTA, 3 mM DTT, 0.45% Triton, 300 mM NaCl, 12 mM MgCl). The paramagnetic particles were blocked in 1X binding buffer with 1% BSA for 30 minutes at room temperature with gentle shaking. The lysed cells collected in FLAG buffer were added to the particles along with 10 μg of Poly DI/DC and incubated for 1 hour at 4°C with gentle shaking. The samples were eluted in 1X Laemmli buffer, 20 nM DTT, and purified PCR water and boiled at 95-100°C for 5 minutes. Once cooled, the supernatants as well as 1% and 10% inputs were used for western blot analysis.

Table 1: Oligonucleotide Primers of Sections of the Human Kisspeptin Promoter. Using the ZIC1 consensus sequence for its protein binding through the JASPAR database, we created primers to create oligonucleotides of sections of the human Kisspeptin promoter. Each of the forward primers have a biotin tag included for more efficient binding. As indicated in the primer name, the position of each refers to the number of base pairs upstream of the transcription start site on the human Kisspeptin promoter that each oligonucleotide represents.

Position	Name	Primer	Sequence
	Zic1_Consensus_ Multimer	Forward Reverse	/5Biosg/CCCGCTGCCCGCTGCCCGCTGCCCGCTGCC GGCAGCGGGCAGCGGGCAGCGGGCAGCGGG
+364	Zic1_364_hkiss	Forward Reverse	/5Biosg/ACGTAGAAAACGACAGGAAGAGGGGAGGAG CTCCTCCCCTCTTCCTGTCGTTTTCTACGT
+1123	Zic1_1123_hkiss	Forward Reverse	/5Biosg/ATGCCGGCAGTCTGCAGGTTAGCACCCCTC GAGGGGTGCTAACCTGCAGACTGCCGGCAT
+1272	Zic1_1272_hkiss	Forward Reverse	/5Biosg/CCAAAACTTCGCTGTGGGGATGATGTGTAC GTACACATCATCCCCACAGCGAAGTTTTGG

RESULTS

Select Mutations Reverse ZIC1 Induction of Kisspeptin Luciferase Expression in KT Cell Lines

ZIC1 was one of the genes identified by our collaborators at Harvard University that could be a possible candidate gene for causing Isolated GnRH Deficiency (IGD). The ZIC1 mutations H134Rfs*21, Y286X, E299K, and S413Y were found as heterozygous mutations in IGD patients that may have led to IGD phenotypes. IGD results from a defect at the level of the hypothalamus in the HPG axis where GnRH is not being properly secreted. ARC and AVPV Kisspeptin neurons are crucial for stimulating GnRH neurons to secrete GnRH into the HPG axis [6]. We used the KTaV and KTaR cell lines, which are lines derived from Kisspeptin neurons, to observe the effects of these mutations on human Kisspeptin luciferase expression [22].

For the Kisspeptin luciferase assays, luminescence was measured for each of the mutations and analyzed with t-tests comparing the mutations to the wildtype ZIC1. We transfected the KTaR and KTaV cells with the wildtype ZIC1 plasmid and each of the plasmids that harbored the ZIC1 mutations. We observed from the luciferase assays that the H134Rfs*21, Y286X, and E299K mutations significantly reversed ZIC1 repression of Kisspeptin luciferase expression in the KTaR cell line (Fig. 3). The S413Y mutation had no significant effect on expression levels of hKiss-luc. In the KTaV cell line, ZIC1 does not induce or repress Kisspeptin luciferase expression. Only the E299K mutation significantly induced Kisspeptin luciferase expression compared to the wildtype ZIC1 (Fig. 4).

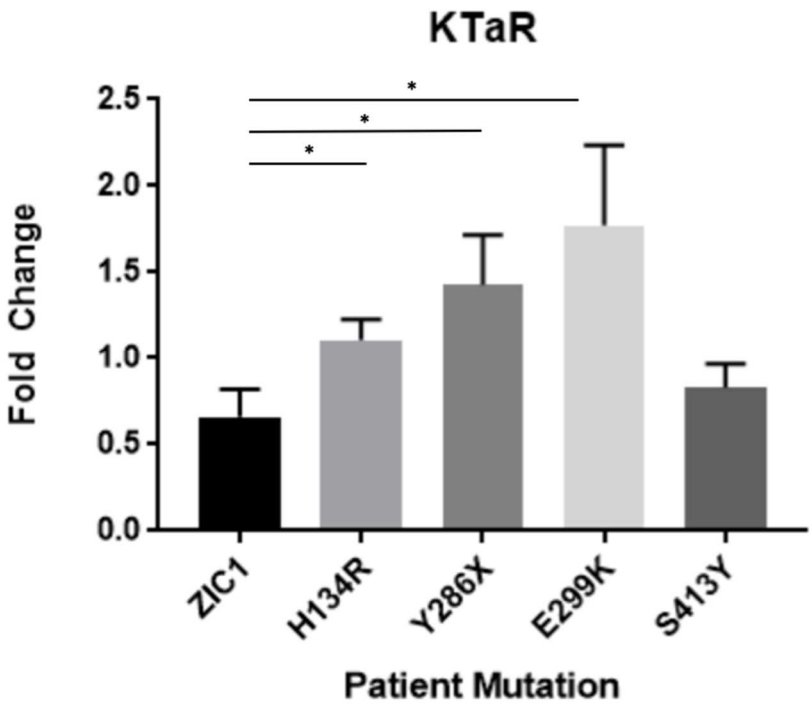


Figure 3. Mutations Reverse ZIC1 Repression of Kisspeptin Luciferase Expression in the KTaR cell line. KTaR cells were transfected with human Kisspeptin luciferase reporter gene along with the wildtype ZIC1 or the ZIC1 mutants. Normally, wildtype ZIC1 suppresses expression of Kisspeptin luciferase in the KTaR cell line. The H134Rfs*21, Y286X, and E299K mutations significantly reversed ZIC1 induction of Kisspeptin luciferase expression (n=9). Error bars represent standard error of the mean. * $p<0.05$.

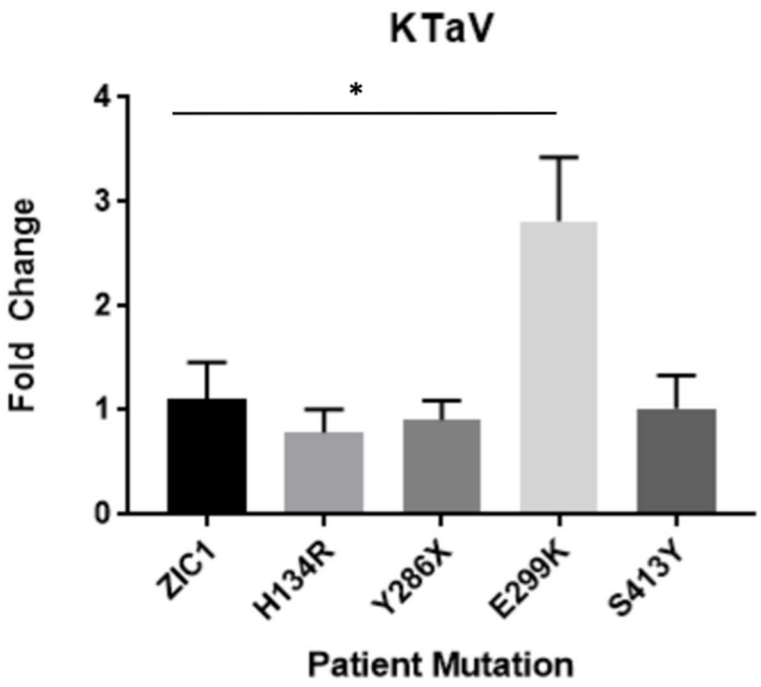


Figure 4. E299K Induces Kisspeptin Luciferase Expression in the KTaV cell line. ZIC1 does not significantly affect the expression of Kisspeptin luciferase in the KTaV cell line, as indicated by a luminescence fold-change of 1. The H134Rfs*21, Y286X, and S413Y mutations show similar fold-changes, indicating they had no effect on Kisspeptin luciferase expression. Only the E299K mutation significantly caused an induction of Kisspeptin luciferase expression compared to wildtype ZIC1 (n=9). Error bars represent standard error of the mean. * $p < 0.05$.

Select Mutations Alleviate ZIC1 Induction of GnRH e/p-Luc Expression in GT1-7 Cell Line

We also measured the effects of ZIC1 and the ZIC1 mutations on GnRH luciferase expression by transfecting the GT1-7 immortalized cell line with the wildtype ZIC1 plasmid and each of the plasmids that harbored the ZIC1 mutations [23]. We performed t-tests comparing the quantified luminescence fold-change of the ZIC1 mutations to the wildtype ZIC1. From the luciferase data, we observed that ZIC1 induces expression of hKiss-luc. We also observed that the H134Rfs*21, Y286X, and E299K mutations significantly reduce ZIC1 induction of GnRH luciferase expression compared to the wildtype ZIC1 plasmid (Fig. 5). Wildtype ZIC1 is able to enhance expression of GnRH e/p-luc, but each of the mutations with the exception of S413Y caused a significant reduction of ZIC1 induction [24, 25].

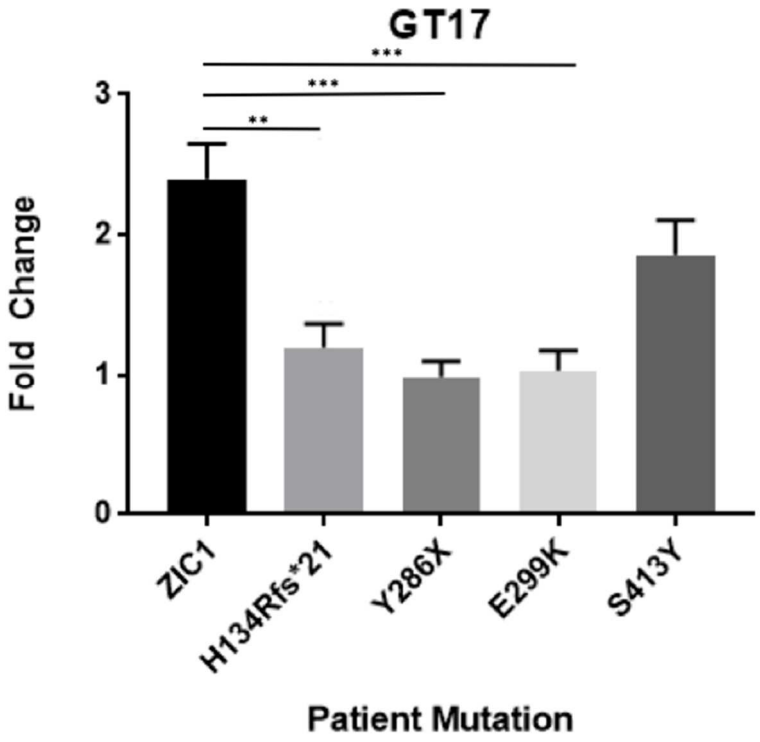


Figure 5. Mutations Alleviate ZIC1 Induction of GnRH e/p Luciferase Expression in the GT1-7 Cell Line. Wildtype ZIC1 induces GnRH e/p luciferase expression in GT1-7 cells, as indicated by a fold-change of 2.5. The mutations H134Rfs*21, Y286X, and E299K significantly alleviated ZIC1 induction of GnRH luciferase expression (n=9). Error bars represent standard error of the mean. ** $p<0.005$. *** $p<0.0005$.

*H134Rfs*21 and Y286X Produce Truncated Proteins*

H134Rfs*21 is a frameshift mutation that codes for a premature stop codon after 21 base pairs. Y286X codes for a premature stop codon at amino acid position 286. In order to determine whether a protein product is produced from the ZIC1 genes harboring truncating mutations, the second endogenous intron of ZIC1 was included in the ZIC1 plasmid. This plasmid was used to make all mutant plasmids. Sequencing was used to verify the successful insertion of the intron, as seen in Figure 6. The PCR primers shown in Table 2 were used to verify that the intron was spliced out, which was confirmed by amplification of cDNA collected from cells transfected with the plasmid (Fig. 7). Furthermore, we used Western Blotting to verify both the presence of and size of the protein. Because an HA epitope was added onto the N terminus of ZIC1, we blotted using an antibody against HA. The E299K and S413Y missense mutations produced proteins that were the same size as proteins produced by the wildtype ZIC1 (Fig. 9). Analysis of western blotting shows bands that correspond to the predicted length of the resultant HA-tagged proteins, given the premature stop codons by H134Rfs*21 and Y286X (Fig. 9).

In addition, Green fluorescence protein (GFP), which was cloned onto the plasmid under the control of a different promoter, was used as a transfection control (Fig. 9). β-actin served as a protein loading control (Fig. 9). To observe total ZIC1 protein produced, we used GeneSys to quantify HA band normalized to GFP band intensity. We observed that the total amount of protein produced by H134Rfs*21 was at least 50 times less than the other mutations (Fig. 8).

Feature	Direction	Type	Location ↓
ZIC1 Exon	>>>	misc_feature	359..368
Intron	>>>	misc_feature	369..1035
ZIC1 Exon	>>>	misc_feature	1036..1045

```
        *   10    *   20    *   30    *   40    *   50    *   60    *   70    *   80    *   90    *
   1 GNAGCCGTGTGTCGGAGCAGAGTATCACATCTGCTTCTGGGAGGAGTGTCCGCGCGAGGGCAAGCCCTTCAAAGCCAAATACAAACTGGTTAACCA
  97 CATCCGCGTGCACACGGGCGAGAAGCCCTTTCCCTGCCCCTTCCCTGGCTGTGGCAAGGTCTTCGCGCGCTCCGAGAATTTAAAGATCCACAAAAG
 193 GACGCACACAGGGGAGAAGCCCTTCAAGTGCGAGTTTGAGGGCTGTGACCGGCGCTTCGCTAACAGCAGCGACCGCAAGAAGCACATGCACGTGCA
 289 CACGAGCGACAAGCCCTATCTTTGCAAGATGTGCGACAAGTCCTACACGCATCCCAGTTCGCTGCGCAAACACATGAAGGTAATCGCCGCACTCTC
 385 GTCGCCCCCTTTGAGGCAGGAGCTCTCTTGGCTCTCGGCTTGGGGTCGGGCGGCGAGTGGCAGACAGGCGGTGGCGGGAGCCAGAGGAAGCGGGAA
 481 GGCAAAGGTTCCACTCAGTGGAACTGGGCAGAGAAGGTAGGGCGGGACCTGGAAAAGGGGATGGGAGTGTCCAAGGCCGTTTTAAATTTTTGGTTT
 577 AATAATAAAAGTTAAAAGAGGCGAGTATGAGCAAAGGCCGCCACTGAATTTGGCCCAGAGCTAGGGCTTTTGCAGGGGGTTGACGCTGCGGAAACT
 673 GCTGCCCTTGCAGCTGCCAGTGAGACGCAAGAATTGGACTCCTGCGCCCAGGCCTGAGCTCTGGCTCACCTAAACATGGACAAGCAAAGTTCAGGG
 769 AAAGAAGTGCAGTCTCTGGCCTCATATCCAGTCTCCCTCTTCCCAGACCTATTTTTCTTTTGTGAGTCTTGGGGACCCTGACTTTCCCAGGAGCAC
 865 GGTTTCTGCAGGCCTGTGCAGAGAAACCAAGCCCAAAGTCCCGCTGATCGGGCCTCACCTGTGTTCAGGGGCTCCAAGGGGTCCAGGAGGAAGGCA
 961 CTCGCGGCCTTCGCCTCTCTAGTCGGCCGCCGCACTGGCTCTTTATGTCCGTAAAACGCGACTTTATTCCTGCAGTCCACGAATCCTCCTCGCAGG
1057 CTCGCAGCCTTCGCGCGCAGCTCTGGCTACGATCCTCCACGCCTCCCACATCGTGTCTCCCTCCACAGACACCCGACCACAGCTCTATCGCCCTCC
1153 TCCTCCGCAGTCCACCACACAGCGCAAAGTGCGCCTTTCTCATTNNGATGNACGTNANTCGGAGAACCCAGCTTCTTTGAACAAGTGGTGAGGCCC
1249 GCGCCGCCTCTCGGAGGCGACC
```

Figure 6. Sequencing to Verify Intron Insertion into ZIC1 Plasmid Constructs. Highlighted in pink is the full 667 bp intron that was input into ZIC1 and each of the ZIC1 mutation plasmids. Highlighted in blue are the ends of the ZIC1 exon in which the intron was cloned. ApE software was used to display the sequencing that was ordered from EtonBio.

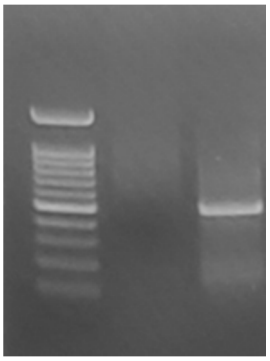


Figure 7. Gel Electrophoresis of cDNA of ZIC1 Plasmid with Inserted Intron. It was confirmed through PCR and conversion to cDNA that the inserted 667 bp intron was successfully spliced out. The first lane contains a 100 bp ladder and the last lane contains the ZIC1 plasmid cDNA. Predicted length with the intron was 1.1 kb. Predicted length after splicing was 500 bp.

Table 2: PCR Primers to Confirm Insertion of the Intron into the ZIC1 plasmids. Each of the ZIC1 plasmids, which include the wildtype and the mutations, had an intron cloned near the end of the gene. The primers listed here were used to verify through PCR that the intron was successfully input.

Name	Primer Sequence	Anneal T (°C)
Zic1_intron_PCR_Forward	GCAAGCCCTTCAAAGCCAAA	55
Zic1_intron_PCR_Reverse	ACGTACCATTCGTTAAAATTGGAAGA	55

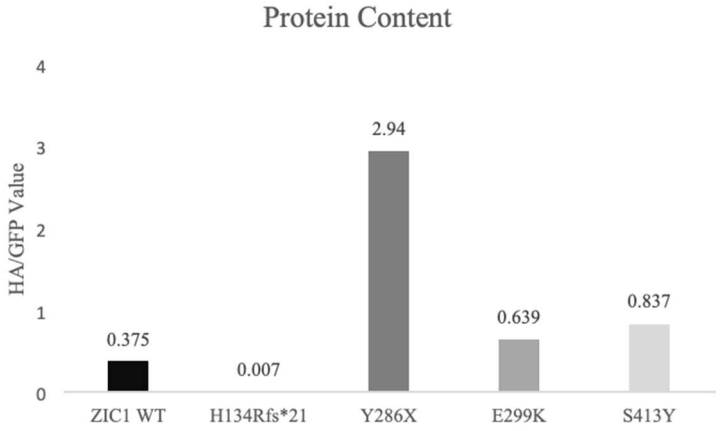


Figure 8. Measured Protein Content from ZIC1 and ZIC1 mutants. To measure total protein content produced by wildtype ZIC1 and the ZIC1 mutants in the KTaR cell line, quantification of HA band intensity values was normalized against GFP band intensity values (n=1). The raw values are displayed above each bar. From the protein content analysis, we observed that H134Rfs*21 produced at least 50 times less protein than the rest of the plasmids.

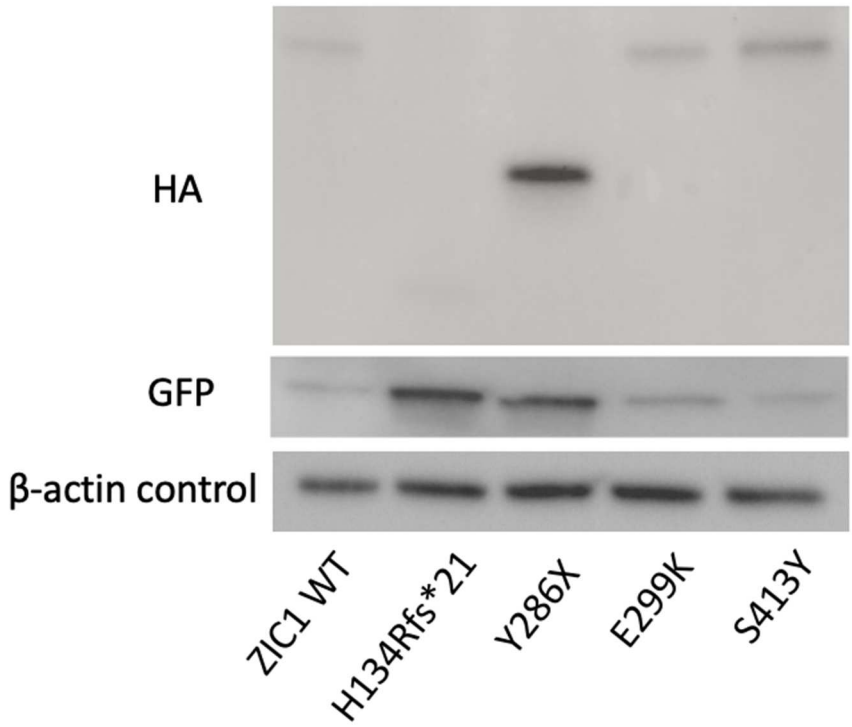


Figure 9. Western Blotting Shows Truncated Proteins for H134Rfs*21 and Y286X. ZIC1 contains an N-terminal HA tag. Therefore, we used antibody against HA to detect the proteins (n=1). The mutations that express premature stop codons, H134Rfs*21 and Y286X, produced visibly truncated proteins compared to the ZIC1 wildtype expression plasmid. We found that H134Rfs*21 also produced less overall protein content than wildtype ZIC1 and the other mutations. GFP was measured as a transfection efficiency control. β-actin was measured as a protein loading control.

*H134Rfs*21 Localizes to Both the Nucleus and Cytoplasm*

ZIC1 is a known transcription factor. To determine whether ZIC1 is a viable and functional transcription factor, we tested if protein is located in the expected part of the cell. We transfected the KTaR and KTaV cells with wildtype ZIC1 with an HA-tag as well as the expression plasmids with an HA-tag harboring each of the ZIC1 mutations. Immunohistochemistry with antibodies against the HA tag was used to detect proteins. Cell nuclei were stained with DAPI to assess the location of protein with respect to the nucleus.

Figures 10 and 11 show that wildtype ZIC1 localizes to the nucleus in both KTaR and KTaV cell lines. The H134Rfs*21 frameshift mutation is dispersed in both the nucleus and the cytoplasm in both cell lines. On the other hand, the Y286X, E299K, and S413Y mutations localize exclusively to the nucleus, indicated by the HA luminescence seen mostly in the nucleus of cells.

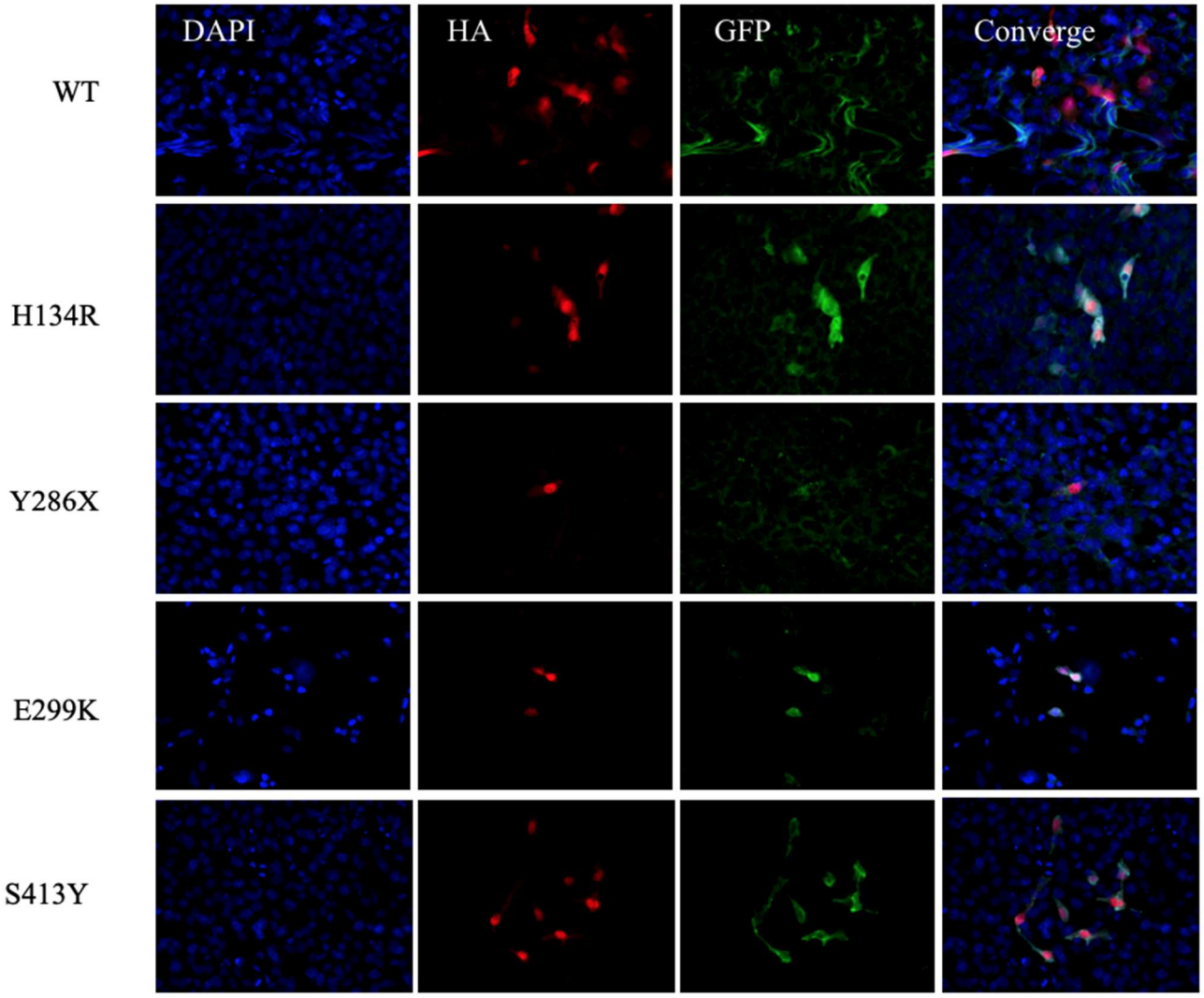


Figure 10. H134Rfs*21 Localizes to Both the Nucleus and Cytoplasm in KTaR Cells. KTaR cells were transfected with ZIC1 with HA epitope and each of the ZIC1 mutations with HA epitope expression plasmids. Immunohistochemistry was performed using anti-HA and anti-GFP primary antibodies. The nuclei of the cells were stained using DAPI. Wildtype ZIC1, Y286X, E299K, and S413Y localize exclusively to the nucleus but H134Rfs*21 localizes to both the nucleus and the cytoplasm, as indicated by the dispersion of luminescence in both of those regions.

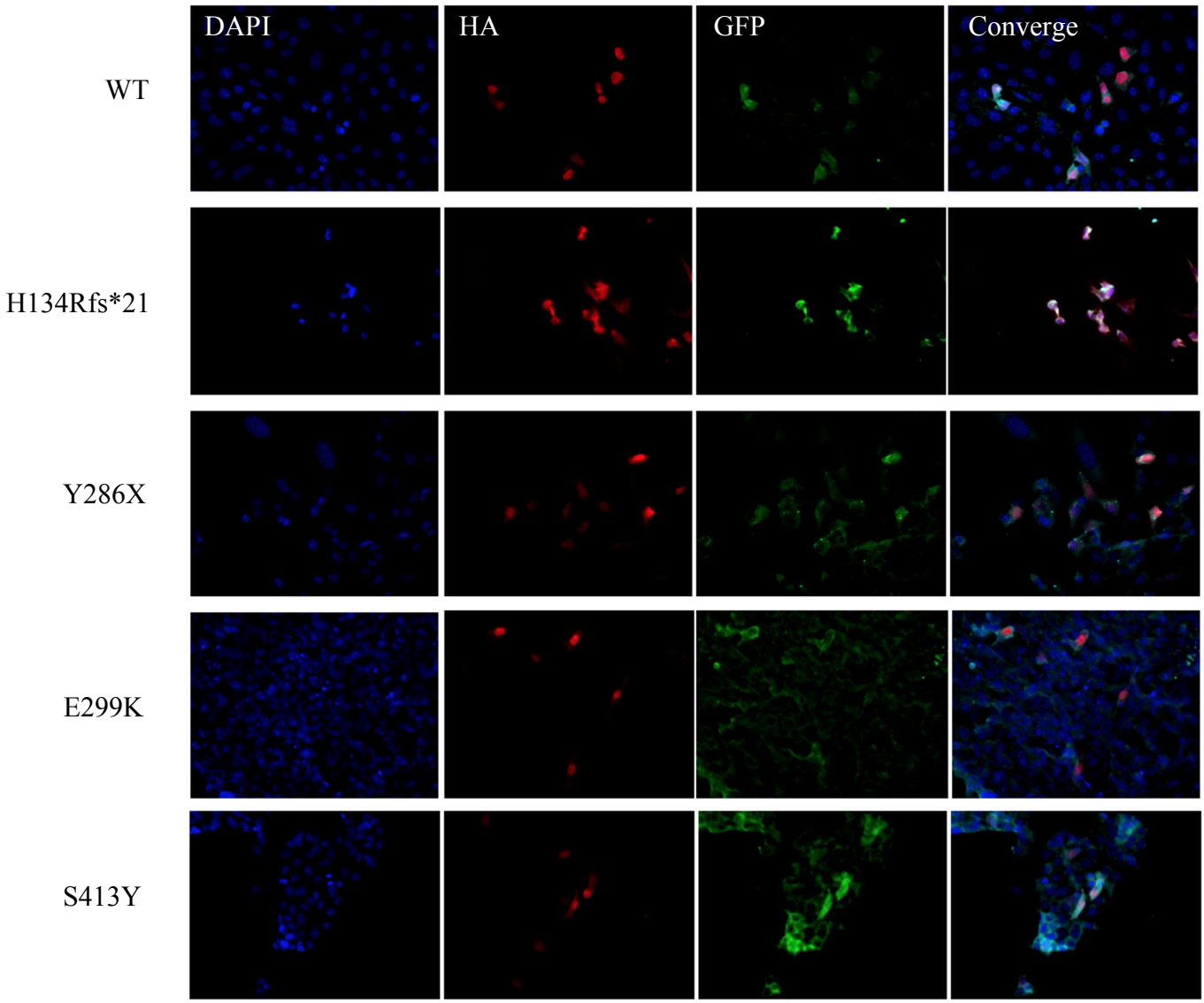


Figure 11. H134Rfs*21 Localizes to Both the Nucleus and Cytoplasm in KTaV Cells. KTaV cells were transfected with ZIC1 with HA epitope and each of the ZIC1 mutations with HA epitope expression plasmids. Immunohistochemistry was performed using anti-HA and anti-GFP primary antibodies. The nuclei of the cells were stained using DAPI. Similar to the KTaR cells, wildtype ZIC1, Y286X, E299K, and S413Y localize to the nucleus but H134Rfs*21 is dispersed in both the nucleus and the cytoplasm, as indicated by the luminescence in both regions.

DISCUSSION

Herein, we tested the ZIC1 heterozygous patient mutations kindly provided by our collaborators at Harvard University and showed their effects on human Kisspeptin and GnRH e/p luciferase expression *in vitro* as a potential cause of Isolated GnRH Deficiency. IGD results from a defect at the level of the hypothalamus in the HPG axis where GnRH is not being properly secreted. ARC and AVPV Kisspeptin neurons are crucial for stimulating GnRH neurons to secrete GnRH into the HPG axis [22]. The KTaV and KTaR cell lines are derived from female murine kisspeptin neurons, making them ideal models for our *in vitro* studies [22]. The GT1-7 cell line is derived from murine GnRH neurons and secretes GnRH, making it an ideal model for this study as well [22]. Quantification of luminescence as a fold change compared to the empty Vector Builder backbone allows us to understand the effect of ZIC1 in regulating the human Kisspeptin luciferase (hKiss-luc) promoter and GnRH enhancer/promoter luciferase (GnRH e/p-luc), which contains GnRH enhancers (position -4199 to -3895, position -3135 to -2980, and position -1863 to -1571 relative to the transcription start site and GnRH minimal promoter (position -173 to +1) [24, 25]. Normally, wildtype ZIC1 represses Kisspeptin luciferase expression in the KTaR cell line and enhances GnRH luciferase expression in the GT1-7 cell line. From analysis of fold-change luminescence, we observed that the H134Rfs*21, Y286X, and E299K mutations when compared to wildtype ZIC1, the mutations reversed the ZIC1 repression of hKiss-luc expression in the KTaR cell line and reversed ZIC1 induction of GnRH e/p-luc expression in the GT1-7 cell line. Contrary to expectation, ZIC1 does not induce or repress Kisspeptin luciferase expression in the KTaV cell line. However, E299K shows a significant increase in fold change compared to wildtype ZIC1, indicating that it is inducing the expression of Kisspeptin luciferase. To reiterate, in IGD, insufficient GnRH is circulating in the HPG axis in

affected individuals due to a defect at the level of the hypothalamus involving either Kisspeptin or GnRH. The alleviation of induction of GnRH e/p luciferase expression by the ZIC1 mutations suggests that they may contribute to the observed phenotype of insufficient circulating GnRH in IGD patients. The S413Y mutation was the only mutation that did not significantly affect human Kisspeptin luciferase or GnRH e/p-luciferase expression compared to the wildtype ZIC1. This may be due to the fact that the KTaR and KTaV cells endogenously express ZIC1 and this expression may have been sufficient enough to mask the phenotype of S413Y.

The ZIC1 H134Rfs*21 frameshift mutation and Y286X nonsense mutation both code for premature stop codons. Insertion of an intron to the plasmids was necessary for these mutated proteins to be expressed as the intron is essential for nonsense-mediated decay to be signaled. By inputting the intron, we are able to determine whether the protein would get made despite the premature stop codon. We used Western Blotting to observe if these mutant proteins yielded a difference in size compared to the wildtype. As expected, the blots showed that ZIC1 H134R*21 produced truncated proteins compared to the wildtype ZIC1 as seen in Figure 8. This suggests that the truncated proteins that are produced may fail to exhibit the DNA binding required for the ZIC1 transcription factor to function normally. There may be a possible dysfunction in the C2H2 Zinc finger protein domains' ability to interact with DNA. Additionally, we quantified total amount of protein produced from ZIC1 and the ZIC1 mutations by dividing the intensity of each HA band over intensity of the respective plasmids' GFP bands, which were a transfection control. We found that there was at least 50 times less protein produced by H134Rfs*21 compared to the other plasmids. The Y286X mutation also produced truncated protein compared to wildtype ZIC1. The other two mutations E299K and S413Y are missense mutations and produced similarly sized proteins as the wildtype. The H134Rfs*21 and Y286X mutations were

shown to significantly reverse ZIC1 repression of Kisspeptin luciferase expression in the KTaR cell line. They also significantly reduce ZIC1 induction of GnRH luciferase expression in our luciferase analysis. The specific mechanisms for ZIC1 binding in the reproductive system are yet to be elucidated. Finding these mechanisms would be a relevant avenue to pursue that would further explain how ZIC1 is able to regulate Kisspeptin and GnRH expression. Previous studies have shown that ZIC1 heterodimerizes with GLI proteins to properly function, so it would be interesting to determine if it performs the same roles within the HPG axis and the loss of those cause IGD phenotypes.

Because ZIC1 is known to be a transcription factor, it requires translocalization into the nucleus to perform regulation of gene transcription. We performed immunohistochemistry using the KTaR and KTaV cells to observe localization of wildtype ZIC1 and each of the ZIC1 mutants. As expected from previous studies, wildtype ZIC1 localizes to the nuclei of cells in both cell lines. The image staining indicates that the H134Rfs*21 frameshift mutant is dispersed in both the nucleus and cytoplasm in the KTaV cell line. The other mutants localized exclusively to the nucleus. This observation suggests that there may be a defect in protein trafficking or localization resulting from only the H134Rfs*21 frameshift mutation. Combined with Western Blot analysis that H134Rfs*21 produced less overall protein, this indicates that H134Rfs*21 may be producing insufficient protein that is dispersed in areas of the cell in which wildtype ZIC1 is not normally found, thus causing the loss of ZIC1 effects on Kisspeptin and GnRH luciferase expression. To further investigate the functions of ZIC1 on Kisspeptin expression, we conducted DNA precipitation assays to test if ZIC1 binds to sites in the human Kisspeptin promoter. We used the consensus binding sequence of ZIC1 to create oligonucleotides of different sections of the human Kisspeptin promoter to observe if ZIC1 would bind to any of those sites.

Our data on the mutations in ZIC1 found from IGD patients suggest that ZIC1 may be a causative gene in IGD. It is interesting that these mutations would cause IGD instead of Dandy-Walker Syndrome, a condition where the cerebellum is malformed and of which has been shown to be caused by heterozygous deletion mutations in ZIC1 and ZIC4 [27]. This could be due to the fact that ZIC1 may interact in an indirect manner on the components of the HPG axis.

To further our understanding of the mechanisms of ZIC1 mutations that cause IGD phenotypes, we can perform DNA precipitation assays using the ZIC1 mutations to observe if they bind or do not bind to the human Kisspeptin promoter. Additionally, we can perform these assays using sections of the GnRH promoter since we observed that the mutations have an effect on GnRH luciferase expression as well. Further investigation to determine the specific mechanisms of ZIC1 on components of the HPG axis and in causing IGD would further our understanding of its relevance in mammalian reproduction.

REFERENCES

1. Thoma ME, McLain AC, Louis JF, King RB, Trumble AC, Sundaram R, Buck Louis GM. Prevalence of infertility in the United States as estimated by the current duration approach and a traditional constructed approach. *Fertil Steril*. Apr 2013;99(5):1324-1331.e1. doi:10.1016/j.fertnstert.2012.11.037

2. Marques P, Skorupskaite K, George JT, Anderson RA. Physiology of GNRH and Gonadotropin Secretion. 2018 Jun 19. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, de Herder WW, Dhatariya K, Dungan K, Grossman A, Hershman JM, Hofland J, Kalra S, Kaltsas G, Koch C, Kopp P, Korbonits M, Kovacs CS, Kuohung W, Laferrère B, McGee EA, McLachlan R, Morley JE, New M, Purnell J, Sahay R, Singer F, Stratakis CA, Trence DL, Wilson DP, editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000–.

3. Harter CJL, Kavanagh GS, Smith JT. The role of kisspeptin neurons in reproduction and metabolism. *J Endocrinol*. 09 2018;238(3):R173-R183. doi:10.1530/JOE-18-0108

4. Chan YM, de Guillebon A, Lang-Muritano M, Plummer L, Cerrato F, Tsiaras S, Gaspert A, Lavoie HB, Wu CH, Crowley WF Jr, Amory JK, Pitteloud N, Seminara SB. GNRH1 mutations in patients with idiopathic hypogonadotropic hypogonadism. *Proc Natl Acad Sci U S A*. Jul 2009;106(28):11703-8. doi:10.1073/pnas.0903449106

5. Wray S, Grant P, Gainer H. Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. *Proc Natl Acad Sci U S A*. Oct 1989;86(20):8132-6. doi:10.1073/pnas.86.20.8132

6. Hu MH, Li XF, McCausland B, Li SY, Gresham R, Kinsey-Jones JS, Gardiner JV, Sam AH, Bloom SR, Poston L, Lightman SL, Murphy KG, O'Byrne KT. Relative Importance of the Arcuate and Anteroventral Periventricular Kisspeptin Neurons in Control of Puberty and Reproductive Function in Female Rats. *Endocrinology*. Jul 2015;156(7):2619-31. doi:10.1210/en.2014-1655

7. Klein CE. The Hypothalamic-Pituitary-Gonadal Axis. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast RC, Gansler TS, Holland JF, Frei E III, editors. *Holland Frei-Cancer Medicine*. 6th ediction. Hamilton (ON): BC Decker; 2003. Available from https://www.ncbi.nlm.nih.gov/books/NBK13386/

8. Balasubramanian R, Crowley WJ, eds. *Isolated Gonadotropin-Releasing Hormone (GnRH) Deficiency*. GeneReviews® [Internet]; 2017. Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Mirzaa G, Amemiya A, editors, ed.

9. Waldstreicher J, Seminara SB, Jameson JL, Geyer A, Nachtigall LB, Boepple PA, Holmes LB, Crowley WF Jr. The genetic and clinical heterogeneity of gonadotropin-releasing hormone deficiency in the human. *J Clin Endocrinol Metab*. Dec 1996;81(12):4388-95. doi:10.1210/jcem.81.12.8954047

10. Balasubramanian R, Crowley WF. Isolated GnRH deficiency: a disease model serving as a unique prism into the systems biology of the GnRH neuronal network. *Mol Cell Endocrinol*. Oct 2011;346(1-2):4-12. doi:10.1016/j.mce.2011.07.012

11. Seminara SB, Hayes FJ, Crowley WF. Gonadotropin-releasing hormone deficiency in the human (idiopathic hypogonadotropic hypogonadism and Kallmann's syndrome): pathophysiological and genetic considerations. *Endocr Rev*. Oct 1998;19(5):521-39. doi:10.1210/edrv.19.5.0344

12. Schwanzel-Fukuda M, Pfaff DW. Origin of luteinizing hormone-releasing hormone neurons. *Nature*. Mar 1989;338(6211):161-4. doi:10.1038/338161a0

13. Mason AJ, Hayflick JS, Zoeller RT, Young 3rd WS, Phillips HS, Nikolics K, Seeburg PH. A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. *Science*. Dec 1986;234(4782):1366-71. doi:10.1126/science.3024317

14. Mason AJ, Pitts SL, Nikolics K, Szonyi E, Wilcox JN, Seeburg PH, Stewart TA. The hypogonadal mouse: reproductive functions restored by gene therapy. *Science*. Dec 1986;234(4782):1372-8. doi:10.1126/science.3097822

15. Twigg SR, Forecki J, Goos JA, Richardson IC, Hoogeboom AJ, van den Ouweland AM, Swagemakers SM, Lequin MH, Van Antwerp D, McGowan SJ, Westbury I, Miller KA, Wall SA; WGS500 Consortium, van der Spek PJ, Mathijssen IM, Pauws E, Merzdorf CS, Wilkie AO. Gain-of-Function Mutations in ZIC1 Are Associated with Coronal Craniosynostosis and Learning Disability. *Am J Hum Genet*. Sep 2015;97(3):378-88. doi:10.1016/j.ajhg.2015.07.007

16. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS Jr, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF Jr, Aparicio SA, Colledge WH. The GPR54 gene as a regulator of puberty. *N Engl J Med*. Oct 2003;349(17):1614-27. doi:10.1056/NEJMoa035322

17. Teles MG, Bianco SD, Brito VN, Trarback EB, Kuohong W, Xu S, Seminara SB, Mendonca BB, Kaiser UB, Latronico AC. A GPR54-activating mutation in a patient with central precocious puberty. *N Engl J Med*. Feb 2008;358(7):709-15. doi:10.1056/NEJMoa073443

18. Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, Boe AF, Boguski MS, Brockway KS, Byrnes EJ, Chen L, Chen L, Chen TM, Chin MC, Chong J, Crook BE, Czaplinska A, Dang CN, Datta S, Dee NR, Desaki AL, Desta T, Diep E, Dolbeare TA, Donelan MJ, Dong HW, Dougherty JG, Duncan BJ, Ebbert AJ, Eichele G, Estin LK, Faber C, Facer BA, Fields R, Fischer SR, Fliss TP, Frensley C, Gates SN, Glattfelder KJ, Halverson KR, Hart MR, Hohmann JG, Howell MP, Jeung DP, Johnson RA, Karr PT, Kawal R, Kidney JM, Knapik RH, Kuan CL, Lake JH, Laramee AR, Larsen KD, Lau C, Lemon TA, Liang AJ, Liu Y, Luong LT, Michaels J, Morgan JJ, Morgan RJ, Mortrud MT, Mosqueda NF, Ng LL, Ng R, Orta GJ, Overly CC, Pak TH, Parry SE, Pathak SD, Pearson OC, Puchalski RB, Riley ZL, Rockett HR, Rowland SA, Royall JJ, Ruiz MJ, Sarno NR, Schaffnit K, Shapovalova NV, Sivisay T, Slaughterbeck CR,

Smith SC, Smith KA, Smith BI, Sodt AJ, Stewart NN, Stumpf KR, Sunkin SM, Sutram M, Tam A, Teemer CD, Thaller C, Thompson CL, Varnam LR, Visel A, Whitlock RM, Wohnoutka PE, Wolkey CK, Wong VY, Wood M, Yaylaoglu MB, Young RC, Youngstrom BL, Yuan XF, Zhang B, Zwingman TA, Jones AR. Genome-wide atlas of gene expression in the adult mouse brain. *Nature*. Jan 2007;445(7124):168-76. doi:10.1038/nature05453

19. Aruga J. The role of Zic genes in neural development. *Mol Cell Neurosci*. Jun 2004;26(2):205-21. doi:10.1016/j.mcn.2004.01.004

20. Inoue T, Ota M, Ogawa M, Mikoshiba K, Aruga J. Zic1 and Zic3 regulate medial forebrain development through expansion of neuronal progenitors. *J Neurosci*. May 2007;27(20):5461-73. doi:10.1523/JNEUROSCI.4046-06.2007

21. Zhong J, Chen S, Xue M, Du Q, Cai J, Jin H, Si J, Wang L. ZIC1 modulates cell-cycle distributions and cell migration through regulation of sonic hedgehog, $PI3_K$ and MAPK signaling pathways in gastric cancer. *BMC Cancer*. 2012;12(290)doi:10.1186/1471-2407-12-290

22. Jacobs DC, Veitch RE, Chappell PE. Evaluation of Immortalized AVPV- and Arcuate-Specific Neuronal Kisspeptin Cell Lines to Elucidate Potential Mechanisms of Estrogen Responsiveness and Temporal Gene Expression in Females. *Endocrinology*. 09 2016;157(9):3410-9. doi:10.1210/en.2016-1294

23. Mellon PL, Windle JJ, Goldsmith PC, Padula CA, Roberts JL, Weiner RI. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron*. Jul 1990;5(1):1-10. doi:10.1016/0896-6273(90)90028-e

24. Whyte DB, Lawson MA, Belsham DD, Eraly SA, Bond CT, Adelman JP, Mellon PL. A neuron-specific enhancer targets expression of the gonadotropin-releasing hormone gene to hypothalamic neurosecretory neurons. *Mol Endocrinol*. Apr 1995;9(4):467-77. doi:10.1210/mend.9.4.7659090

25. Iyer AK, Miller NL, Yip K, Tran BH, Mellon PL. Enhancers of GnRH transcription embedded in an upstream gene use homeodomain proteins to specify hypothalamic expression. *Mol Endocrinol*. Oct 2010;24(10):1949-64. doi:10.1210/me.2010-0156

26. Fornes O, Castro-Mondragon JA, Khan A, van der Lee R, Zhang X, Richmond PA, Modi BP, Correard S, Gheorghe M, Baranašić D, Santana-Garcia W, Tan G, Chèneby J, Ballester B, Parcy F, Sandelin A, Lenhard B, Wasserman WW, Mathelier A. JASPAR 2020: update of the open-access database of transcription factor binding profiles. *Nucleic Acids Res*. 01 2020;48(D1):D87-D92. doi:10.1093/nar/gkz1001

27. Grinberg I, Northrup H, Ardinger H, Prasad C, Dobyns WB, Millen KJ. Heterozygous deletion of the linked genes ZIC1 and ZIC4 is involved in Dandy-Walker malformation. *Nat Genet*. Oct 2004;36(10):1053-5. doi:10.1038/ng1420