

**THE CHARACTERIZATION AND POTENTIAL
FUNCTIONAL ROLE OF *WDR81*,
A NOVEL ZEBRAFISH GENE, ASSOCIATED WITH
CEREBELLAR ATAXIA, MENTAL RETARDATION AND
DYSEQUILIBRIUM SYNDROME (CAMRQ) IN HUMANS**

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By
Füsün Doldur-Ballı
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**THE CHARACTERIZATION AND POTENTIAL FUNCTIONAL ROLE OF *WDR81*,
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HUMANS**

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April, 2016

We certify that we have read this dissertation and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy.

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Abstract

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Cerebellar ataxia, mental retardation and dysequilibrium syndrome (CAMRQ) is a neurodevelopmental disorder. The gene encoding WD repeat containing protein 81 (*WDR81*) was reported to be associated with CAMRQ2 [MIM 610185]. Human and mouse studies indicated the potential importance of *WDR81* in neurodevelopment. The first aim in this study was to characterize the transcript and to reveal the expression profile of *wdr81* in zebrafish. The second aim was to perform the initial characterization of *wdr81* morphants. *In silico* analysis indicated that the conserved domains are shared in human, mouse and zebrafish orthologous proteins, implying a conserved function of *WDR81* in three species. The characterization of the transcript revealed that *wdr81* possessed one ORF and one 5'UTR structure. The predicted sequence for 3'UTR was confirmed along with detection of some variants and an insertion site in samples from ten developmental timepoints and in several adult tissues. This region was not detected in kidney, intestine and gills, which might be pointing out an alternative polyadenylation event. *wdr81* appeared to be maternally supplied. 5 hpf and 18 hpf were detected as crucial timepoints regarding *wdr81* expression. Expression of *wdr81* was found to be increased in the eye and brain regions at 18 hpf and 48 hpf. *wdr81* was found to be ubiquitously expressed in the

adult zebrafish. The expression of *wdr81* in the adult brain and eye was detected in several regions including retinal layers, presumptive Purkinje cells and some proliferative zones. The splice blocking morpholino which targets the exon 2-intron 2 junction of *wdr81* worked at 3 tested doses; 2 ng, 4 ng and 8 ng. The effect of the *wdr81* morpholino was detected to add the intron, which is downstream of the target exon, to the transcript and introduce a stop codon. Preliminary results indicated a significant reduction in the head sizes at a ratio of 3.88% (p:0.027) in the *wdr81* morphant group compared to uninjected group and *gbx2* expression was observed to be higher in *wdr81* morphants compared to the control groups.

In short, findings of this study emphasize the significance of *wdr81* in neurodevelopment and suggest a potential role in neuronal proliferation. This study also serves as a basis for future functional studies.

Keywords: *wdr81*, zebrafish, RACE, gene expression, morpholino

Özet

İNSANLARDAKİ SEREBELLAR ATAKSİ, ZEKA GERİLİĞİ VE DENGESİZLİK SENDROMU İLE İLİŞKİLENDİRİLEN VE ZEBRABALIĞINDA YENİ BİR GEN OLAN *WDR81*'İN KARAKTERİZASYONU VE MUHTEMEL İŞLEVSEL ROLÜ

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Serebellar ataksi, zeka geriliği ve dengesizlik sendromu nörogelişimsel bir hastalıktır. WD tekrarı içeren protein 81'i kodlayan genin bu sendrom ile ilişkilendirildiği daha önce rapor edilmiştir. *WDR81* geninin sinir sisteminin gelişim sürecindeki potansiyel önemi insan ve fare çalışmalarıyla ortaya konmuştur. Bu çalışmada ilk amaç, *wdr81*'in zebrabalığında karakterize edilmesi ve ifade profilinin gösterilmesiydi. İkinci amaç *wdr81* morfantlarının öncül karakterizasyonunun yapılmasıydı. *In siliko* analizler insan, fare ve zebrabalığına ait ortolog proteinlerin korunmuş domeynleri paylaştığını gösterdi, bu durum da *WDR81*'in işlevinin bu üç türde korunmuş olabileceğine işaret etmektedir. Transkriptin karakterizasyonu, *wdr81*'in bir açık okuma çerçevesine ve bir 5'UTR yapısına sahip olduğunu gösterdi. 3'UTR'nin öngörülen dizisi, bazı varyantların ve bir içine yerleştirme (insertion) bölgesinin varlığı tespit edilmekle birlikte, teyit edildi. Bu içine yerleştirme (insertion) bölgesi on gelişimsel evreye ait örnekte ve çeşitli yetişkin dokularında tespit edilmiş olup böbrek, bağırsak ve solungaçlarda bulunmadı. Bu durumun da alternatif poliadenilasyon işlemine işaret ettiği öngörülmektedir. *wdr81*'in yumurta hücresi tarafından sağlanan transkriptler arasında olduğu tespit edildi. *wdr81*'in ifadesi açısından döllenme sonrası 5. ve 18. saatler kritik evreler olarak saptandı. Döllenme sonrası 18. ve 48. saat evrelerinde *wdr81* ifadesinin göz ve beyin bölgelerinde

artmış olduğu gözlemlendi. *wdr81*'in test edilen yetişkin dokularının tümünde ifade edildiği, yetişkin göz ve beyin dokularında ise retina katmanları, muhtemel Purkinje hücreleri ve bazı proliferatif alanların da bulunduğu çeşitli bölgelerde ifade edildiği belirlendi. Uçbirleştirmeyi (splicing) etkileyen morfolino *wdr81* genine ait ekzon 2-intron 2 bölgesini hedefleyecek şekilde tasarlandı ve denenen üç dozun (2 ng, 4 ng ve 8 ng) da çalıştığı tespit edildi. *wdr81* morfolinonun etkisinin, hedef ekzonun aşağı yönündeki intronu transkripte dahil etmek olduğu ve bu dizinin de durdurma kodonu içerdiği tespit edildi. Öncül sonuçlar, *wdr81* morfant grubuna ait kafa bölgelerinin ölçümünde, enjeksiyon yapılmayan gruba kıyasla %3,88 oranında anlamlı (p: 0,027) bir düşüş olduğu sonucunu verdi. Ayrıca, *gbx2* ifadesi *wdr81* morfant grupta kontrol gruplarına kıyasla daha yüksek gözlemlendi.

Özetle, bu çalışmaya ait bulgular *wdr81*'in nörogelişimsel önemini anlamakta katkıda bulunmuştur ve bu gen için nöronların çoğalmasına (proliferasyonu) ilişkin olası bir rol önermektedir. Elde edilen veriler aynı zamanda ileride yapılacak fonksiyonel çalışmalar için de temel teşkil edecektir.

Keywords: *wdr81*, zebrabalığı, RACE, gen ifadesi, morfolino



To Mevlüt and Yaren Rüya...

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Table of Contents

ABSTRACT.....	III
ÖZET.....	V
ACKNOWLEDGEMENTS.....	VIII
TABLE OF CONTENTS.....	X
LIST OF FIGURES.....	XIII
LIST OF TABLES.....	XVI
ABBREVIATIONS.....	XVII
CHAPTER 1. INTRODUCTION	1
1.1. CEREBELLAR ATAXIA, MENTAL RETARDATION AND DYSEQUILIBRIUM SYNDROME.....	1
1.1.1. CAMRQ ASSOCIATED GENES.....	2
1.1.1.1. VERY LOW DENSITY LIPOPROTEIN RECEPTOR (<i>VLDLR</i>).....	2
1.1.1.2. WD REPEAT CONTAINING PROTEIN 81 (<i>WDR81</i>).....	3
1.1.1.3. CARBONIC ANHYDRASE VIII (<i>CA8</i>).....	8
1.1.1.4. ATPASE, AMINOPHOSPHOLIPID TRANSPORTER, CLASS I, TYPE 8A, MEMBER 2 (<i>ATP8A2</i>).....	10
1.2. ZEBRAFISH AS A MODEL ORGANISM.....	11
1.2.1. ZEBRAFISH CENTRAL NERVOUS SYSTEM.....	15
1.2.1.1. ZEBRAFISH EMBRYO.....	15
1.2.1.2. ADULT ZEBRAFISH.....	16
1.3. CELL PROLIFERATION IN ZEBRAFISH NERVOUS SYSTEM	17
1.3.1. NEUROGENESIS IN ZEBRAFISH.....	18
1.3.1.1. ZEBRAFISH EMBRYO.....	18
1.3.1.2. ADULT ZEBRAFISH.....	19
1.3.2. GLIOGENESIS IN ZEBRAFISH.....	19
1.3.2.1. ZEBRAFISH EMBRYO.....	20
1.3.2.2. ADULT ZEBRAFISH.....	20
1.4. SYNAPTOGENESIS IN ZEBRAFISH.....	21
1.5. AIM AND SCOPE OF THIS STUDY.....	23
CHAPTER 2. MATERIALS AND METHODS.....	25
2.1. METHODS.....	25
2.1.1. ZEBRAFISH AND EMBRYOS.....	25
2.1.2. BIOINFORMATICS ANALYSIS.....	25

2.1.3. CHARACTERIZATION OF THE <i>WDR81</i> TRANSCRIPT.....	26
2.1.3.1. AMPLIFICATION OF THE OPEN READING FRAME OF <i>WDR81</i>	27
2.1.3.2. RAPID AMPLIFICATION OF cDNA ENDS (RACE).....	28
2.1.3.2.1. CHARACTERIZATION OF THE 5'RACE PRODUCT.....	28
2.1.3.2.2. CHARACTERIZATION OF THE 3'RACE PRODUCT.....	30
2.1.4. ANALYSIS OF THE EXPRESSION OF <i>WDR81</i> IN WILD TYPE ZEBRAFISH.....	32
2.1.4.1. QUANTITATIVE REAL-TIME PCR (QRT-PCR).....	32
2.1.4.2. <i>IN SITU</i> HYBRIDIZATION EXPERIMENTS.....	34
2.1.4.2.1. WHOLE MOUNT <i>IN SITU</i> HYBRIDIZATION (WMISH) ON EMBRYOS.....	34
2.1.4.2.2. <i>IN SITU</i> HYBRIDIZATION (ISH) ON BRAIN AND EYE TISSUES.....	35
2.1.5. MORPHOLINO MICROINJECTIONS TO KNOCKDOWN <i>WDR81</i>	36
2.1.5.1. <i>WDR81</i> MORPHOLINO DOSE CURVE.....	37
2.1.5.2. INITIAL CHARACTERIZATION STUDIES.....	38
2.1.5.2.1. HEAD SIZE MEASUREMENT.....	38
2.1.5.2.2. PHENOTYPE CHARACTERIZATION WITH WMISH METHOD.....	39
2.2.MATERIALS.....	40
2.2.1. GENERAL CHEMICALS, REAGENTS AND ENZYMES.....	40
2.2.2. GENERAL MATERIALS AND EQUIPMENTS.....	41
2.2.3. BUFFERS AND SOLUTIONS.....	42
2.2.4. MOLECULAR SIZE MARKERS AND PLASMID VECTORS.....	42
CHAPTER 3. RESULTS.....	43
3.1. ZEBRAFISH ORTHOLOGUE OF <i>WDR81</i>.....	43
3.2. ZEBRAFISH <i>WDR81</i> TRANSCRIPT POSSESSES ONE ORF.....	45
3.3. CHARACTERIZATION OF 5' END OF <i>WDR81</i> VERIFIED THE PREDICTION AND AN INSERTION SITE WAS DETECTED IN THE 3'END OF SOME TISSUES	46
3.4. EXPRESSION OF <i>WDR81</i> IS INCREASED AT 5 HPF AND 18 HPF DURING DEVELOPMENT AND IT IS ENRICHED IN THE EYE AND BRAIN OF EMBRYOS.....	53
3.5. SPATIAL EXPRESSION OF <i>WDR81</i> AND ITS DETECTION IN PLOLIFERATION ZONES.....	61
3.6. INITIAL CHARACTERIZATION OF MORPHANTS.....	68
3.6.1. DOSE CURVE OF <i>WDR81</i> MORPHOLINO.....	68
3.6.2. INITIAL RESULTS FROM PHENOTYPE CHARACTERIZATION OF <i>WDR81</i> MORPHANTS.....	67

CHAPTER 4. DISCUSSION.....	79
CHAPTER 5. FUTURE PERSPECTIVES.....	88
REFERENCES	90
APPENDIX A –BUFFERS AND SOLUTIONS.....	111
APPENDIX B –MOLECULAR SIZE MARKERS.....	117
APPENDIX C-CLONING VECTORS.....	119
APPENDIX D–MULTIPLE SEQUENCE ALIGNMENT OF <i>DANIO RERIO</i> (ZEBRAFISH) WDR81 AMINO ACID SEQUENCE WITH WDR81 SEQUENCES FROM VARIOUS SPECIES	121
APPENDIX E–TBLASTN SEARCH FOR WDR81.....	131



List of Figures

FIGURE 1: QUADRUPEDAL GAIT IN A MALE IN 1914 (LEFT) AND IN A FEMALE PATIENT NOWADAYS (RIGHT).....	1
FIGURE 2: BRAIN MORPHOLOGY OF A CONTROL INDIVIDUAL AND A PATIENT WERE ANALYZED VIA MAGNETIC RESONANCE IMAGING (MRI).....	5
FIGURE 3: PHYLOGENETIC TREE OF PROTEIN SEQUENCES OF WDR81 IN VERTEBRATES.....	7
FIGURE 4: LATERAL VIEWS OF A FEMALE (F) AND A MALE (M) ADULT ZEBRAFISH.....	12
FIGURE 5: FIVE DPF WILD TYPE ZEBRAFISH EMBRYO.....	16
FIGURE 6: ILLUSTRATION WHICH COMPARES THE BRAIN SIZES OF HUMAN, MOUSE AND ZEBRAFISH.....	17
FIGURE 7: ZEBRAFISH <i>wdr81</i> GENE, ILLUSTRATION OF ITS GENOMIC STRUCTURE AND BINDING REGIONS OF PRIMERS.....	44
FIGURE 8: THE CONSERVED DOMAINS AND THE STRUCTURAL ORGANIZATION OF ZEBRAFISH WDR81, PREDICTED <i>IN SILICO</i>	45
FIGURE 9: ALIGNMENT OF THE PROTEIN SEQUENCES OF HUMAN, MOUSE AND ZEBRAFISH WDR81.....	47
FIGURE 10: DIAGRAM SHOWING THE SPLICE VARIANTS OF HUMAN <i>WDR81</i> , DERIVED FROM ENSEMBL.....	51
FIGURE 11: DIAGRAM SHOWING THE SPLICE VARIANTS OF MOUSE <i>Wdr81</i> , DERIVED FROM ENSEMBL.....	52
FIGURE 12: DIAGRAM SHOWING THE ZEBRAFISH <i>wdr81</i> TRANSCRIPT, DERIVED FROM ENSEMBL.....	52
FIGURE 13: AGAROSE GEL ELECTROPHORESIS IMAGE OF THE PCR PRODUCTS OF THE REACTIONS, WHICH ZEBRAFISH <i>wdr81</i> OPEN READING FRAME WAS CHARACTERIZED.....	53
FIGURE 14: THE ILLUSTRATION OF THE STRATEGY IN THIS STUDY TO OBTAIN 5'RACE PRODUCT OF <i>wdr81</i> BY PERFORMING A NESTED PCR FOLLOWING A TOUCH DOWN PCR.....	54
FIGURE 15: AGAROSE GEL ELECTROPHORESIS IMAGE OF THE 5'RACE PRODUCT OF <i>wdr81</i> TRANSCRIPT.....	54
FIGURE 16: THE SCHEMATIC ILLUSTRATION OF THE ORDER OF SEQUENCES IN PLASMIDS EXPECTED TO BE OBSERVED IN THE SANGER SEQUENCING OF 5'RACE PRODUCT OF <i>wdr81</i>	55

FIGURE 17: A CLCBIO MAIN WORKBENCH SOFTWARE ANALYSIS DIAGRAM SHOWING A REPRESENTATIVE SANGER SEQUENCING RESULT OF 5'RACE PRODUCT WITH A 6 BP SHORTER 5'UTR THAN THE PREDICTION.....	55
FIGURE 18: THE ILLUSTRATION OF THE STRATEGY IN THIS STUDY TO OBTAIN 3'RACE PRODUCT OF <i>WDR81</i> BY AMPLIFYING RACE-READY cDNAs AS THREE OVERLAPPING PCR PRODUCTS.....	56
FIGURE 19: AGAROSE GEL ELECTROPHORESIS IMAGE OF THE OVERLAPPING AMPLICONS OBTAINED FROM THE EXPERIMENT IN WHICH 3'END OF THE ZEBRAFISH <i>WDR81</i> WAS CHARACTERIZED.....	58
FIGURE 20: ALIGNMENT OF THE CLONES OF THE INSERTION SITE DETECTED IN 3'UTR OF ZEBRAFISH <i>WDR81</i> TRANSCRIPT REVEALED ITS LENGTH AS 266 BP.....	60
FIGURE 21: AGAROSE GEL ELECTROPHORESIS IMAGE OF THE AMPLICONS OBTAINED FROM THE REACTIONS IN WHICH PRESENCE OF THE INSERTION SITE WAS TESTED IN SAMPLES FROM DEVELOPMENTAL STAGES AND IN ADULT TISSUES.....	61
FIGURE 22: RELATIVE EXPRESSION OF <i>WDR81</i> AT TEN DEVELOPMENTAL STAGES.....	63
FIGURE 23: SPATIO-TEMPORAL EXPRESSION OF <i>WDR81</i> DURING EARLY DEVELOPMENT REVEALED VIA WMISH.....	64
FIGURE 24: TRANSVERSE SECTIONS OBTAINED FROM THE HEAD REGIONS OF WMISH SPECIMENS FROM 3 DEVELOPMENTAL STAGES.....	65
FIGURE 25: RELATIVE EXPRESSION OF <i>WDR81</i> IN TEN ADULT TISSUES.....	66
FIGURE 26: DISTRIBUTION OF THE SIGNAL OF RIBOPROBE REVEALED THE EXPRESSION OF <i>WDR81</i> IN THE ADULT BRAIN AREAS AND EYE TISSUE.....	67
FIGURE 27: AGAROSE GEL ELECTROPHORESIS OF THE PCR PRODUCTS FROM A REACTION SHOWING <i>WDR81</i> EXPRESSION BETWEEN 1-48 HPF TIMEPOINTS.....	70
FIGURE 28: ILLUSTRATION OF THE LOCATION OF THE TARGET OF THE MORPHOLINO SEQUENCE (SHOWN IN RED) WHICH WAS DESIGNED TO KNOCK DOWN <i>WDR81</i>	71
FIGURE 29: ILLUSTRATION SHOWING THE LOCATION OF THE PRIMER PAIR, WHICH WAS DESIGNED TO REVEAL THE EFFECT OF <i>WDR81</i> MORPHOLINO ON SPLICING.....	73
FIGURE 30: AGAROSE GEL ELECTROPHORESIS OF THE PCR PRODUCTS FROM A REACTION SHOWING THE EFFECT OF THREE DOSES OF <i>WDR81</i> MORPHOLINO ON SPLICING.....	74
FIGURE 31: A REPRESENTATIVE PICTURE (TAKEN WITH A LEICA MZ10F MICROSCOPE) OF AN EMBRYO, WHOSE HEAD SIZE AND BODY LENGTH WAS MEASURED VIA LEICA APPLICATION SUITE 4.3 (LASv 4.3) SOFTWARE.....	75
FIGURE 32: COMPARISON OF <i>GBX2</i> EXPRESSION AMONG 3 EXPERIMENT GROUPS, WHICH WERE 2 NG <i>WDR81</i> MORPHOLINO INJECTED (A-C), 2 NG STANDARD NEGATIVE CONTROL MORPHOLINO INJECTED (D, E) AND UNINJECTED (F) EMBRYOS.....	78

FIGURE 33: A TWENTY FOUR HOURS POST FERTILIZATION ZEBRAFISH EMBRYO WHICH WAS INCUBATED WITH BRDU FOR 24 HOURS	89
FIGURE B.1: PUC MIX MARKER, 8, SEPERATED ON A 1.7% AGAROSE GEL (SM0303, FERMENTAS).....	117
FIGURE B.2: MASSRULER DNA LADDER, SEPERATED ON A 1% AGAROSE GEL (SM0403, THERMO SCIENTIFIC).....	118
FIGURE C.1: PCR4-TOPO CLONING VECTOR (INVITROGEN).....	119
FIGURE C.2: PGEM-T EASY CLONING VECTOR (PROMEGA).....	120
FIGURE D.1: MULTIPLE SEQUENCE ALIGNMENT OF <i>DANIO RERIO</i> (ZEBRAFISH) WDR81 AMINO ACID SEQUENCE WITH WDR81 SEQUENCES FROM VARIOUS SPECIES.	121



List of Tables

TABLE 1: SUMMARY OF THE REPORTED CASES WITH CAMRQ1.....	4
TABLE 2 : SUMMARY OF THE REPORTED CASES WITH CAMRQ3.....	10
TABLE 3: PRIMER PAIRS DESIGNED TO CHARACTERIZE THE OPEN READING FRAME OF <i>WDR81</i>	27
TABLE 4: PRIMER SETS DESIGNED TO OBTAIN THREE OVERLAPPING AMPLICONS IN ORDER TO CHARACTERIZE 3'UTR OF <i>WDR81</i>	31
TABLE 5: PRIMER PAIRS AND PROBES TO BE USED IN QRT-PCR.....	33
TABLE 6: VARIANTS DETECTED IN THE 3'RACE PRODUCTS OF 24 HPF EMBRYO AND ADULT BRAIN <i>WDR81</i> EXCEPT THE INSERTION SITE WHICH WAS FOUND TO BE 266 BP LONG.....	59
TABLE 7: INTENSITIES OF THE BANDS OF PCR PRODUCTS FROM FIGURE 21.....	62
TABLE 8: SURVIVAL RATES OF THE EMBRYOS FROM EXPERIMENT GROUPS IN THE <i>WDR81</i> MORPHOLINO DOSE CURVE STUDY.....	72
TABLE 9: SURVIVAL RATES OF THE EMBRYOS FROM EXPERIMENT GROUPS IN THE HEAD SIZE MEASUREMENT STUDY.....	76
TABLE E.1: TBLASTN SCORES OF ZEBRAFISH <i>WDR81</i>	131
TABLE E.2: LIST OF THE HITS OBTAINED AS A RESULT OF TBLASTN SEARCH OF ZEBRAFISH <i>WDR81</i>	131

Abbreviations

ANOVA, Analysis of variance	ISH, <i>In situ</i> hybridization
ATP8A2, ATPase, aminophospholipid transporter, class I, type 8A, member 2	MFS, Major facilitator superfamily
BA6, Brodmann area 6	MLF, Medial longitudinal fascicle
BEACH, Beige and Chediak-Higashi	ORF, Open reading frame
BrdU, 5-bromo-2'-deoxyuridine	P15, Post natal day 15
CA8, Carbonic anhydrase VIII	qRT-PCR, Quantitative real-time PCR
CAMRQ, Cerebellar Ataxia, Mental Retardation and Dysequilibrium Syndrome	RACE, Rapid amplification of cDNA ends
CNS, Central nervous system	RoP, Rostral primary motor neuron
DIG, Digoxigenin	SGZ, Subgranular zone
Dpf, Days post fertilization	SVZ, Subventricular zone
ENU, <i>N</i> -ethyl- <i>N</i> -nitrosourea	UTR, Untranslated region
GABA, Gamma-aminobutyric acid	VLDLR, Very low density lipoprotein receptor
<i>gbx2</i> , gastrulation brain homeobox 2	WD40, ~40 amino acid structural motifs, often ending with a Tryptophan-Aspartic acid (W-D) dipeptide
GFAP, glial fibrillary acidic protein	WDR81, WD repeat containing protein 81
Hpf, Hours post fertilization	ZFIN, The Zebrafish model organism database

Chapter 1. Introduction

1.1. Cerebellar Ataxia, Mental Retardation and Dysequilibrium Syndrome

Neurodevelopmental disorders are associated with mutations which might affect numerous cellular mechanisms leading to impairment of growth and development of the nervous system. These type of impairments during the neurodevelopmental processes give rise to neurological or psychiatric diseases¹. Schizophrenia, autism spectrum disorder, fragile-X and Rett syndrome are a few examples of such neurodevelopmental disorders²⁻⁵. Cerebellar ataxia, mental retardation and dysequilibrium syndrome (CAMRQ) is also a neurodevelopmental disorder. The inheritance pattern is autosomal recessive and it is a rare condition, which is characterized by mental retardation, cerebellar ataxia and dysarthric speech with or without quadrupedal gait⁶⁻¹⁴. Quadrupedal gait, ie. walking on all fours, in humans is also referred to as Unertan Syndrome^{6,15} and was first observed in 1914 in the Black Sea Region¹⁶ (Figure 1). CAMRQ is associated with specific mutations and these mutations have been shown in consanguineous families with one exception¹⁷. Homozygosity mapping, linkage analysis and targeted next generation sequencing of homozygous regions revealed genetic heterogeneity in this syndrome.



Figure 1. Quadrupedal gait in a male in 1914 (left)¹⁶ (by courtesy of Prof. Dr. Üner Tan) and in a female patient nowadays (right)¹⁸ (Reprinted with permission from Taylor and Francis Group, <http://www.tandfonline.com>).

1.1.1. CAMRQ Associated Genes

VLDLR (very low-density lipoprotein receptor), *WDR81* (WD repeat containing protein 81), *CA8* (carbonic anhydrase VIII) and *ATP8A2* (ATPase, aminophospholipid transporter, class I, type 8A, member 2) have been reported to be associated with CAMRQ1 (MIM: 224050)^{8–10,13,17,19–21}, CAMRQ2 (MIM: 610185)^{6–8,12}, CAMRQ3 (MIM: 613227)^{11,22,23} and CAMRQ4 (MIM: 615268)¹⁴, respectively. The CAMRQ associated genes are mapped to chromosomes 8, 9, 13 and 17; *CA8* to chromosome 8q12, *VLDLR* to chromosome 9p24, *ATP8A2* to chromosome 13q12 and *WDR81* to chromosome 17p13.

1.1.1.1. Very Low Density Lipoprotein Receptor (VLDLR)

VLDLR is a gene made up of 19 exons²⁴ and encodes a receptor protein which is a member of the low-density lipoprotein receptor family²⁵. It is an integrative element in the reelin pathway and functions in guiding neuroblast migration in the developing cerebral cortex and cerebellum^{26,27}. ApoER2 (Apolipoprotein E Receptor 2) and *VLDLR*, which are reelin receptors, are both required for the coordination of alignment of Purkinje cell subsets in the developing cerebellum^{28,29}. Reelin mediates cortical layer formation and dendrite development in hippocampus via *VLDLR*/ApoER2-Dab1 (Disabled 1) pathway³⁰. Recent studies showed that *VLDLR* is actively involved in regulation of formation and development of dendritic spines^{31,32}. In addition, *VLDLR* is expressed in synapses, both presynaptically and postsynaptically. Knockdown of *VLDLR* is reported to significantly decrease the synaptophysin puncta number and also to decrease glutamate receptor subunits such as GluN1 levels and levels of GluA1 at the cell surface³². Besides its role in the reelin signaling pathway, *VLDLR* fulfills critical functions in triglyceride metabolism. It has an effect on the uptake of VLDL triglycerides in peripheral tissues³³. It is also shown that the expression of *VLDLR* is essential in promoting adipocyte differentiation³⁴. The summary of the reported cases with *VLDLR*-associated CAMRQ (CAMRQ1) and the information about the *VLDLR* mutations are given in Table 1.

The common morphological abnormalities in the brains of the patients with CAMRQ1 are the hypoplasia of cerebellum^{8,10,13,17,19–21} and moderate gyral simplification of the cerebral cortex^{8,10,13,17,20}. The pons was observed particularly small in some patients^{8,13,17,21} while mild hypoplasia of the corpus callosum was observed by Turkmen *et al.* (2008)¹⁹. Moheb *et al.* (2008) was unable to obtain any MRI (magnetic resonance imaging) or CT (computerized tomography) scans of the brains from any patients, and for this reason the brain abnormalities of the patients in this study could not be evaluated⁹.

1.1.1.2. WD Repeat Containing Protein 81 (WDR81)

The gene encoding WD repeat containing protein 81 (*WDR81*) was found to be mutated in some CAMRQ patients. It is a missense mutation and it lies in exon 1 of the *WDR81* isoform 1, *WDR81* p.P856L. The most significant morphological changes in the CAMRQ patients carrying this mutation were significant decline in the volumes of cerebellum and corpus callosum. Additional analysis showed that morphological differences in the precentral gyrus and Brodmann areas BA6, BA44 and BA45 took place in the patients' brains¹² (Figure 2). When researchers investigated the neuro-ophthalmic aspect of CAMRQ, they observed that four patients who carry the missense mutation in *WDR81* gene had downbeat nystagmus and two male patients among four patients had also bilateral temporal disc pallor and ring-shaped macular atrophy³⁵. A similar phenotype to CAMRQ was obtained with a mutant mouse line *nur5*. The *N*-ethyl-*N*-nitrosourea (ENU) induced mutation in this model was a missense mutation, L1349P, and it was also located in the predicted major facilitator superfamily domain of the *Wdr81* protein as in patients. This homozygous missense mutation caused loss of Purkinje cells, which was detected at P28 (young adult) and loss of photoreceptor cells, which was detected on P15 (infant) the earliest tested timepoints in this mouse model³⁶.

The mitochondrial defects in the dendrites of Purkinje cells was detected in mutant mice at P21 (juvenile) and this finding led the conclusion that the mitochondrial abnormalities are followed up by death of Purkinje cells during development³⁶.

Table 1. Summary of the reported cases with CAMRQ1 (Adapted from Ali *et al.* 2012²⁰)

	Boycott <i>et al.</i> 2005	Moheb <i>et al.</i> 2008	Turkmen <i>et al.</i> 2008	Ozcelik <i>et al.</i> 2008	Ozcelik <i>et al.</i> 2008	Boycott <i>et al.</i> 2009	Kolb <i>et al.</i> 2010	Ali <i>et al.</i> 2012	Dixon-Salazar <i>et al.</i> 2012
Mutation	Homozygous deletion of the <i>VLDLR</i> gene	Homozygous c.1342 C>T (p.R448X)	Homozygous c.2339delT (p.I780TfsX3)	Homozygous c.769C.T (p.R257X)	Homozygous c.2339delT (p.I780TfsX3)	Compound heterozygous c.1561G>C + c.1711_1712dupT (p.D521H + p.Y571LfsX7)	Homozygous deletion, which begins in the 5'UTR and continues through a part of exon 1, includes exons 2,3,4 and a part of exon 5	Homozygous c.2117G>T (p.C706F)	Homozygous c.1247_53delGTTACAA (p.G1246fsX1305)
Ethnicity	Hutterite	Iranian	Turkish	Turkish	Turkish	Caucasian	Turkish	United Arab Emirates (Omani)	Turkish
Consanguinity	Present	Present	Present	Present	Present	Not present	Present	Present	Present
Ambulation	Delayed	No ability to walk independently	Delayed-Quadrupedal	Delayed-Quadrupedal	Delayed-Quadrupedal	Delayed	Delayed-Bipedal	Delayed-Bipedal	Delayed
Number of patients in the study	10	8	3	5	3	1	2	5	2

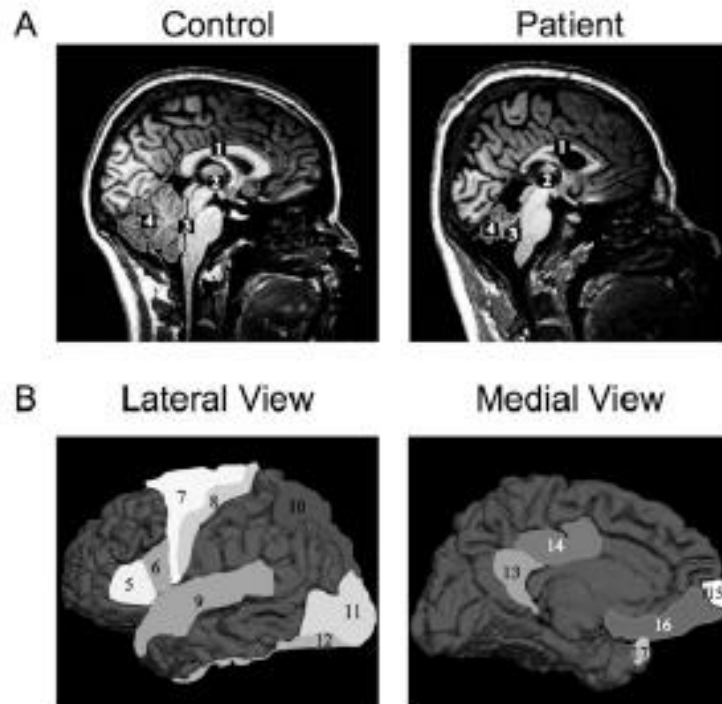


Figure 2. Brain morphology of a control individual and a patient were analyzed via magnetic resonance imaging (MRI). The patient was reported to be affected from *WDR81* associated CAMRQ (CAMRQ2, MIM: 610185). (A) A healthy control individual (left) and a patient (right) were scanned. The areas, which were assigned with numbers indicate regions where differences in volumes are distinguishable: corpus callosum (1), third ventricle (2), fourth ventricle (3), and cerebellum (4). (B) Lateral and medial views of a reference cortex on which the significantly affected morphometric parameters are numbered: BA45 (5), BA44 (6), BA6 (7), precentral (8), superior temporal (9), superior parietal (10), lateral occipital (11), fusiform (12), isthmus cingulated (13), posterior cingulated (14), frontal pole (15), medial orbitofrontal (16), and temporal pole (17)¹². Reprinted with permission from Cold Spring Harbor Laboratory Press.

WDR81 gene was shown to be mutated in cancer and it appears that it locates in a metabolically important loci. *WDR81* gene was reported to be mutated and expressed in >10% of the evaluated 23 colorectal cancer cell lines³⁷. Transethnic meta-analysis of European ancestry and Japanese genome-wide association studies revealed that

SERPINF2-WDR81 loci as one of the six significant loci for serum albumin³⁸. Serum albumin level is metabolically important because it is inversely associated with cardiovascular risk and mortality risk^{39,40}.

WDR81 was shown to be expressed in all of the tested human tissues and its level was the highest in cerebellum and corpus callosum among brain regions¹². Expression of *Wdr81* was detected in Purkinje cell neurons, photoreceptor cells, deep cerebellar nuclei neurons and neurons of brainstem among the central nervous system neurons in wild-type adult mouse. Localization of *Wdr81* was observed in the mitochondria of Purkinje cells using electron microscopy. *Wdr81* expression was also detected in all of the evaluated adult tissues³⁶. Gulsuner *et al.* also revealed that *Wdr81* expression was higher in the Purkinje cell neurons and molecular layer of cerebellum in mouse embryonic brain¹².

The function of WDR81 is not currently known, however it can be predicted *in silico* with information about the domains of the protein. The presence of conserved domains in proteins may lead to the indication of their function as well. Moreover, conservation of the identical domains in homologous proteins from different species sheds light on the critical importance of the protein of interest at several evolutionary levels. WDR81 protein is conserved among vertebrates, the mutation also hits a conserved domain of the protein of interest¹² (Figure 3). The putative domains of human and mouse WDR81 proteins are Beige and Chediak-Higashi (BEACH) domain, a major facilitator superfamily (MFS) domain and six WD40 repeat domains. Both proteins are predicted to be transmembrane proteins^{12,36}. A BEACH-domain containing proteins are proposed to perform as scaffold proteins. They are mostly large proteins and they take place in membrane-related events. These events include vesicle fusion and fission, so that they might function in vesicular transport, apoptosis, receptor signaling, formation of synapses, autophagy and membrane dynamics⁴¹. WD40 domain is given this name because of the length of the repeats of 40 aminoacids and the repeats often end with the conserved Tryptophan-Aspartic acid (WD) dipeptide.

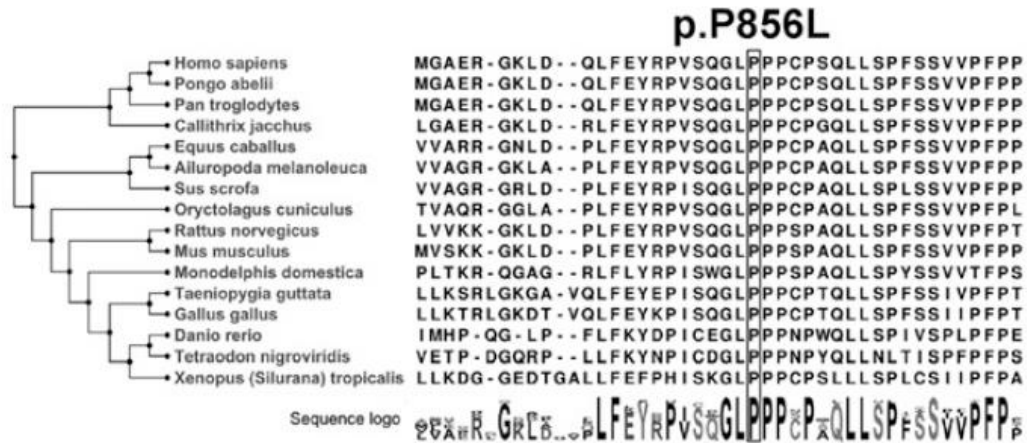


Figure 3. Phylogenetic tree of protein sequences of WDR81 in vertebrates. The box implies the amino acid, mutated in CAMRQ patients¹². Reprinted with permission from Cold Spring Harbor Laboratory Press.

WD40 domain-containing proteins take place in numerous cellular functions such as cell cycle control, signal transduction, regulation of transcription, apoptosis, chromatin dynamics, vesicular trafficking and cytoskeletal assembly. The WD40 domains of BEACH domain-containing proteins serve as a scaffold for protein-protein or protein-DNA interactions^{41,42}. The MFS transporters function in response to chemiosmotic ion gradients and they transport small molecules. They are single-polypeptide secondary carriers and act as uniporters, symporters or antiporters⁴³. Taken together, the WDR81 protein might have an importance related to both the central nervous system and the metabolism. The findings from mouse and human studies indicate that *WDR81* might be a critical gene in neurodevelopment, but the exact function of the gene is still not fully understood.

The family of proteins named as WDR and followed by a number, is composed of almost 100 proteins in human genome. Some of them were reported to be associated with diseases. The association of *WDR10* with cranioectodermal dysplasia 1 [MIM 218330]⁴⁴, *WDR11* with hypogonadotropic hypogonadism 14 with or without anosmia [MIM 614858]⁴⁵, *WDR19* with Senior-Loken syndrome 8 [MIM 616307]^{46,47}, *WDR34*

with short-rib thoracic dysplasia 11 with or without polydactyly [MIM 615633]^{48,49}, *WDR35* with cranioectodermal dysplasia 2 [MIM 613610]⁵⁰ and short-rib thoracic dysplasia 7 with or without polydactyly [MIM 614091]⁵¹, *WDR36* with primary open angle glaucoma [MIM 609887]⁵², *WDR56* with short-rib thoracic dysplasia 2 with or without polydactyly [MIM 611263]^{53,54}, *WDR60* with short-rib thoracic dysplasia 8 with or without polydactyly [MIM 615503]⁵⁵, *WDR62* with microcephaly 2, primary, autosomal recessive, with or without cortical malformations [MIM 604317]^{56–60} were reported. Dolichocephaly and high forehead were observed in cranioectodermal dysplasia as a result of abnormal development of the cranium and skeleton⁴⁴. Anosmia was associated with the absence or underdevelopment of olfactory bulbs and their nerve fibers⁶¹. Hypogonadism was found to be related with the failure in migration of neurons which synthesize gonadotrophin releasing hormone during development⁶². Senior-Loken syndrome 8 is a progressive disorder characterized by retinal and renal failure⁶³. Impairment in the organs such as brain, eye, heart, kidneys, liver, pancreas, intestines, and genitalia as well as cleft lip/palate might accompany the short-rib thoracic dysplasia 11 with or without polydactyly condition⁶⁴. Primary open angle glaucoma was characterized by the defect in the vision, optic nerve damage and increased intraocular pressure⁶⁵. Primary microcephaly was characterized by mental retardation and brain size, which is smaller than normal⁵⁶. The brain malformations found to be related with *WDR62* mutations were pachygyria^{56,57}, underdevelopment of corpus callosum^{57,59}, polymicrogyria^{58,59}, schizencephaly, subcortical heterotopia⁵⁹, microlissencephaly, band heterotopias and dysplastic cortex⁵⁶. Thus, mutations in several genes encoding WD40 domain containing proteins, similar to *WDR81*, were shown to be associated with brain and eye malformations during development.

1.1.1.3. Carbonic Anhydrase VIII (CA8)

Another CAMRQ associated gene *CA8* is comprised of 9 exons²⁴ and encodes a protein which has a critical function in motor control⁶⁶. It has been classified as a member of the carbonic anhydrase gene family because of the sequence similarity, however the gene product of *CA8* does not possess the enzymatic activity to catalyze the reaction of

hydration of carbon dioxide reversibly⁶⁷. CA8 binds to IP₃R1 (inositol 1,4,5 triphosphate receptor type 1) and binding of CA8 to IP₃R inhibits binding of IP₃ to the receptor by decreasing its affinity. Expression of both CA8 and IP₃R1 is abundant in Purkinje cells and they co-localize in the cytoplasm, dendrites and axons. This might explain why the sensitivity of IP₃R1 to IP₃ for IP₃-induced Ca⁺² release in this layer is low⁶⁸. Expression of CA8 in human fetal brain was detected in neuroprogenitor cells in the subventricular zone and in the neurons, which migrate to the cortex. In the adult human, CA8 expression was found out in the neural cell bodies in most regions of the central nervous system, such as cerebrum, diencephalon, cerebellum, pons and medulla⁶⁹. The “waddles” (*wdl*) mouse is an animal model of *Car8* null mutation⁶⁶. Car8 is the homologous protein of CA8 in mouse which shows a 98% identity⁷⁰. Ataxia and appendicular dystonia comprised the phenotype of the *wdl* mouse and the gait disorder continued throughout life-time⁶⁶. CA8 overexpression in neuronal cell lines under staurosporine induced apoptotic stress reduced the cell death and overexpression of CA8 in neuronal cell lines and mouse cerebellar granule neurons increased neuronal migration and invasion. CA8 downregulation decreased cell migration and invasion ability and resulted in abnormal Ca⁺² release in cerebellar granule neurons. Knockdown of *ca8* in zebrafish was achieved by using morpholino injection and *ca8* morphants at 3 days post fertilization exhibited a decrease in motility⁷¹. Knockdown of *ca8* via microinjection of morpholino oligonucleotides in another study resulted in neuronal death and malformations in cerebellum and muscle. Body axis was curved and motor functions and coordination were defective, showing a similar phenotype to human condition⁷². Also, CA8 expression has been found to be increased in non-small lung cancer and colorectal cancer^{73,74}.

The summary of the reported cases with CA8-associated CAMRQ (CAMRQ3) and the information about the CA8 mutations are given in Table 2. Among the three publications summarized in Table 2, only Kaya *et al.* (2011) provided results of brain imaging studies on patients. The MRI study showed variable volume loss in cerebellum and vermis in patients in this study²².

Table 2. Summary of the reported cases with CAMRQ3

	Turkmen <i>et al.</i> 2009	Kaya <i>et al.</i> 2011	Najmabadi <i>et al.</i> 2011
Mutation	Homozygous c.298T>C (p.S100P)	Homozygous c.484G>A (p.G162R)	Homozygous chr8:61297790 C>T (p.R237Q)
Ethnicity	Iraqi	Saudi	N/A
Consanguinity	Present	Present	Present
Ambulation	Quadrupedal	Delayed-without quadrupedal gait	N/A
Number of patients in the study	4	7	4

N/A: not applicable, information not provided in the article.

1.1.1.4. ATPase, Aminophospholipid Transporter, Class I, Type 8A, Member 2 (ATP8A2)

ATP8A2 (ATPase, aminophospholipid transporter, class I, type 8A, member 2) is made up of 37 exons²⁴ and encodes a protein, which is a member of P4 ATPase protein family⁷⁵. This protein family facilitates transport of phospholipids from exoplasmic leaflet to cytosolic leaflet and thereby are involved in formation of the asymmetry of the cell membrane. They are also called lipid flippases and they take roles directly or indirectly in dynamics of cytoskeleton, signaling and metabolism of lipids, cell division and membrane trafficking⁷⁶. *ATP8A2* is expressed in brain, retina and testis among evaluated human tissues. Expression of *ATP8A2* in the brain was detected in both fetal and adult tissues⁷⁷. In adult human brain, all the tested regions showed expression of the gene and the highest level of expression was found to be in cerebellum¹⁴. In mouse, *Atp8a2* expression was detected in brain, spinal cord, retina and testis^{78,79}. The function of *Atp8a2* was revealed as to transfer aminophospholipids (phosphatidylserine and phosphatidylethanolamine) from exoplasmic to cytosolic leaflet in an ATP-dependent manner⁷⁸. Overexpression and downregulation studies on *Atp8a2*, in conjunction with the results from the overexpression and downregulation of *Cdc50a*, demonstrated that *Atp8a2* in synergy with *Cdc50a* functions in axon elongation of neurons which also

indicates a critical role in rat hippocampal neuronal differentiation⁸⁰. A mutant mouse model, *wobbler lethal*, which has ataxia and neurodegeneration, was shown to carry loss of function mutations in *Atp8a2* gene. It was concluded *Atp8a2* is associated with axon degeneration and neurodegenerative disease⁷⁹. Disruption of the *ATP8A2* gene was also reported to be associated with severe mental retardation and hypotonia in a patient⁷⁷.

ATP8A2-associated CAMRQ (CAMRQ4) was reported in a consanguineous family from Turkey. Four CAMRQ patients were detected in the family, however DNA from one patient could not be included in the study. Patients showed delayed ambulation and have quadrupedal gait⁸. The index patient was unable to walk at the time of the study. A missense mutation in *ATP8A2* (c. 1128 C>G, p.I376M) was found out to be associated with the condition. Moderate hypoplasia of inferior cerebellum, corpus callosum and cerebral cortex were observed in the patients¹⁴.

1.2. Zebrafish as a Model Organism

The model organism used in the current work is the zebrafish (*Danio rerio*) (Figure 4). George Streisinger and his colleagues established zebrafish as a genetic model system 35 years ago⁸¹. Zebrafish have become a promising model organism for scientists in developmental biology, neurophysiology, biomedical research and ethology fields⁸². Comparison of human reference genome and zebrafish reference genome revealed that one clear zebrafish orthologue gene exists for approximately 70% of human genes⁸³. It is a vertebrate with an integrated nervous system and possesses common organs and tissues like brain and spinal cord⁸⁴. Zebrafish produce large batches of externally fertilized and transparent embryos that enable observation of development with microscopy techniques. Moreover, embryos develop at a fast rate and key developmental events take place earlier and faster compared to mammals. For example neurogenesis begins around 10 hours post-fertilization (hpf), synaptogenesis and the first behaviors of embryos begin around 18 hpf and hatching is observed around 52 hpf^{82,84}. After approximately 2 days of development, zebrafish embryo has its internal organs, eyes, ears and a brain, which has been already divided into

compartments. Thus the embryo has formed all of the common main characteristics of a vertebrate body by this timepoint⁸⁵.



Figure 4. Lateral views of a female (F) and a male (M) adult zebrafish. *wt: wild type, L: left side of the bodies. Scale bar indicates 2 mm⁸⁶. Reprinted with permission from Elsevier.*

Various molecular genetic techniques have been devised and used in zebrafish research and new techniques are continuing to emerge over time. For example, transgenic zebrafish lines, which express a fluorescent protein in specific types of neurons, are useful in studying the development of nervous system and neural circuits⁸⁷. Bacterial artificial chromosome (BAC) mediated transgenesis is managed by using BAC which is engineered with homologous recombination to express fluorescent reporter in target cells⁸⁸. Although the efficiency of BAC mediated transgenesis is low, this technique is advantageous in obtaining the reporter genes and regulatory sequences together in the construct instead of putting effort in laborious subcloning work^{87,89}. The efficiency of integration of the DNA injected to the zebrafish embryos increases based on the activity of the transposases, such as sleeping beauty⁹⁰ and tol-2⁹¹ transposases. Employing transgenes under control of heat-inducible promoters provides conditional control of gene expression^{92,93}. Using the adapted form of the yeast galactose inducible⁹⁴ and Cre-loxP systems⁹⁵, which efficiently works in mouse models, also provide conditional control of gene expression and permanent labeling of cell lineages in zebrafish models.

Targeted mutation is managed by zinc finger nucleases (ZFNs)⁹⁶ and TAL effector nucleases (TALENs)^{97,98} in zebrafish. A double strand break induced by these nucleases

is repaired by non-homologous end joining. This often results in insertion or deletions and ultimately a loss-of-function phenotype is obtained⁹⁶⁻⁹⁸. A recent method, Clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas9 is reported to be more efficient than ZFN and TALEN methods in terms of germline transmission. This system is a genome editing tool, already used in various model organisms and has advantages over other mutagenesis methods by its simplicity in design, being practical to use and enabling to target more than one genes at the same time⁹⁹.

Morpholino antisense technology is used in loss-of-function experiments. Morpholino sequences knock down the gene of interest either by targeting the RNA splicing or the translation initiation. These sequences are stable and introduced to the embryos preferably at one-cell stage however their effect lasting for up to seven days and the concentration is diluted as cell division continues. Possible off-target effects needs to be evaluated as well. If apoptosis is triggered as an off-target effect of the morpholino, co-injection of p53 morpholino might be considered^{89,100}. ENU mutagenesis is utilized to provide random mutagenesis also in zebrafish¹⁰¹ and Targeted Induced Local Lesions in Genomes (TILLING) method can reveal the mutations caused by ENU¹⁰².

Applying morpholino antisense technology was preferred in the present study since establishment of this technique would be faster compared to other genetic manipulation tools. The data obtained with the transient knockdown of the gene of interest might be investigated further by employing stable knockout systems. Besides, the effects of knockdown of *wdr81* gene in zebrafish is possible to be searched starting from early developmental stages up to seven days post fertilization, which encompasses the critical timepoints of neurodevelopment. This provides an important advantage when it is considered that CAMRQ is a neurodevelopmental disease.

Zebrafish was used as a model organism to investigate cerebellar disorders. Knockdown of ataxin-7 via morpholino microinjection was applied by producing mild and severe phenotypes in order to study spinocerebellar ataxia 7 (SCA7). SCA7 was characterized by loss of Purkinje cells and granule cells of cerebellum and rod-cone photoreceptors.

While severe phenotype ended up with increased lethality of embryos and developmental defects, moderate phenotype led the observations close to the human condition. The differentiation of photoreceptors, Purkinje cells and granule cells was prevented via partial depletion of ataxin-7. Moreover, the phenotypes could be rescued by using human transcript, which might be evaluated as an evidence of conserved function¹⁰³. Another study in which involvement of a gene to a condition was searched by utilizing morpholino technique demonstrated cerebellar and cerebral atrophy. Knockdown of *clpb*, which encodes caseinolytic peptidase B protein homolog, led to this phenotype and proved involvement of the gene of interest to 3-methylglutaconic aciduria, progressive brain atrophy, intellectual disability, congenital neutropenia, cataracts and movement disorder. This phenotype was also rescued via injection of human transcript¹⁰⁴. Microinjection of sorting nexin 14 (*snx14*) translation blocking morpholino to embryos of a zebrafish line, which expresses GFP in the hindbrain, showed decrease in the intensity of the signal from GFP. Microinjection of the morpholino to embryos of a wild type strain showed decreased number of Purkinje cells. Data from both experiments propose that *snx14* was necessary for hindbrain and formation and survival of Purkinje cells. Rescue was achieved by using the human transcript¹⁰⁵. Epistatic interaction of two genes was investigated in association with ataxia, dementia and hypogonadotropism. *rnf216*, encoding a ubiquitin E3 ligase and *otud4*, encoding a deubiquitinase were silenced separately in zebrafish and impairments in the cerebellum, optic tectum and eye were obtained. Coinjection of morpholinos targeting both genes showed a more severe phenotype. All the phenotypes were rescued with human transcript¹⁰⁶. Homozygous mutant zebrafish, carrying loss of function mutation in *qars*, which encodes glutaminyl tRNA synthetase, demonstrated a phenotype similar to human patients. The phenotype was composed of small brain, neurodegeneration and small eye size¹⁰⁷. A transgenic zebrafish line was generated in order to examine whether excitability of motor neurons were affected by spinocerebellar ataxia type 13 (SCA13) associated mutations. Human dominant mutation in the Kv3.3 voltage-gated K⁺ channel was expressed in zebrafish and this mutation was found to be associated with the loss in locomotion and decreased excitability¹⁰⁸. Another research group utilized from several transgenic lines of zebrafish in order to screen mutations, which affect the development of cerebellum¹⁰⁹. Hence, zebrafish became an attractive model organism for studying human diseases. Rescuing morphants with human

transcripts helps proving that the function of the gene of interest was conserved through evolution as well. Zebrafish community is also capable of generating stable models such as transgenic lines or mutants. These models might be used to examine the cellular changes underlying the diseases further.

1.2.1. Zebrafish Central Nervous System

Zebrafish central nervous system (CNS) will be discussed under two sections: the first “1.2.1.1. Central Nervous System in Zebrafish Embryos” and the second “1.2.1.2. Central Nervous System in Adult Zebrafish” in order to review information about both the development of the CNS and the developed CNS, respectively.

1.2.1.1 Central Nervous System in Zebrafish Embryos

The neuroectoderm needs to be specified on the dorsal part of the embryo via neural induction in order to form neural plate. The neural plate is transformed into a neural tube following a combination of signaling and morphogenetic movements. The anterior region of the neural tube forms the brain and the posterior region forms the spinal cord^{110,111}.

Emergence of some critical structures related with zebrafish CNS are earlier than that of mammals. The brain rudiment appears at sphere stage (blastula, 4-4.33 hpf), primary motor neurons appear at 1-4 somites stage (segmentation, 10.33-11.66 hpf), neural tube appears at 10-13 somites stage (segmentation, 14-16 hpf), cerebellum appears at 26+ somites stage (segmentation, 22-24 hpf), spinal cord appears at prim-5 (pharyngula, 24-30 hpf) and immature eye appears at 5-9 somites stage (segmentation, 11.66-14 hpf)¹¹².

All of the main components of the brain have formed by 5 days post fertilization (dpf)⁸⁵. The size of the larval brain at 5 dpf facilitates microscopic research *in vivo*. Its thickness is around 500 micrometers and the length is 1.5 millimeters (mm), makes all neurons possible to be studied with this technique⁸² (Figure 5).

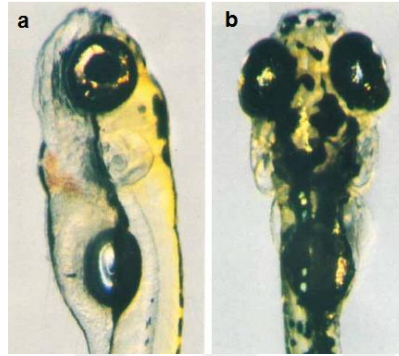


Figure 5. Five dpf wild type zebrafish embryo. a) Lateral view b) dorsal view¹¹³.
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1.2.1.2 Central Nervous System in Adult Zebrafish

The regions of the zebrafish adult brain are telencephalon (olfactory bulbs, *ventralis telencephali*, *area dorsalis telencephali*, telencephalic tracts and commissures), diencephalon (*area praeoptica*, epithalamus, dorsal thalamus, ventral thalamus, posterior tuberculum, hypothalamus, synencephalon, pretectum, diencephalic tracts and commissures), mesencephalon (*tectum opticum*, *torus semicircularis*, tegmentum), rhombencephalon (cerebellum, medulla oblongata), *medulla spinalis* and brain stem¹¹⁴. One of the most striking features of adult zebrafish brain is the dominance of the optic tectum in the dorsal midbrain, as also observed in other teleosts. One disadvantage of studying with zebrafish telencephalon is eversion of this region dorsally, which is not present in other vertebrates (Figure 6). This feature, partly causes difficulties in finding homologous regions between fish and mammalian forebrain regions⁸⁵.

The length of the adult zebrafish brain is around 4.5 mm and its thickness is between 0.4 -2 mm. The zebrafish brain size and number of neurons enable research with advanced microscopy techniques such as multiphoton microscopy and 3D electron microscopy (Figure 6). Since activity analysis and connectivity pattern studies possess size restrictions from a circuit neuroscience point of view⁸².

1.3. Cell Proliferation in Zebrafish Nervous System

The differentiated cells of the central nervous system originate from multipotent neuroepithelial stem cells. These stem cells give rise to mature and functional neurons or glia after the processes called neurogenesis and gliogenesis, respectively¹¹⁵. Because of this phenomenon, cell proliferation in zebrafish central nervous system will be discussed under two titles of “1.3.1. Neurogenesis in Zebrafish” and “1.3.2. Gliogenesis in Zebrafish”.

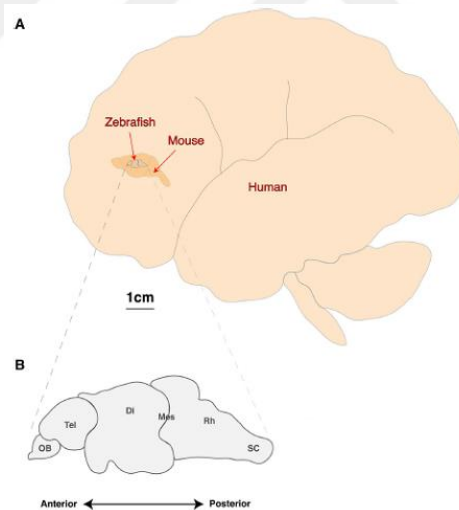


Figure 6. Illustration which compares the brain sizes of human, mouse and zebrafish. (A) Comparison of the lateral views of the adult brains from three species to scale. (B) Major brain areas of zebrafish in a linear plane. OB, olfactory bulb; Tel, Telencephalon; Di, Diencephalon; Mes, Mesencephalon; Rh, Rhombencephalon; sc, Spinal cord¹¹⁶ (By courtesy of Associate Professor Philippe Mourrain). The small size of the zebrafish brain enables observation of the whole brain under microscopy, especially in neuroscience research.

1.3.1. Neurogenesis in Zebrafish

Neurogenesis is defined as the process which first starts with neural induction and ends up with differentiated functional neurons¹¹⁷. Neurogenesis in zebrafish will be discussed under two sections: the first “1.3.1.1. Neurogenesis in Zebrafish Embryos” and the second “1.3.1.2. Neurogenesis in Adult Zebrafish”.

1.3.1.1. Neurogenesis in Zebrafish Embryos

Neural induction, which is defined as specification of the neuroectoderm, is required before neurogenesis. Neural induction is directed by extrinsic and intrinsic factors. Indeed, the interplay of extrinsic bone morphogenetic protein (BMP), wingless-integrated (Wnt) and fibroblast growth factor (Fgf) family members and intrinsic factors (SRY-box containing genes B1 (SoxB1) family members) decides the vertebrate neural induction. After the neuroectoderm is specified, it forms the neural plate¹¹¹. The neural plate is converted into the neural tube as a result of the process called as neurulation. In contrast with most vertebrates, zebrafish generates the neural keel. The neural plate is firstly transformed into a solid structure in zebrafish instead of folding the lateral edges of the neural plate and forming a tube with a lumen by fusing the edges in the dorsal midline. However the typical neural tube is obtained after the cells in the center detach. Despite the differences, fish and mammals produce very similar neural tubes^{111,118}.

Neurogenesis take place in two stages in zebrafish: primary neurogenesis and secondary neurogenesis. Primary neurogenesis starts at late gastrulation and proceeds during embryogenesis. This process yields early-born, big neurons which possess long axons. The brain epiphyseal and post-optic clusters, Rohon-Beard sensory neurons, Mauthner cells, and the three types of primary spinal motoneurons (the rostral (RoP), middle (MiP) and and caudal (CaP) are produced as a result of primary neurogenesis. The primary neurons form the first functional neuronal scaffold at embryonic and early larval development. Axonogenesis starts to appear between 14-24 hpf¹¹⁹. Secondary

neurogenesis, which is also called postembryonic neurogenesis starts at around 2 dpf in zebrafish. This second stage of neurogenesis turns the primary system into a network which gets more fine-tuned and complex¹²⁰. Neuronal migration, differentiation and survival are the critical steps of producing functional neurons after the cells leave mitosis and multipotent progenitors determine particular cell types¹¹⁹.

1.3.1.2. Neurogenesis in Adult Zebrafish

Although adult mammals have a restricted neurogenic capacity, teleosts including zebrafish possess a larger potential for neurogenesis. Two neurogenic areas in adult mammalian brain are the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus¹²¹. Neurogenic regions in the adult zebrafish brain are more widespread and there are regions homologous to mammalian SVZ and SGZ. The number of newly born cells in the adult zebrafish brain is approximately 6000 cells during every 30 minutes¹¹¹.

The zebrafish has become an attractive model for adult neurogenesis studies in recent years both because of the extensive capacity of neurogenesis and its advantages over mammalian models. In contrast to mammalian neural tissue, zebrafish brain does not exhibit scar formation after an injury. The zebrafish is a promising model organism to ultimately devise a therapeutic approach for humans by providing the mechanisms of the plasticity and of regeneration by suppressing the scar formation¹¹¹.

1.3.2. Gliogenesis in Zebrafish

Gliogenesis, which is defined as the generation of mature glia cell types, in zebrafish will be discussed under two sections: the first is “1.3.2.1. Gliogenesis in Zebrafish Embryos” and the second is “1.3.2.2. Gliogenesis in Adult Zebrafish”.

1.3.2.1. Gliogenesis in Zebrafish Embryos

Glial cell types in central nervous system of mammals are macroglia (astrocytes, oligodendrocytes and ependymocytes) and microglia. Schwann cells, satellite glial cells, and enteric glia constitute the glial cell types in the peripheral nervous system of mammalian organisms. The glial cell types in zebrafish have not been described profoundly yet, however there is an accumulating data about the similarities between the glial cells of fish and mammals⁸⁹.

Glial cells appear at 26+ somites stage (segmentation, 22-24 hpf) in zebrafish¹¹². Oligodendrocytes, in both mammals and zebrafish, appear to originate from the ventral regions of the neural tube. Oligodendrocyte precursor cells migrate to the appropriate regions and divide in the central nervous system. They, then leave the cell cycle and cover axons with myelin sheath^{89,115}. Schwann cells of both mammalian organisms and zebrafish generate from neural crest tissues and the developmental process they pass through is similar. During the process, they migrate to and eventually cover peripheral axons with myelin sheath⁸⁹.

Radial glia cells and a subpopulation of astrocytes serve as neural stem cells in the adult mammalian brain. Neurons, ependymal cells, astrocytes, intermediate progenitor cells that give rise to neurons (nIPCs), and intermediate progenitor cells that give rise to oligodendrocytes (oIPCs) originate from the neuroepithelial-radial glia-astrocyte line¹²².

1.3.2.2. Gliogenesis in Adult Zebrafish

As previously mentioned under “1.3.2.1. Zebrafish Embryo” section, zebrafish possesses mammalian counterparts of oligodendrocytes and Schwann cells. Expression analysis showed that satellite and enteric glia of zebrafish are also similar to their

mammalian counterparts^{123,124}. Ependymal cells have not been detected in zebrafish and low number of cells are detected with the known star shaped morphology of astrocytes. Radial glia cells appear to carry the characteristics of ependymal cells and astrocytes and to fulfill their functions. A large number of radial glia cells in zebrafish express glial fibrillary acidic protein (GFAP), glutamine synthase and aquaporin-4. GFAP and glutamine synthase are markers often used to detect astrocytes in mammalian model organisms and aquaporin-4 is a water channel found on astrocytes⁸⁹. Microglia are present in zebrafish. The origin of microglia cells is mesoderm¹²⁵ and they are mononuclear phagocytes, which function in the defense of the central nervous system^{126,127}. In both mammals and zebrafish this glial cell type takes place in the parenchyma of the central nervous system¹²⁶.

Gliogenesis continues in the adult zebrafish as neurogenesis does; radial glia cells and a subpopulation of astrocytes can re-enter the cell cycle and can give rise to macroglia cell types as well as to neurons¹²⁸. The radial glia cell population reduces in the adult mammalian brain, but still in the neurogenic areas of the brain, which are the subventricular zone (SVZ) of the lateral ventricle in telencephalon and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus, some radial glia cells serve as neural stem cells. In contrast to mammalian adult brain, zebrafish holds the large numbers of radial glia cells both in the late embryonic development and during adulthood⁸⁹.

1.4. Synaptogenesis in Zebrafish

Synaptogenesis is a multi-step process to form synapses. This process is critical in neurodevelopment and has a continuing significance in learning, memory, plasticity, cognition and adaptation throughout adulthood^{129,130}. Synapses are the contact sites of neurons where one transfers signals to another. These signals might be either electrical or chemical. Chemical synapses are more common in the vertebrate nervous system. Electrical signals, in the form of action potential, start at the axon hillock and then move

through the axon of the neuron, arrive at the presynaptic terminal and this triggers a release of neurotransmitters into the synaptic cleft. This is where electrical signal is converted into a chemical one. The neurotransmitters bind to their receptors at the membrane of the postsynaptic neuron. The signal is converted back to electrical one within the postsynaptic dendrite. Excitatory chemical synapses are asymmetric structures, and inhibitory chemical synapses are symmetric structures and occur between neurons and other neurons, muscles or glands¹³⁰.

Synaptic vesicle fusion does not take place randomly, active zones are specialized sites for synaptic vesicles to dock and fuse with the plasma membrane in the presynaptic terminal. Another specialized region in the postsynaptic membrane which is occupied with the receptors, secondary messenger molecules and voltage-gated ion channels is called postsynaptic density. The size, organization and thickness of the active zone and postsynaptic density of the synapses in the central nervous system depend on three parameters: type of the synapse, function of the synapse and efficacy of the synapse. Type of the synapse is defined by the type of the released neurotransmitter. The synapse might be classified into three groups based on their functions: excitatory, inhibitory or modulatory. Efficacy of the synapses is determined by its reliable or unreliable, continuous or sporadic activity¹³⁰.

Synaptogenesis in neurodevelopment starts in embryonic stages and continues through early postnatal stage. It is closely related with neuronal differentiation and formation of the neural circuits. The genes which encode synaptic proteins are activated a short time after the neurons differentiate and form their axons and dendrites. The axons and dendrites make some contacts and form synapses. These initial synapses are frequently transient. A set of molecules and proteins (such as receptors and cell surface adhesion molecules) and signaling process (specialization of active zone and postsynaptic density) are required for synapse formation. Eventually, the decision on keeping or eliminating a synapse is determined based on its activity both during neurodevelopment and processes going on in mature brain¹³⁰.

Synaptogenesis starts at around 18 hpf in zebrafish⁸⁴. Zebrafish embryos are very suitable for *in vivo* time-lapse imaging since they are small in size and transparent and they develop externally and quickly. The central nervous system of the embryo is simpler than higher organisms. Research on synaptogenesis in order to answer fundamental questions using zebrafish embryos have been established by employing imaging techniques (confocal and multiphoton microscopy) and the genetic techniques which utilize fluorescent labeling of neuronal proteins¹³¹. Zebrafish mutants also serve to unravel the mechanisms underlying synaptogenesis. For example embryos of a motility mutant *sofa potato* revealed that the paralysis is because of the lack of nicotinic acetylcholine receptors. The synaptic transmission is defective and postsynaptic complex formation is not complete¹³². Neuromuscular junctions are also extensively used models of synaptogenesis studies in adult zebrafish. A zebrafish mutant line, which carries a homozygous null mutation in growth differentiation factor 6a (*gdf6a*) gene, was grown to adulthood. Nine months old *gdf6a*^{-/-} adult zebrafish were found to develop impaired neuromuscular junction. Mean volume of the synapse was higher and the volume of presynaptic motoneuron was around 3 fold higher than the wild-type siblings in the mutants. These findings also serve as clues to the mechanisms of neuromuscular degenerative diseases¹³³.

1.5. Aim and Scope of This Study

Human and mouse studies indicate the potential importance of *WDR81* gene in neurodevelopment and metabolism. We conducted research on the orthologue of mammalian *WDR81* gene in zebrafish since this was a novel gene and this model organism is ideally suited to observe changes during development when the gene is knocked down. The observation of the changes during development when the protein product is depleted might indicate the causality and progression of CAMRQ. The hypothesis of this study is “*wdr81* is a critical gene in neurodevelopment as suggested for human and mouse orthologs and this can be proven by detecting the conserved domains and shared expression sites which will lead to reveal a conserved function among species”. Our first aim in this study to test this hypothesis was to characterize the *wdr81* transcript and to reveal the temporal and spatial expression of the gene of

interest. Our second aim was to introduce the initial characterization of *wdr81* morphants. The findings from our work are expected to give rise to an understanding of the potential role of *wdr81* during development.

In order to fulfill our first aim in this study, we characterized the 5' untranslated region (UTR) and 3'UTR of *wdr81* transcript, amplified the open reading frame (ORF) of the transcript, and investigated the temporal and spatial expression of *wdr81* in zebrafish. We also aligned the sequences of human, mouse and zebrafish putative WDR81 proteins. This alignment showed that identical conserved domains are predicted in all three orthologous proteins, indicating that the function of the protein of interest might be conserved in these three species as well.

In order to address our second aim in this study, we performed morpholino microinjections to zebrafish embryos. Morpholino microinjections were performed to knockdown *wdr81* and to observe the development of the zebrafish embryos in the absence of a functional protein product of *wdr81*. We measured the head size of 4.25-4.5 dpf embryos by taking body length into consideration. This measurement would indicate a result related to the global changes in the development of the brain, such as a microcephaly phenotype. Another initial characterization study was carried out using whole mount *in situ* hybridization (WMISH) method with a cerebellum marker (*gbx2*) on *wdr81* morphants to see how the expression of a cerebellum related gene is affected by knockdown of *wdr81*. The findings from our work will provide a basis to fully discover the function of *wdr81* along with the future research.

Chapter 2. Materials and Methods

2.1. Methods

2.1.1. Zebrafish and Embryos

AB strain of zebrafish (*Danio rerio*) was used as a model organism in the experiments. Embryos from pairs were collected in petri dishes and raised in E3 medium⁸⁵. The zebrafish and embryos were kept under 14 hours light and 10 hours dark cycle at 28°C in the zebrafish facility of the Department of Molecular Biology and Genetics, Bilkent University, Ankara, Turkey. The Bilkent University Local Animal Ethics Committee (HADYEK) approved the protocol for experiments in this study in which zebrafish samples are used. The approval date was 10 October 2010 and the protocol number was 2010/31.

2.1.2. Bioinformatics Analysis

The homologous protein sequences of WDR81 from human, mouse and zebrafish were aligned and compared in order to detect the presence and location of the conserved domains. The aminoacid sequences of human WDR81 (encoded by the transcript ENST00000409644), mouse Wdr81 (encoded by the transcript ENSMUST00000173320) and zebrafish wdr81 (encoded by the transcript ENSDART00000156621) were derived from Ensembl database²⁴ and aligned with Clustal Omega¹³⁴. The file obtained as a result of the alignment was submitted to ESPript 3.0 software¹³⁵. This software highlights the similar sequences at specific locations. The locations of BEACH and WD40 domains, which are predicted by SMART database¹³⁶ and MFS domain, which is predicted by CLC software (CLC Main Workbench software package, CLC Bio Inc) were detected on the ESPript 3 output file.

TMpred software was used to calculate the membrane spanning regions of the zebrafish *wdr81*¹³⁷. The splice variants of *WDR81* of human (ENSG00000167716), mouse (ENSMUSG00000045374) and zebrafish (ENSDARG00000079702) genes were derived from Ensembl²⁴. The *WDR81* protein sequences of the species were aligned by using Clustal Omega¹³⁴. The accession numbers for *WDR81* orthologs were XP_002918082 (*Ailuropoda melanoleuca*), JAB47838 (*Callithrix jacchus*), XP_002664681 (*Danio rerio*), XP_001502383 (*Equus caballus*), XP_415806 (*Gallus gallus*), XP_007485816 (*Monodelphis domestica*), NP_620400 (*Mus musculus*), XP_002718930 (*Oryctolagus cuniculus*), XP_003952981 (*Pan troglodytes*), XP_002826860 (*Pongo abelii*), NP_001157281.1 (*Homo sapiens*), NP_001127832 (*Rattus norvegicus*), JAA74278 (*Sus scrofa*), XP_012424209 (*Taeniopygia guttata*), CAG08933 (*Tetraodon nigroviridis*), XP_002937192 (*Xenopus tropicalis*). A similar list of orthologous proteins were also used by Gulsuner *et al.* (2011) in order to prepare the phylogenetic tree of protein sequences of *WDR81* in vertebrates¹² (Figure 3). Zebrafish *wdr81* (encoded by the transcript ENSDART00000156621) amino acid sequence was submitted to the tblastn database of NCBI¹³⁸ in order to detect the rate of similarities between *wdr81* and other genes encoding WD repeat containing proteins in zebrafish genome.

2.1.3. Characterization of the *wdr81* Transcript

The aim was the characterization of zebrafish *wdr81* transcript in order to reveal whether there are more than one transcripts as in human and mouse. In order to fulfill this aim, the open reading frame and cDNA ends of the transcript of interest were described. The transcript prediction released by Ensembl (ENSDART00000156621) was used as a model for designing primers in these experiments since *wdr81* was not characterized previously.

2.1.3.1. Amplification of the Open Reading Frame of *wdr81*

Thirty 24 hours post fertilization (hpf) zebrafish embryos and pooled brains of five 10 month old male zebrafish were collected. Total RNA of embryos and brains were isolated with a Trizol reagent (15596018, Ambion, USA) and genomic DNA was removed using DNase (AM1907, Turbo DNA free, Ambion, USA). Five hundred nanogram (ng) of total RNA was used to synthesize cDNA (04379012001, Transcriptor First Strand cDNA Synthesis Kit, Roche, USA). Ten primer pairs, which produce overlapping amplicons and cover the open reading frame (ORF) of *wdr81* were designed (Table 3).

Table 3. Primer pairs designed to characterize the open reading frame of *wdr81*
(Reprinted from Doldur-Balli et al., 2015¹³⁹)

		Primer Sequence	Annealing Temperature	Expected Amplicon Size (bp)
Pair 1	F	5'-GCAAACAGTGCAGAGCTTCTT-3'	60 °C	897
	R	5'-GCTGCTCATCAACTGCAATATC-3'		
Pair 2	F	5'-CCTATCCACCTGCTCAGCTC-3'	60 °C	874
	R	5'-AACATGGCTGCATAGCACAG-3'		
Pair 3	F	5'-CCAGATCTTGGTGGACCAGT-3'	60 °C	909
	R	5'-TCTGTGAAGCATGGCAGTTC-3'		
Pair 4	F	5'-TTGGTTTGGTTGTGTCTCCA-3'	60 °C	875
	R	5'-ATACCAGGCCGCATAAACAG-3'		
Pair 5	F	5'-CTCCATCATGCACTGGACAC-3'	60 °C	920
	R	5'-CCAACTGTTGTTGCTCCAGA-3'		
Pair 6	F	5'-GCCACATCTTCTGGCAAAGT-3'	55 °C	979
	R	5'-TGAGTACCACAGCACCCAAA -3'		
Pair 7	F	5'-GAGACCAGACTGCAAGACCAG-3'	55 °C	698
	R	5'-ACTTGCTCGTTCGTGGTAAGA-3'		
Pair 8	F	5'-GCAGAGTGCACATACCTGGA-3'	60 °C	704
	R	5'-TGGAAGTGGAAGTGGGAGTC-3'		
Pair 9	F	5'-AACAGGACCTTCCACGTAGC-3'	60 °C	896
	R	5'-TGTGCTGTCCAGAATGGAGT-3'		
Pair 10	F	5'-CACAAGCCACTCCACCAGTA-3'	60 °C	429
	R	5'-CGCGGAGGTTGTAAGTTCTC-3'		

Negative control templates, which do not include reverse transcriptase enzyme in the cDNA synthesis reaction (-RT), were added to the experiment in order to eliminate the possibility of genomic DNA contamination. The amplicons obtained as a result of amplification of ORF were loaded to a 1% agarose gel.

2.1.3.2. Rapid Amplification of cDNA Ends (RACE)

The rapid amplification of cDNA ends (RACE) method was utilized in order to characterize 5' UTR (untranslated region) and 3'UTR of zebrafish *wdr81*. The samples were DNase treated (RNase free DNase set, 79254, Qiagen, Germany) total RNA (RNeasy Mini Kit, 74104, Qiagen, Germany) from forty 24 hpf embryos and pooled brains of three 10 month old male zebrafish.

2.1.3.2.1. Characterization of the 5'RACE Product

The GeneRacer™ kit (L1500-01, RLM-RACE, Invitrogen, USA) was used to define the 5' end of *wdr81*. The experiments were performed by following the manual from the kit. Twenty-four hpf embryo and adult brain total RNA samples were treated with a few enzymatic steps before being converted into RACE-ready cDNAs. The enzymatic steps are composed of treatment with calf intestinal phosphatase (CIP) and tobacco acid pyrophosphatase (TAP) and ligation with an RNA oligonucleotide. Briefly, CIP enzyme clears away the RNA structures, which are not capped, by removing the 5' phosphates and then TAP renders full length mRNA with a 5' phosphate group by removing the 5'cap structure. Then, RNA oligonucleotide (GeneRacer™ RNA oligo), whose sequence is known, is ligated to the full-length mRNA with the 5' phosphate group. RNA oligonucleotide is ligated to serve as a binding site for the GeneRacer™ 5' primer and GeneRacer™ 5' nested primer. The mRNA samples, which have the GeneRacer™ RNA Oligo in the 5' upstream region, were converted to RACE-ready cDNAs with Cloned AMV reverse transcriptase and the GeneRacer™ oligo dT primer. A touch

down PCR was performed to amplify the RACE-ready cDNA with a primer pair composed of GeneRacer™ 5' Primer (5'-CGACTGGAGCACGAGGACACTGA-3') and a gene specific primer (wdr81_Racer-5E2, 5'-ACAGTTTCTGCAGGGCTTGACGAAC-3'). The reaction conditions of touch down PCR were: 30 sec. at 94 °C, 30 sec. at 94 °C, 40 sec. at 72 °C, 45 sec. at 68 °C for 5 cycles; 30 sec. at 94 °C, 40 sec. at 71 °C, 45 sec. at 68 °C for 5 cycles; 30 sec. at 94 °C, 40 sec. at 70 °C, 45 sec. at 68 °C for 25 cycles; and 5 min. at 68 °C. The embryo and brain amplicons obtained from the touch down PCR were diluted at a 1:10000 ratio and used as templates for a nested PCR. Nested PCR following touch down PCR was performed to produce a specific *wdr81* 5'RACE product (Figure 14). A nested PCR using a primer pair composed of GeneRacer™ 5' nested primer (5'-GGACACTGACATGGACTGAAGGAGTA-3') and a gene specific nested primer (wdr81_Racer-5E2_Nested, 5'-CTGCATATGGCTGCACATGAGTC-3') was performed under the following reaction conditions: 30 sec. at 94 °C; 30 sec. at 94 °C, 40 sec. at 72 °C, 50 sec. at 68 °C for 30 cycles; and 5 min. at 68 °C. PCR products were loaded to a 1% agarose gel, run and separated using electrophoresis. One specific 5'RACE product of *wdr81* from embryo and brain samples were excised from the gel and purified (K220001, Pure-Link Quick Gel Extraction and PCR Purification Combo Kit, Invitrogen, USA). The purified DNA fragments from embryo and brain samples were cloned (45-0071, Topo TA Cloning Kit for Sequencing, Invitrogen, USA) and single colonies, that grew under both ampicillin and kanamycin treated conditions, were picked up. Plasmids isolated from the selected colonies (K2100-11, Purelink Quick Plasmid Miniprep Kit, Invitrogen, Germany) were analyzed with restriction endonuclease digestion (EcoRI, ER0271, Fermentas) and the insert containing plasmids were sequenced. The Sanger sequencing results were examined with CLCBio Main Workbench software package (CLCBio Inc).

2.1.3.2.2. Characterization of the 3'RACE Product

The GeneRacer™ kit (L1500-01, RLM-RACE, Invitrogen, USA) was used to define the 3' end of *wdr81*. The experiments were performed according to the manual from the kit. The total RNA isolated from 24 hpf embryo and adult brain were converted to RACE-ready cDNA with cloned AMV reverse transcriptase and the GeneRacer™ Oligo dT primer. While synthesizing cDNA, the GeneRacer™ Oligo dT primer introduces a sequence to the 3' downstream of the full length mRNA, which serves a binding site for GeneRacer™ 3' Primer. The RACE-ready cDNA samples from embryo and adult brain were amplified with 3 primer pairs, which were designed to obtain 3 overlapping amplicons to ultimately obtain 3' RACE product of *wdr81* (Table 4, Figure 18). Negative control reactions, without a template, were included to the experiments as well. The steps of the reaction 1 with the primer pair 1 (Table 4) were: 40 sec. at 98 °C; 10 sec. at 98 °C, 30 sec. at 68 °C, 35 sec. at 72 °C for 30 cycles; and 5 min. at 72 °C. The steps of the reaction 2 with primer pair 2 (Table 4) were: 40 sec. at 98 °C; 10 sec. at 98 °C, 30 sec. at 64 °C, 40 sec. at 72 °C for 30 cycles; and 5 min. at 72 °C. The steps of the reaction 3 with primer pair 3 (Table 4) were: 2 min. at 95 °C; 30 sec. at 95 °C, 30 sec. at 61 °C, 60 sec. at 72 °C for 30 cycles; and 7 min. at 72 °C. The PCR products of these three experiments were run on a 1% agarose gel and a single band per reaction was observed, then the PCR products were sent to Sanger sequencing. The sequencing results were examined with CLCBio Main Workbench software package (CLCBio Inc).

Although the reactions 1 and 3 gave PCR products as predicted, the PCR product of the reaction 2 was observed to be larger than the predicted size. This result indicated presence of an insertion site within the PCR product obtained with primer pair 2 (Table 4). A new primer pair was designed to characterize the insertion site by amplifying it within a narrower frame. RACE-ready cDNA from the same source as used in reactions 1-3 was used as a template in a PCR experiment with this new primer pair, which was composed of a forward (5'-CATTATTATCTCCAGACATTCCAA-3') and a reverse primer (5'-TGAGGGAATTAGCGAACCAT-3'). The reaction conditions of this amplification were: 40 sec. at 98 °C; 10 sec. at 98 °C, 30 sec. at 58 °C, 30 sec. at 72 °C

for 30 cycles; and 5 min. at 72 °C. This experiment was designed to observe a 250 base pair (bp) long PCR product if the insertion site is not present in the sample and if at the same time the predicted sequences flanking the insertion site are present.

Table 4. Primer sets designed to obtain three overlapping amplicons in order to characterize 3'UTR of *wdr81* (Reprinted from Doldur-Balli et al., 2015¹³⁹)

	Primer	Primer Sequence	Expected amplicon size, bp
Pair 1	F (Pair1F_Drwdr81_3 RACE)	5'-CTGACAACGGTGCCATCAGG-3'	717
	R (Pair1R_Drwdr81_3 RACE)	5'-TTCAGGACCATCCCATTGCATA-3'	
Pair 2	F (Pair2F_Drwdr81_3 RACE)	5'-CTGTATCCACGTCAATGGAGCGTAA-3'	757
	R (Pair2R_Drwdr81_3 RACE)	5'-GAAGCATTTGTTCAATGTACGTTCCGGTA-3'	
Pair 3	F (Pair3F_Drwdr81_3 RACE)	5'-CATTTATGGTTCGCTAATTCCCTCAA-3'	634
	R (GeneRacer™ 3' Primer)	5'-GCTGTCAACGATACGCTACGTAACG-3'	

Firstly, embryo and brain samples were amplified with this primer pair and the amplicons were cloned (A1360, pGEM-T Easy Vector System I, Promega, USA). Single white colonies generated based on blue/white screening were picked up. The plasmids from the selected colonies were isolated (K2100-11, Purelink Quick Plasmid Miniprep Kit, Invitrogen, Germany) and 3 plasmids per sample type (ie. 24 hpf embryo and adult brain) were sent to Sanger sequencing. The results of the sequencing were examined with CLCBio Main Workbench software package (CLCBio Inc). Then the

presence of the insertion site in samples from different developmental timepoints and different adult tissues was planned to test. In order to fulfill this purpose, cDNAs of 10 adult tissues and of samples from 10 developmental timepoints were amplified with the same primer pair. The cDNA samples from the same sources were amplified with a house keeping gene primer pair (a beta-actin gene primer pair-forward, 5'-ATTGCTGACAGGATGCAGAAG-3' and reverse, 5'-GATGGTCCAGACTCATCGTACTC-3')¹⁴⁰ to observe whether the samples were present and intact. The PCR products of this experiment were run on a 1% agarose gel. The band intensities of the amplicons were measured by using Image J program¹⁴¹.

2.1.4. Analysis of the Expression of *wdr81* in Wild Type Zebrafish

The expression of *wdr81* in wild type adult zebrafish and during developmental stages were studied with quantitative real-time pcr (qRT-PCR) and *in situ* hybridization methods.

2.1.4.1. Quantitative Real-Time PCR (qRT-PCR)

The temporal expression of *wdr81* was studied with the samples from 10 developmental timepoints. Thirty embryos from 1 hpf, 5 hpf, 10 hpf, 18 hpf, 24 hpf, 48 hpf, 72 hpf and 5 dpf, eight larvae from 15 dpf and eight juvenile fish from 35 dpf stages were collected. Spatial expression of *wdr81* was studied with 10 adult tissues; brain, testis, heart, kidney, liver, intestine, eye, gills, tail and muscle. Each tissue was pooled from five 10 month old male zebrafish. A Trizol reagent (15596018, Ambion, USA) and a homogenizer (Bullet Blender, Next Advance, Storm 24) were utilized to isolate total RNA from all samples. The isolated total RNA samples were treated with DNase (AM1907, Turbo DNA free, Ambion, USA) and 500 nanogram (ng) of RNA, after removal of genomic DNA, were used in cDNA synthesis reaction (05081955001,

Transcriptor High Fidelity cDNA Synthesis Kit, Roche, Germany). The Roche Light-Cycler 480 System was employed to perform the qRT-PCR experiments.

The cDNA samples were used at 1:4 dilution ratio and 5 microliters (µl) of diluted cDNA was used as a template in each reaction. The primers and probes designed to detect *wdr81* expression were selected from Universal Probe Library Assay Design Center, which provides zebrafish specific assays (Table 5, Figure 7). A primer pair and a probe to detect β-actin expression were added as well (Table 5). The experiments were planned to include 400 nanomolar (nM) of forward and reverse primers and 200 nM of probes in a 20 µl reaction volume. The experiments were carried out in duplicates on each plate and were repeated 3 times and included negative control samples, -RT and without a template. The reaction steps were composed of 10 min. at 95 °C; 10 sec. at 95 °C, 30 sec. at 60 °C, 1 sec. at 72 °C for 45 cycles; and 30 sec. at 40 °C. The Ct values were derived from LCS480 software (Roche, Germany) and the $2^{-\Delta\Delta C_t}$ method was applied to calculate the fold changes for comparison. The ΔC_t value of each reaction was obtained by subtracting the Ct value of β-actin reaction from the Ct value of *wdr81* reaction. Fold changes in expression were calculated according to the ΔC_t value of 15 dpf larva in order to obtain relative temporal expression profile and of brain in order to obtain relative spatial expression profile. $2^{-\Delta\Delta C_t}$ formula was applied on Excel program (Microsoft Office Excel 2007) and standard error of the mean (SE) was calculated using Graphpad¹⁴². The graphics of the relative temporal and spatial expression including +standard error were prepared with GraphPad¹⁴².

Table 5. Primer pairs and probes to be used in qRT-PCR (Reprinted from Doldur-Balli et al., 2015¹³⁹).

Gene	Primers	Sequence	Probes
<i>wdr81</i>	F	5'-TCTCATGCAGGGAGTATCACA-3'	Probe 46 (cat. no.04688066001, Roche)
	R	5'-AGGTGTCTGCTCAACGGAAT-3'	
β-Actin	F	5'-GCCTGACGGACAGGTCAT-3'	Probe 104 (cat. no.04692225001, Roche)
	R	5'-ACCGCAAGATTCCATACCC-3'	

2.1.4.2. *In situ* Hybridization Experiments

The expression of *wdr81* both on whole mount embryos from several developmental stages and on adult tissues, brain and eye, was examined using a gene specific probe.

2.1.4.2.1. Whole Mount *In Situ* Hybridization (WMISH) on Embryos

The spatio-temporal expression of *wdr81* on whole mount embryos from 6 hpf, 10 hpf, 18 hpf, 24 hpf, 48 hpf and 72 hpf timepoints was investigated with an RNA probe. The probe was prepared by amplifying the region of *wdr81* transcript between primer pair 5 (5F) and primer pair 6 (6R) from Table 3. This region is corresponding to the site of mutation in patients. The template for probe preparation was 24 hpf embryo cDNA and the reaction steps for amplification were: 2 min. at 95 °C, 30 sec. at 95 °C, 30 sec. at 62 °C, 2 min. at 72 °C for 35 cycles; and 7 min. at 72 °C. The amplicon was observed in a 0.8% agarose gel, the single band was excised from the gel and the DNA fragment was purified (D4007, Zymoclean Gel DNA Recovery Kit, USA). The fragment was cloned into a pGEM-T Easy vector (A1360, pGEM-T Easy Vector System I, Promega, USA) and single white colonies which grew based on blue/white screening were picked up. The plasmids were analyzed with restriction endonuclease digestion (NspI, ER1472, Fermentas) to observe the presence and orientation of the insert. The selected plasmid was linearized with NdeI (ER0582, Fermentas) and SalI (ER0645, Thermo Scientific) double digestion in order to use it as a template in antisense probe synthesis. The linearized plasmid was excised from a 0.8% agarose gel and was purified (D4007, Zymoclean Gel DNA Recovery Kit, USA) and the probe synthesis reaction was set up with T7 enzyme mix (AM1320, MaxiScript SP6/T7 *In Vitro* Transcription Kit, Ambion, USA) and DIG labeling mix (11277073910, DIG RNA Labelling Mix, Roche, Germany).

The embryos from the timepoints mentioned above were collected, fixed in 4% paraformaldehyde (FB001, Invitrogen IC Fixation Buffer, Invitrogen, USA) and saved in 100% methanol after dehydration steps with increasing percentages of methanol. Embryos can stay in 100% methanol at -20°C until they are used in the experiment. The rehydration, bleaching, proteinase K digestion, post fixation, hybridization, probe wash, antibody incubation (anti-digoxigenin-AP, Fab fragments, 11093274910, Roche, Germany) and staining steps (BM purple, 11442074001, Roche, US) were performed by following the previously described protocol¹⁴³ with some modifications. The images of embryos from 6 hpf, 10 hpf, 18 hpf, 24 hpf, 48 hpf and 72 hpf stages were obtained with Zeiss Stereomicroscope Discovery V220 (Carls Zeiss, Germany) at 72x, 130x, 105x, 61x, 50x and 46x magnifications, respectively. The images of head regions of embryos from 18 hpf, 48 hpf and 72 hpf stages were obtained at 150x, 118x and 100x magnifications, respectively. Transverse sections at 20 micrometer (µm) thickness were collected from the head regions of 18 hpf, 48 hpf and 72 hpf embryos, which were WMISH specimens, with a cryostat (CM 1850, Leica) and the images of the sections were obtained with a brightfield upright microscope (Fluorescent and DIC equipped upright microscope, Zeiss, Germany).

2.1.4.2.2. *In Situ* Hybridization (ISH) on Brain and Eye Tissues

The expression of *wdr81* was investigated on coronal sections at 10 µm thickness from brain and eye tissues. A cryostat (CM 1850, Leica, Germany) was used to obtain sections from the tissues of a wild type 10 month old adult male. The antisense probe was synthesized as explained in the “2.4.2.1. Whole Mount *In Situ* Hybridization (WMISH) on Embryos” section and the same plasmid construct was used in preparation of sense probe. The plasmid was linearized with NcoI (FD0573, Thermo Fisher Scientific) and ApaI (ER1411, Thermo Fisher Scientific) double digestion in order to use it as a template in sense probe synthesis. The linearized plasmid was excised from a 0.8% agarose gel and was purified (D4007, Zymoclean Gel DNA Recovery Kit, USA). The probe synthesis reaction was set up with SP6 enzyme mix (AM1320, MaxiScript

SP6/T7 *In Vitro* Transcription Kit, Ambion, USA) and DIG labeling mix (11277073910, DIG RNA Labelling Mix, Roche, Germany).

The experiment was performed as previously described¹². The sections were fixed in 4% paraformaldehyde (FB001, Invitrogen IC Fixation Buffer, Invitrogen, USA), acetylated, and incubated with the probes at 60 °C overnight. Following these steps, the probe was washed, the tissue sections were incubated with antibody (anti-digoxigenin-AP, Fab fragments, 11093274910, Roche, Germany) and stained with the solution including 4-Nitro blue tetrazolium chloride solution (NBT) (11383213001, Roche, Germany), 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (BCIP) (11383221001, Roche, Germany) and levamisole (SP-5000-18, Vector Lab). The images of the sections were taken with a brightfield upright microscope (Fluorescent and DIC equipped upright microscope, Zeiss, Germany).

2.1.5. Morpholino Microinjections to Knockdown *wdr81*

Morpholino antisense oligos contain morpholine rings instead of deoxy ribose or ribose rings in DNA and RNA, respectively and contain adenine, cytosine, guanine and thymine bases. They contain uncharged phosphorodiamidate intersubunit bonds instead of negatively charged phosphodiester bonds, which take place in the nucleic acids. The length of a morpholino sequence is generally 25 oligos. The morpholino oligos are used to knockdown a gene of interest and they are introduced to the yolk region of zebrafish embryos via microinjection. They might be designed to target the translation initiation site, pre-mRNA splicing or maturation of microRNAs¹⁴⁴. Since targeting translation initiation site of a maternally supplied transcript might end up with a more severe phenotype and because of lack of an efficient antibody to detect the effect of a translation initiation site targeting morpholino on protein level, a splice blocking morpholino was ordered to knock down *wdr81* in zebrafish. Its sequence was 5'-CACTTGTTCAAACCTTACCTAATAGT-3' which targets the exon intron junction between exon 2 and intron 2 (Figure 28). Whether *wdr81* is maternally supplied or not

was detected by amplifying cDNA samples from 1 hpf, 2 hpf, 3 hpf, 6 hpf, 12 hpf, 24 hpf and 48 hpf timepoints and their –RT negative control samples with a primer pair. This primer pair was composed of a forward primer with 5'-ACAAGCAGCAAACAGTGCAG-3' sequences (wdr81_5UTR_B) and a reverse primer with 5'-TGTCTGCTCAACGGAATCTG-3' sequences (wdr81_5UTR_C). The reaction steps of amplification were: 30 sec. at 94 °C; 30 sec. at 94 °C, 40 sec. at 58 °C, 35 sec. at 68 °C for 35 cycles; and 5 min. at 68 °C. The amplicons were run on a 1% agarose gel.

2.1.5.1. *wdr81* Morpholino Dose Curve

Three doses of the morpholino antisense oligonucleotide sequence, which was designed to target splicing of *wdr81* transcript, were tested. Two nanograms (ng), 4 ng and 8 ng doses of *wdr81* morpholino were evaluated. Microinjection of the same doses of standard negative control morpholino were also performed in order to observe the specific effect of *wdr81* morpholino on splicing. The 24 hpf morpholino injected and uninjected control group embryos were collected after recording their survival rates. Microinjection of *wdr81* morpholino and negative control morpholino to zebrafish embryos, which were collected from the same clutch, was applied when they were at 1-4 cell stage. Total RNAs from the three experiment groups of each dosage were isolated with a Trizol reagent (AM9738, Ambion, USA) and cDNAs were synthesized (04 379 012 001, Transcriptor First Strand cDNA Synthesis Kit, Roche, Germany) using 500 ng of DNase treated (AM1907, Turbo DNA free, Ambion, USA) total RNA. The cDNA samples of embryos from three experiment groups of three doses were amplified with the a primer pair whose sequences were 5'-CAGAACCAGACAGCAAA-3' for forward primer (Mo_wdr81_E2F) and 5'-CCAAGTTTTGCAGACAACCA-3' for reverse primers (Mo_wdr81_E3R) (Figure 29). The reaction conditions were: 2 min. at 95 °C, 30 sec. at 95 °C, 30 sec. at 61 °C, 50 sec. at 72 °C for 35 cycles; and 5 min. at 72 °C. The amplicons were run on a 1% agarose gel and on a 4% agarose gel.

2.1.5.2. Initial Characterization Studies

We set up two experiments in order to detect the effect of *wdr81* morpholino microinjection on zebrafish embryos. We searched for a possible microcephaly phenotype and tested the morphants with gastrulation brain homeobox 2 (*gbx2*) probe hybridization using WMISH method.

2.1.5.2.1. Head Size Measurement

Since *wdr81* is suggested to be a neurodevelopmentally important gene and the gene knockdown is expected to affect the central nervous system, in a parallel study while establishing a morpholino dose curve, head size measurements were carried out on morphant embryos¹⁴⁵. Golzio *et al.*, (2012) performed a measurement of the distance between the convex edges of the cornea of two eyes and of somitic length on the experiment groups in order to find out changes in head size, which are not caused by developmental delay. In our study, 8 ng. *wdr81* morpholino and 8 ng. standard negative control morpholino were injected to embryos and an uninjected control group was separated. The experiment groups were composed of equal number of embryos from the same fish pair. The injections were applied to the yolk regions of embryos between 1-4 cell stages. The embryos, which comprise experiment groups were anesthetized at the timepoint between 4.25-4.5 dpf. The images of the embryos were taken with the same parameters every time by using a Leica MZ10F microscope. The distance between the convex edges of the cornea of two eyes and the body length of the experiment groups were measured with software (Leica Application Suite 4.3, LASv 4.3) by a researcher who was blind to the experimental groups. The body lengths of the embryos were measured from the tip of the mouth to the tail, where pigments end (Figure 31). Significant differences in the measurements between groups was tested by applying one-way ANOVA and Tukey's posthoc test. One-way ANOVA and the following Tukey's posthoc test were performed by using SPSS program (IBM, Turkey). Standard

error of the mean was also calculated by the same program. The survival rates of the embryos in the experiment were recorded.

2.1.5.2.2. Phenotype Characterization With WMISH Method

Another phenotypic characterization study following morpholino antisense knockdown was to compare the expression of *gbx2* in the three experimental groups. Expression of *gbx2* is established in the literature as being crucial for development of both midbrain and anterior hindbrain¹⁴⁶. Two ng. morpholino microinjection was performed in the one-cell stage embryos. An uninjected control group among the siblings was separated as well. Two dpf *wdr81* morpholino injected embryos, 2 ng. standard negative control morpholino injected embryos and uninjected control group embryos were fixed in 4% paraformaldehyde (FB001, Invitrogen IC Fixation Buffer, Invitrogen, USA) and saved in 100% methanol after dehydration steps. The experiment was performed as explained in the “2.4.2.1. Whole Mount *In Situ* Hybridization (WMISH) on Embryos” section. The plasmid construct carrying the *gbx2* insert was a kind gift from Assoc. Prof. Gunes Ozhan. The plasmid was linearized with BamHI (FD0055, ThermoFisher Scientific) digestion in order to use it as a template in antisense probe synthesis. The linearized plasmid was excised from a 0.8 % agarose gel and purified (D4007, Zymoclean Gel DNA Recovery Kit, USA). The probe synthesis reaction was set up with T7 enzyme mix (AM1320, MaxiScript SP6/T7 *In Vitro* Transcription Kit, Ambion, USA) and DIG labeling mix (11277073910, DIG RNA Labelling Mix, Roche, Germany).

2.2. Materials

2.2.1. General Chemicals, Reagents and Enzymes

Chemicals in general use were agar (214010, Bacto Agar), agarose biomax (144503PR, Prona), agarose reducta (085501PR, Prona), ampicillin sodium salt (A0839, Biochemica), beta mercaptoethanol (M3148, Sigma-Aldrich), bovine serum albumin fraction V (10 735 094 001, Roche), 5 bromo 4 chloro 3 indolyl beta-D galactoside (X-gal, A4978, Applichem), calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 327 607, Carlo Erba), calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, A361521 215, Merck), Chaps (C9426, Sigma-Aldrich), citric acid anhydrous (C0759, Sigma-Aldrich), diethyl pyrocarbonate (DEPC, D5758, Sigma), D-sucrose (A1125, Applichem), ethanol (32221, Sigma-Aldrich), ethidium bromide (E-7637, Sigma, MO), ethyl 3-aminobenzoate methanesulfonate (E10521, Sigma-Aldrich), ethylenediaminetetraacetic acid (EDTA, A3562, Applichem), ficoll type 400 (F4375, Sigma-Aldrich), formamide (F7503, Sigma-Aldrich), glacial acetic acid (272225, Sigma-Aldrich), glycerol (GL00262500, Scharlau), heparin sodium salt from porcine intestinal mucosa (H3393, Sigma-Aldrich), Hepes free acid (0511, Amresco), hydrochloric acid (HCl, 07102, Riedel de Haen), hydrogen peroxide (H_2O_2 , 18312, Sigma-Aldrich), isopropanol (34137, Sigma-Aldrich), isopropyl-beta-D-thiogalactopyranoside (IPTG, A4773, Applichem), kanamycin sulfate (A1493, Applichem), magnesium chloride dihydrate ($\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$, A146417 052, Applichem), magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 105833.1000 Merck), magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, M2773, Sigma), manganese (II) chloride dihydrate ($\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 105917.1000, Merck), methanol (32213, Sigma-Aldrich), methylene blue (M9140, Sigma-Aldrich), mineral oil (M8410, Sigma-Aldrich), nuclease free water (AM9937, Ambion), OCT (4583, Tissue-Tek), phenol red (P0290, Sigma), pipes (A1079.0100, Applichem), polyvinylpyrrolidone (PVP, P5288, Sigma-Aldrich), potassium chloride (KCl, 12636, Sigma-Aldrich), potassium hydroxide (KOH, 105012.1000, Merck), potassium phosphate monobasic (KH_2PO_4 , 04243, Sigma-Aldrich), pronase E (protease from *Streptomyces griseus* type XIV, P5147, Sigma), proteinase K (P2308, Sigma-Aldrich), sodium chloride (NaCl, 13423, Sigma-Aldrich), sodium citrate tribasic ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 5,5\text{H}_2\text{O}$, 6858-44-2, Carlo Erba), sodium

hydroxide (NaOH, 06203, Riedel de Haen), sodium phosphate dibasic dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 04272, Sigma-Aldrich), triethanolamine HCl (TEA-HCl, T1502, Sigma), tris HCl (T3253, Sigma), trizma base (T1503, Sigma Aldrich), tryptone (1612.00, Conda Pronadisa), tween-20 (0777, Amnesco) and yeast extract (1702.00, Conda Pronadisa).

Reagents used in the experiments were bakers yeast RNA (R6750, Sigma), BAN phase separation reagent (BN191, Molecular Research Center), blocking reagent (11 096 176 001, Roche), DNA loading dye (R0611, ThermoFisher Scientific), deoxynucleotide (dNTP) mix (R1121, Fermentas), fetal bovine serum (10270, Gibco), light cycler 480 probes master (04707494001, Roche), lithium chloride solution (L7026, Sigma), magnesium chloride for PCR (25 mM MgCl_2 , 00051040, Fermentas), mounting solution (Clear mount, 008010, Life Tech), salmon sperm DNA (AM9680, Thermo Scientific) and torula RNA (R6625, Sigma).

Enzymes routinely used in the experiments were Fast Start High Fidelity kit (03553400001, Roche), One Taq Hot Start (M0484S, New England Biolabs), Phusion HS Flex (M0536, New England Biolabs) and Taq polymerase (M0267S, New England Biolabs).

2.2.2. General Materials and Equipments

Materials used in the experiments were capillaries to prepare microinjection needles (BF100-50-10, Sutter Instrument Company), microloader (5242956003, Eppendorf), mold for microinjection (TU-1, Adaptive Science Tools), stage micrometer (35037, Olympus) and superfrost plus slides (631-0108, VWR).

Equipments routinely used in the experiments were centrifuges (Biofuge Pico, Heraeus Instrument and GS15R Beckman), gel documentation equipment (Vilber Lourmat), gel

running tank (Midicell EC350), heat block (SHT1, Stuart Scientific Test Tube Heater), hybridization incubator (combi-H12, FinePCR), incubator (WTB binder), microinjection system (Femtojet, Eppendorf), NanoDrop 1000, PCR Thermal cycler (TC 512, Techne and 2720 Thermal Cycler, Applied Biosystems), pipette puller (P30, Sutter Instrument Company), power supply (Pac 200 Biorad), vortex (Genie Scientific Industries) and water bath (Nüve bath).

2.2.3. Buffers and Solutions

Ingredients of the buffers and solutions used in this study are mentioned in Appendix A.

2.2.4. Molecular Size Markers and Plasmid Vectors

Molecular size markers (pUC mix 8, SM0303, Fermentas and MassRuler DNA Ladder, SM0403, Thermo Scientific) used in this study are given in Appendix B. The maps of the plasmid vectors (pCR4-Topo, Invitrogen and pGEM-T Easy, Promega) are given in Appendix C.

Chapter 3. Results

3.1. Zebrafish Orthologue of WDR81

WD repeat containing protein 81 was shown to be highly conserved among vertebrates (Figure 3 and Appendix D)¹². Based on the report of Ensembl database, the highest identity of zebrafish putative wdr81 was detected with its orthologues from orangutan (*Pongo abelii*) among primates (61%), Guinea pig (*Cavia porcellus*) and kangaroo rat (*Dipodomys ordii*) among rodents (58%), megabat (*Pteropus vampyrus*) among Laurasiatheria (59%), Chinese softshell turtle (*Pelodiscus sinensis*) among Sauropsida (71%) and cod (*Gadus morhua*) and stickleback (*Gasterosteus aculeatus*) among fish (79%)²⁴. Clustal Omega alignment was utilized to detect the degree of identity of WDR81 sequences among three species. The results of alignment showed that human WDR81 and mouse Wdr81 carry 56.94% and 56.68% identity with the putative wdr81 protein in zebrafish, respectively¹³⁴. The mutation site in human patients, proline at residue 856, was also shown to be conserved among various species of vertebrates (Appendix D). *wdr81*, which is one copy and not duplicated, is mapped to chromosome 15 in zebrafish genome. The transcript of zebrafish *wdr81* gene (wdr81-001, ENSDART00000156621) is made up of 12 exons containing open reading frame (ORF) and putative UTRs (Figure 7). The length of this novel transcript is predicted as 8249 base pairs (bp) and it encodes a predicted protein composed of 2065 amino acid residues²⁴.

The conserved domains of the zebrafish putative wdr81 protein were predicted as BEACH, MFS and WD40 repeat domains by the SMART database¹³⁶ and CLC Main Workbench software. The BEACH domain was detected to reside between the 337th–598th residues, the MFS domain was predicted locate between the 951st–1513th residues and seven WD40 repeats were concluded to take place between 1758th–1797th, 1807th–1844th, 1850th–1889th, 1892nd–1936th, 1939th–1977th, 1980th–2017th and 2027th–2065th residues of zebrafish wdr81. The number of transmembrane regions of the protein were

predicted to be six¹³⁷. These regions were predicted to reside between 665th–686th, 1031st–1055th, 1413th–1435th, 1463rd–1483rd, 1690th–1712th and 1920th–1943rd (Figure 8 and 9). The same domains and the presence of membrane spanning regions were also predicted in human WDR81 and mouse Wdr81 proteins^{12,36}.

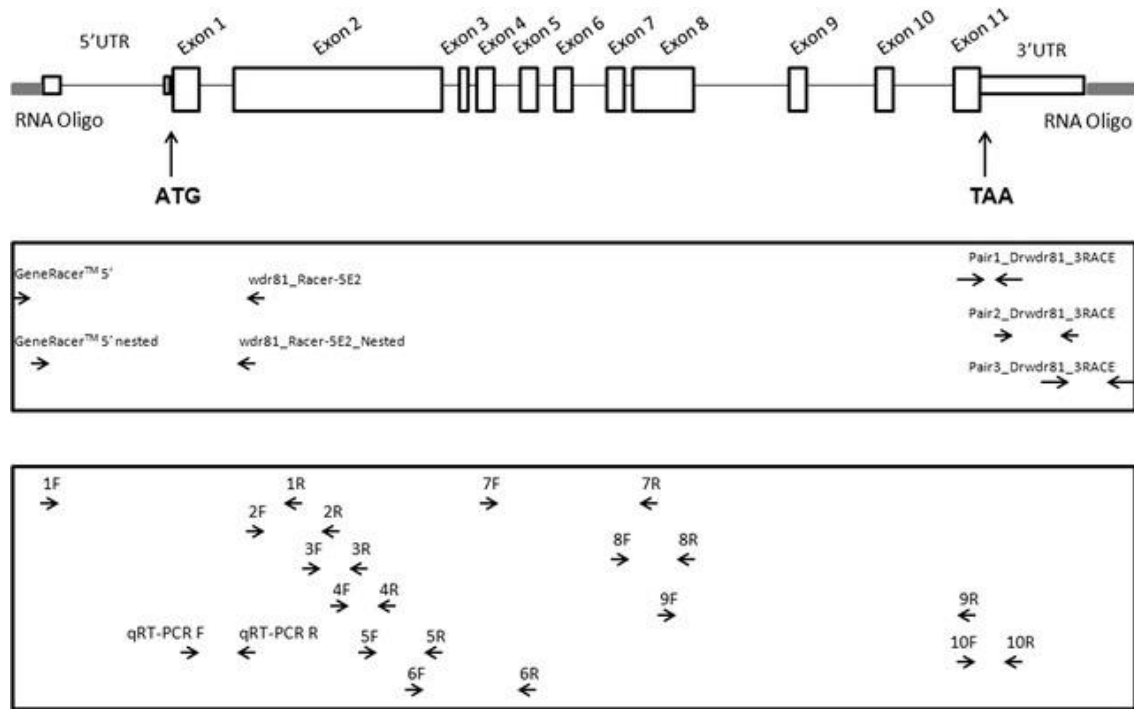


Figure 7. Zebrafish *wdr81* gene, illustration of its genomic structure and binding regions of primers. Sequences of *wdr81*-001 (ENSDART00000156621), which was the prediction from Ensembl database, was utilized as a model. Boxes indicate the exons, lines indicate the introns and gray boxes stand for the RNA oligonucleotides (RNA oligo), which were ligated to the mRNA by the use of RACE kit. The approximate locations of the primers for RACE experiment were indicated with arrows in the upper box. The binding regions of the primer pairs designed to characterize the open reading frame and the primer pair to be used in the qRT-PCR were depicted as arrows in the below box¹³⁹ (Reprinted from Doldur-Balli et al., 2015¹³⁹).

The alignment file of human, mouse and zebrafish WDR81 orthologous proteins obtained from Clustal Omega was submitted to ESPript 3 software. The location of the domains were highlighted on the generated file. This study indicated that the most

conserved domain was the BEACH domain among human, mouse and zebrafish. A difference in the number of WD40 repeat domains were detected; seven WD40 repeat domains were predicted in zebrafish *wdr81* whereas the number of predicted WD40 repeat domains were six in human and mouse (Figure 9).

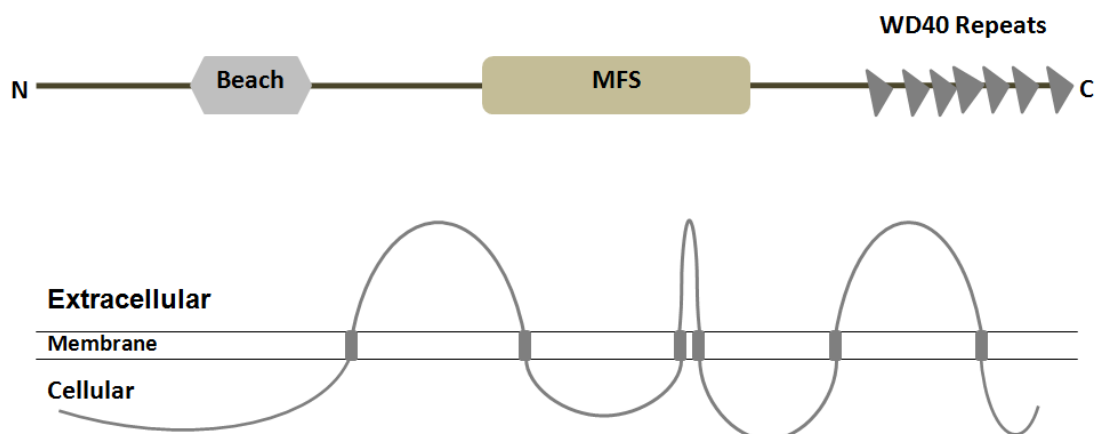


Figure 8. The conserved domains and the structural organization of zebrafish *wdr81*, predicted *in silico*. The predictions of BEACH, MFS and WD40 repeat domains and being a transmembrane protein were also shared for the human and mouse *WDR81* proteins¹³⁹ (Reprinted from Doldur-Balli et al., 2015¹³⁹).

3.2. Zebrafish *wdr81* Transcript Possesses One ORF

A search on the splice variants of human *WDR81* exhibited nine protein coding transcripts which are *WDR81*-001 (ENST00000409644), *WDR81*-002 (ENST00000309182), *WDR81*-004 (ENST00000446363), *WDR81*-005 (ENST00000419248), *WDR81*-007 (ENST00000455636), *WDR81*-009 (ENST00000468539), *WDR81*-010 (ENST00000418841), *WDR81*-014 (ENST00000575206) and *WDR81*-201 (ENST00000437219) (Figure 10). Mouse had three protein coding transcripts of *Wdr81*, which are *Wdr81*-001 (ENSMUST00000117392), *Wdr81*-003 (ENSMUST00000132442) and *Wdr81*-201 (ENSMUST00000173320) (Figure 11)²⁴. *wdr81*-001 (ENSDART00000156621) was

predicted to be one transcript of zebrafish *wdr81* (Figure 12)²⁴ and I investigated the presence or absence of splice variants experimentally, since more than one transcript were predicted for orthologous genes from human and mouse^{12,24,36}.

In order to characterize the transcript of zebrafish *wdr81*, I firstly focused on the ORF and obtained 10 overlapping amplicons using the primer pairs mentioned in Table 3 and cDNAs from 24 hpf embryo and adult brain as templates. The location of the primers designed to amplify ORF were given in Figure 7. They cover the ORF and the experiment was designed so that absence of an exon, because of alternative splicing process, would be observed since common regions would exist between consecutive amplicons. Observing one PCR product per reaction with both 24 hpf embryo template and adult brain template pointed out that zebrafish *wdr81* transcript possesses one ORF (Figure 13).

3.3. Characterization of 5' End of *wdr81* Verified the Prediction and an Insertion Site Was Detected in the 3' End of Some Tissues

The 5' end and 3' end of the zebrafish *wdr81* transcript were characterized in order to observe possible transcript variants regarding these regions. The RACE method was utilized in order to characterize the UTRs of *wdr81*. The gene specific primers and the primers included in the kit (GeneRacer™ primers) were employed (Figure 7, top box). RACE-ready cDNAs from 24 hpf embryo and adult brain were amplified with a touch down PCR and then the amplicons were used as a template in a nested PCR (Figure 14). A single DNA band was obtained from each reaction revealing that *wdr81* transcript possesses one 5'UTR (Figure 15). The experiment was designed so that the PCR products would include the 5'UTR, exon 1 and initial sequences of exon 2 of *wdr81* (Figure 16). The amplicons were sequenced after cloning. Sanger sequencing results of 5'RACE product of *wdr81* verified the predictions. The 5'UTR of *wdr81* was found to be 264 bp long and the prediction was 6 bp longer than it, at the 5' end (Figure 17).

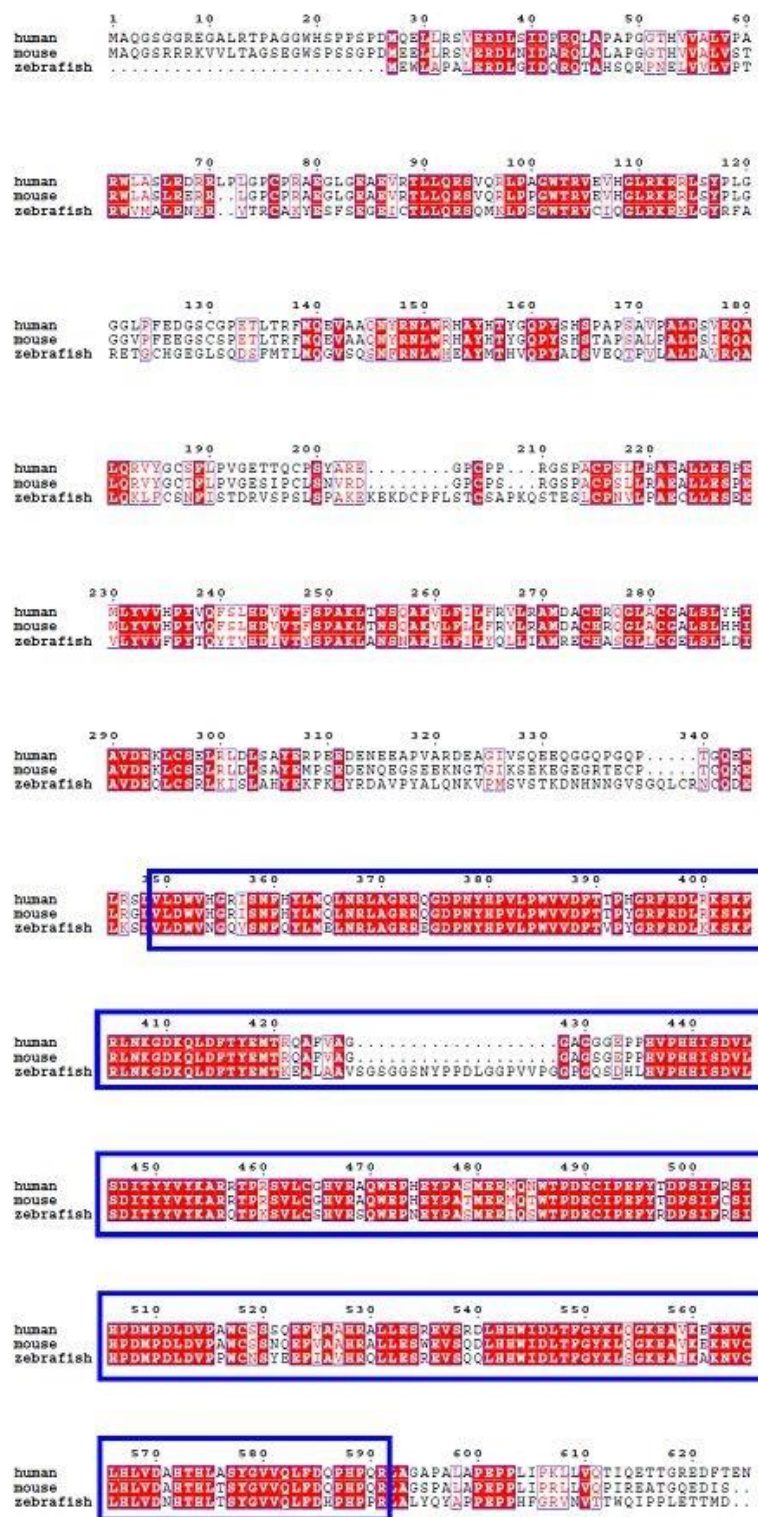


Figure 9. Alignment of the protein sequences of human, mouse and zebrafish WDR81. The conserved amino acid residues in three species were coloured with red background. The blue boxes emphasize the location of the predicted BEACH domain¹³⁹ (Reprinted from Doldur-Balli et al., 2015¹³⁹).

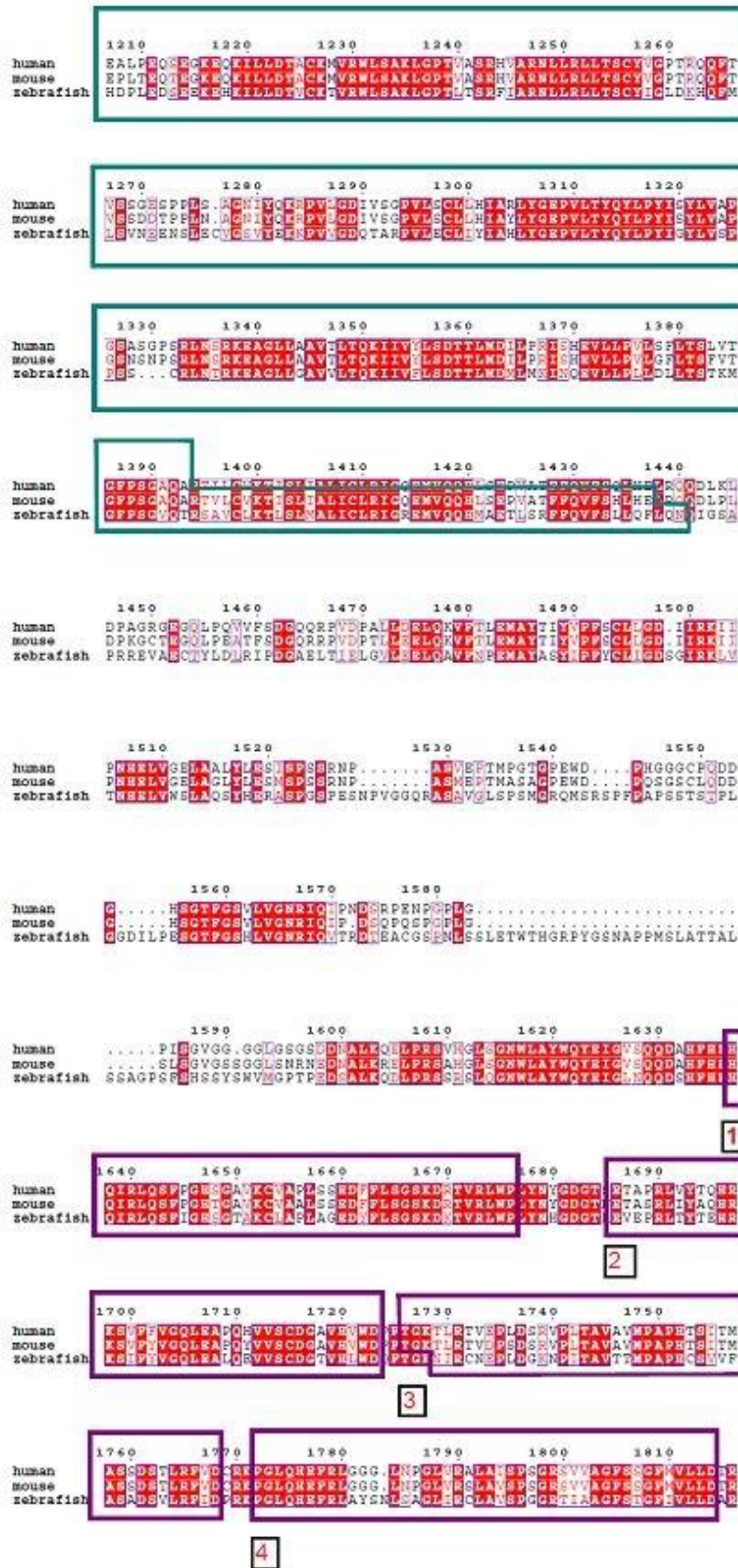


Figure 9. (continued) The purple boxes emphasize the location of the predicted WD40 repeat domains. WD40 repeat domains were numbered¹³⁹.

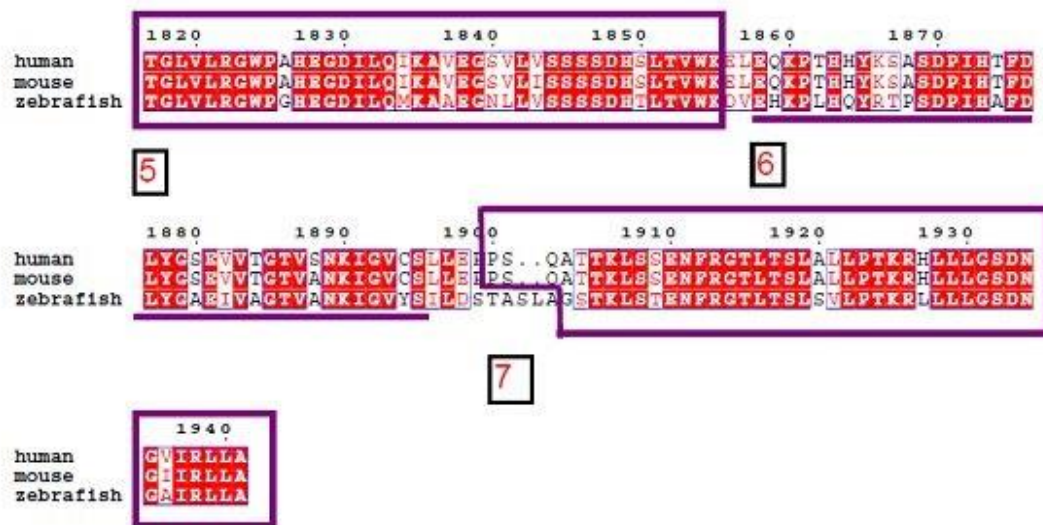


Figure 9. Number 6 was predicted to be present only in zebrafish *wdr81*¹³⁹.

The strategy in this study to obtain 3'RACE product of *wdr81* was to amplify the RACE-ready cDNAs from 24 hpf embryo and adult brain as 3 overlapping PCR products. The experiment was designed so that the PCR products would include the 3'UTR and exon 11 of *wdr81* (Figure 18). A single DNA band was obtained from each reaction at expected sizes as a result of the reactions with primer pairs 1 and 3 from Table 4, revealing that *wdr81* transcript has one 3'UTR. The reaction with primer pair 2 from Table 4 also gave a single DNA band from each reaction however it was longer than expected, which pointed out presence of an insertion site. Obtaining a single DNA band from each reaction with primer 2 also showed that there is one *wdr81* transcript regarding the 3'UTR structure (Figure 19). The PCR products of the reactions were sent to Sanger sequencing, the results of the reactions with primer pairs 1, 2 and 3 (Table 4) confirmed the predictions along with several nucleotide variants and indels (Table 6). In addition an insertion site in amplicon 2 was detected (Figure 19b). The location of the insertion site was detected between the 7564th and 7565th nucleotides of cDNA of *wdr81*. This site was amplified with a new primer, which was designed to characterize the insertion site by amplifying it within a narrower frame. The templates were again from 24 hpf embryo and adult brain. The PCR products were sent to Sanger sequencing after cloning.

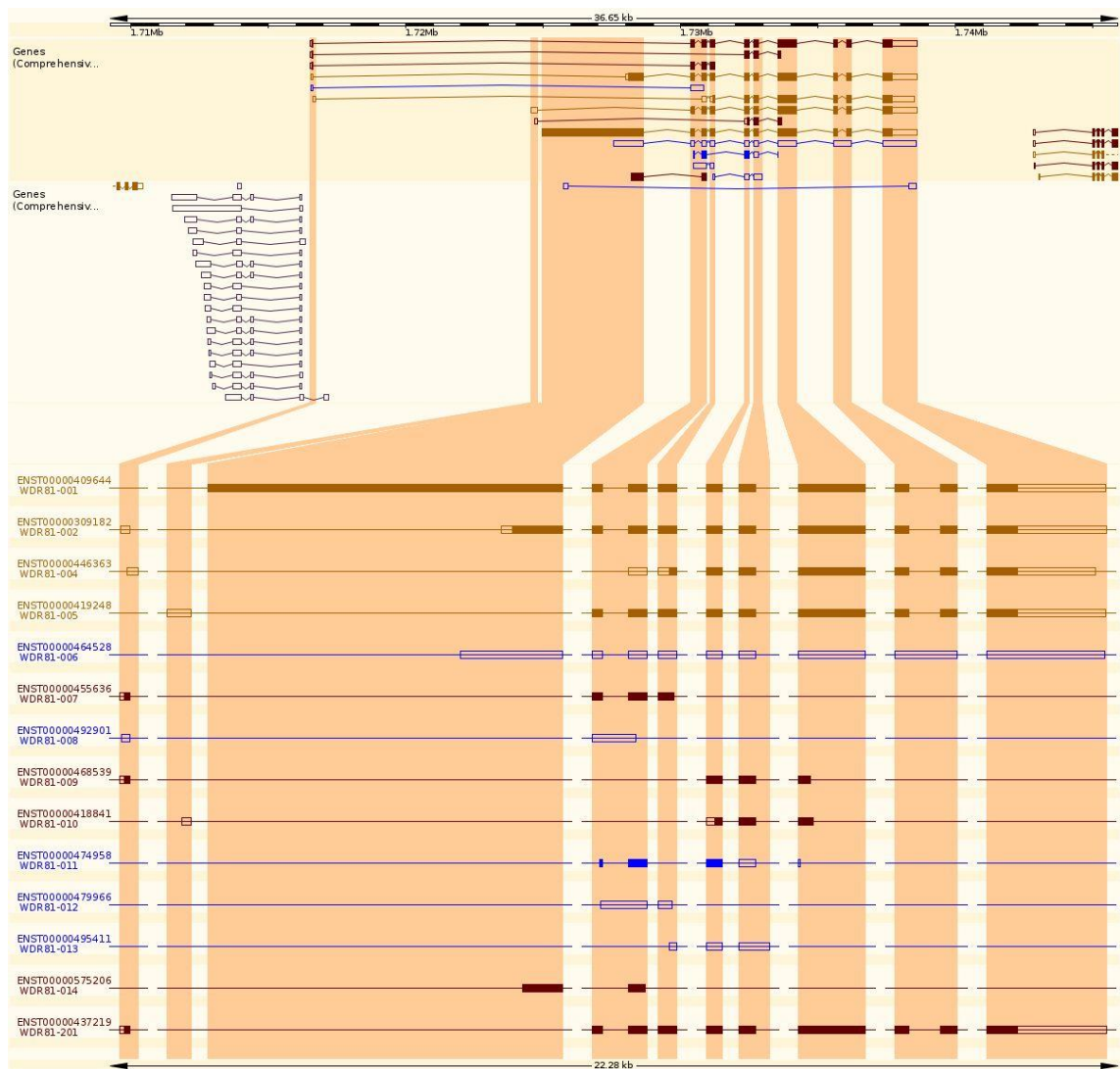


Figure 10. Diagram showing the splice variants of human *WDR81*, derived from Ensembl²⁴. Human *WDR81* encodes 9 protein coding splice variants.

Three plasmids per sample (embryo and adult brain), obtained from single colonies were aligned in order to obtain the sequence of the insertion site. Plasmids 1-1, 1-5 and 1-8 were from 24 hpf sample origin, plasmids 2-3, 2-4 and 2-6 were from adult brain origin. A 266 bp. long insertion site was revealed as a result of sequencing and alignment (Figure 20). Detection of the 266 bp long insertion site in both embryo and brain samples brought up a question about the presence of the same insertion site in other adult tissues and samples from other developmental stages.

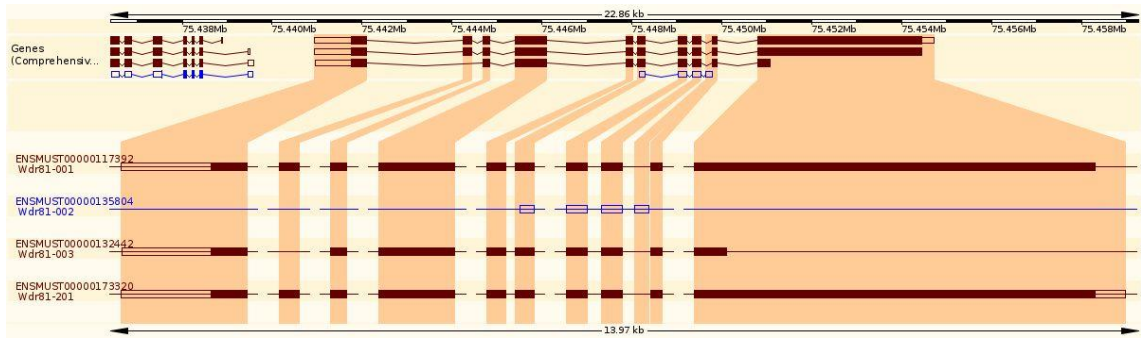


Figure 11. Diagram showing the splice variants of mouse *Wdr81*, derived from Ensembl²⁴. Mouse *Wdr81* encodes 3 protein coding splice variants.

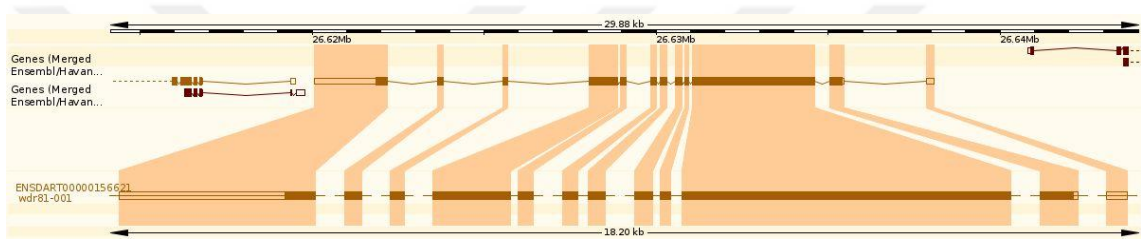


Figure 12. Diagram showing the zebrafish *wdr81* transcript, derived from Ensembl²⁴. Zebrafish *wdr81* is predicted to encode one protein coding transcript.

Reactions with all of the evaluated samples from ten different development timepoints, including 24 hpf embryo sample, demonstrated the presence of the insertion. Reactions with the adult tissues; brain, testis, heart, liver, eye, tail and muscle also demonstrated the presence of the insertion however reactions with kidney, intestine and gills did not give any PCR products (Figure 21). The band intensities of the amplicons in Figure 21 were measured (Table 7). The intensity of the band amplified from the 35 dpf juvenile zebrafish had the lowest intensity among the other developmental timepoint samples (Table 7a). The comparison of the band intensities of the amplicons obtained from adult tissues resulted in observing the highest and nearly equal values from the brain and the testis, followed by the values from the eye. The band intensities obtained from the amplification of the insertion region in heart and muscle samples were nearly equal, and were followed by that of tail and liver, respectively (Table 7b).



Figure 13. Agarose gel electrophoresis image of the PCR products of the reactions, which zebrafish *wdr81* open reading frame was characterized. a) 24 hpf embryo cDNA template was amplified. b) Adult brain cDNA template was amplified. Amplicons obtained with primer pairs 1-10 were loaded to the lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19, respectively. The reaction with –RT controls, which were the same primer pairs were loaded to the lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20. Lanes marked as M include pUC mix DNA Marker (SM0301, Thermo Scientific) (Reprinted from Doldur-Balli et al., 2015¹³⁹).

As a conclusion, characterization of the 5'end of *wdr81* indicated that there is one 5'UTR structure of the transcript and there are not any other transcript variants regarding 5'UTR. In addition, detection of the insertion site in 3'UTR in most of the evaluated tissues might be a result of an alternative polyadenylation process among tissues and so that various lengths of 3'UTR might be observed.

3.4. Expression of *wdr81* is Increased at 5 Hpf and 18 Hpf During Development and is Enriched in the Eye and Brain of Embryos

Expression of *wdr81* during various developmental stages was investigated by employing both qRT-PCR and WMISH methods. The primer pairs, qRT-PCR F and R, whose locations were depicted in Figure 7 were utilized in quantification of expression values at ten developmental timepoints.

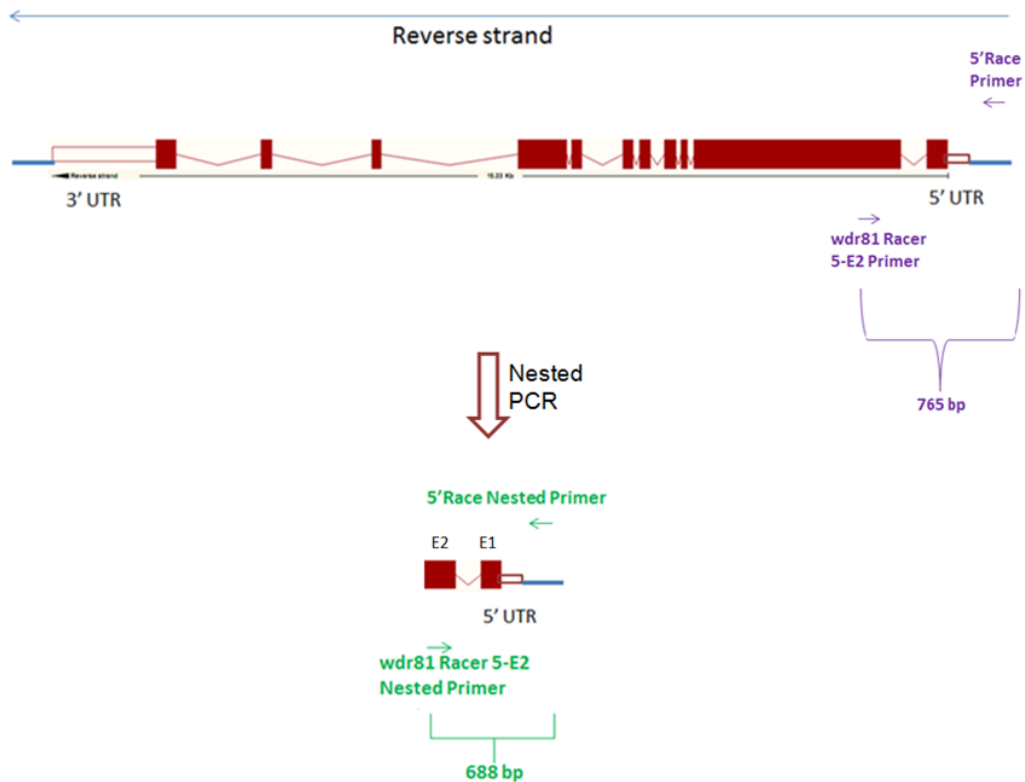


Figure 14. The illustration of the strategy in this study to obtain 5'RACE product of *wdr81* by performing a nested PCR following a touch down PCR. The experiment was designed so that the 5'RACE product of *wdr81* would include 5'UTR, exon 1 and initial sequences of exon 2.

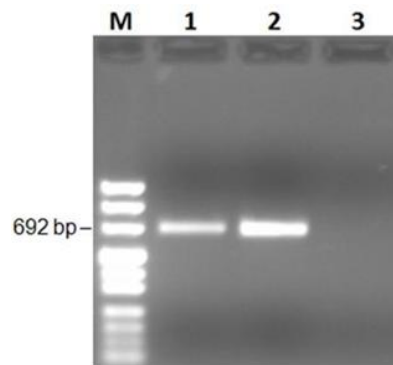


Figure 15. Agarose gel electrophoresis image of the 5'RACE product of *wdr81* transcript. A single DNA band was obtained from each reaction revealing that *wdr81* transcript possesses one 5'UTR. Lane 1: *wdr81* 5'RACE product from 24 hpf embryo template; lane 2: *wdr81* 5'RACE product from adult brain template; lane 3: control reaction which did not include any templates. Lane marked as M included pUC mix marker 8 (SM0301, Thermo Scientific) (Reproduced from Doldur-Balli et al., 2015¹³⁹)

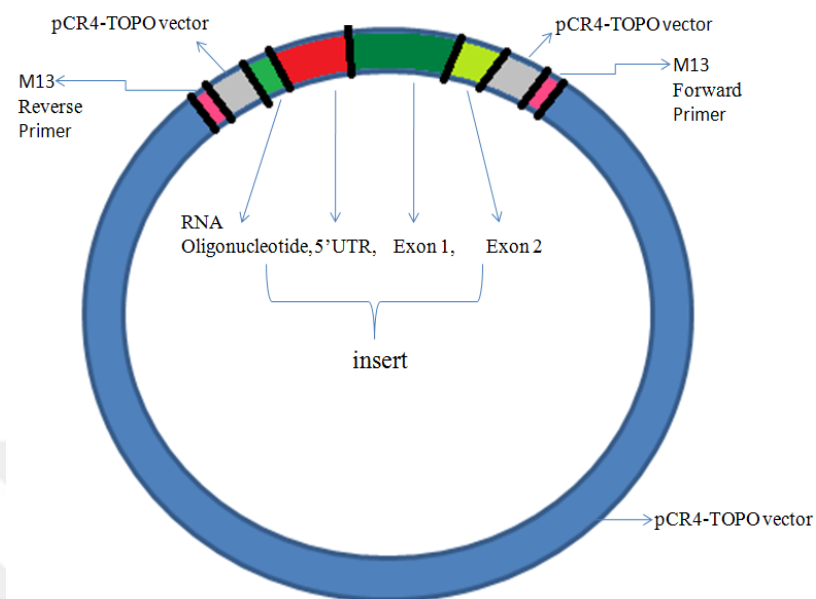


Figure 16. The schematic illustration of the order of sequences in plasmids whose inserts are 5'RACE product of *wdr81*.

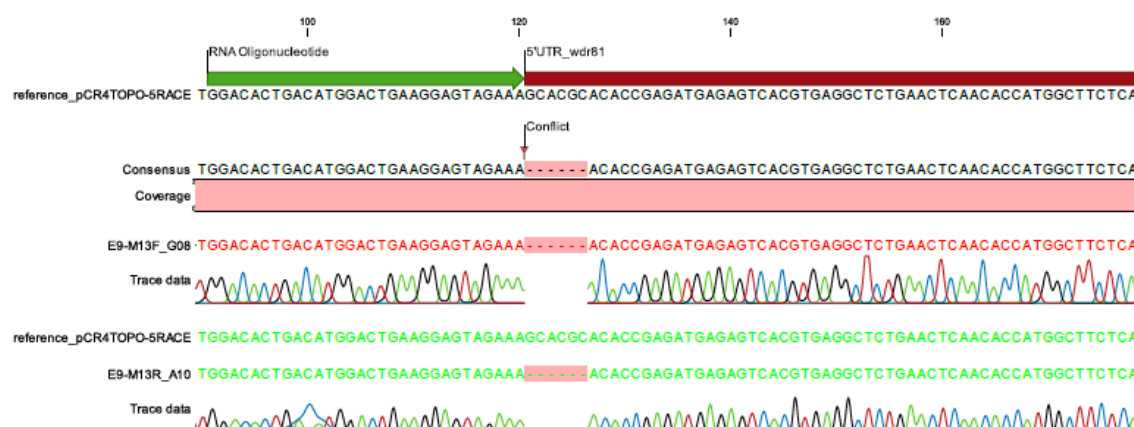


Figure 17. A CLCBio Main Workbench software analysis diagram showing a representative Sanger sequencing result of 5'RACE product with a 6 bp shorter 5'UTR than the prediction.

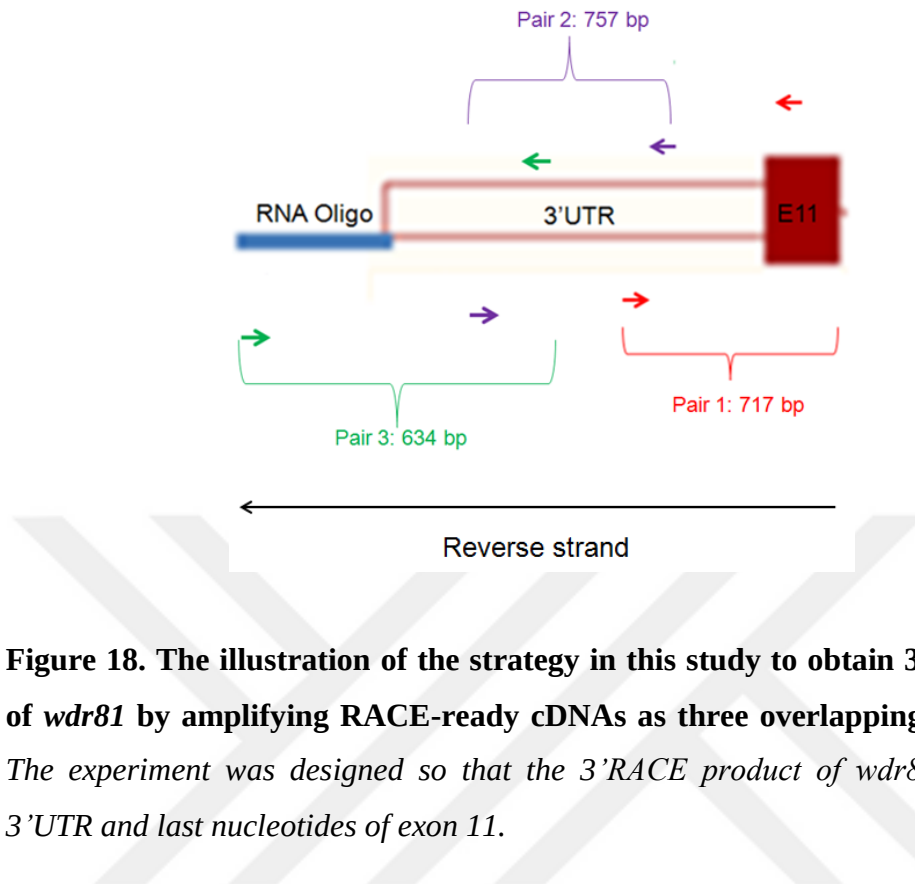


Figure 18. The illustration of the strategy in this study to obtain 3'RACE product of *wdr81* by amplifying RACE-ready cDNAs as three overlapping PCR products. The experiment was designed so that the 3'RACE product of *wdr81* would include 3'UTR and last nucleotides of exon 11.

Relative temporal expression analysis exhibited that there was expression at all timepoints, which were 1 hpf, 5 hpf, 10 hpf, 18 hpf, 24 hpf, 48 hpf, 72 hpf, 5 dpf, 15 dpf and 35 dpf and the level of expression was higher at 1 hpf, 5 hpf and 18 hpf stages (Figure 22). Zygotic expression in zebrafish was reported to start at 3 hpf^{147,148}. Observation on the relative temporal expression of *wdr81* led to a conclusion that the expression level was high at 1 hpf and 5 hpf timepoints which are before and after the beginning of zygotic expression, respectively. Observing the high level of expression at 1 hpf also emphasized that *wdr81* was maternally supplied. A dramatic decline was detected at the level of expression at 10 hpf timepoint and a clear raise was observed at 18 hpf development stage. The level of expression again dropped down at 24 hpf and similar low levels of expression continued during the rest of the timepoints (Figure 22). 5 hpf and 18 hpf appeared to be crucial timepoints related with *wdr81* expression during development of zebrafish in regard to the stages after transition to zygotic expression. Results of WMISH experiment were generally in parallel with the qRT-PCR results.

An RNA probe, which was designed to detect the region corresponding to the mutation site in patients, was synthesized. The length of the probe was 1.7 kb.

The probe was synthesized by amplifying the region between the forward primer of the primer pair 5 and the reverse primer of the primer pair 6, which were previously designed to amplify the open reading frame of *wdr81* (Table 3, Figure 7 lower box). Differential expression of *wdr81* at six developmental stages (6 hpf, 10 hpf, 18 hpf, 24 hpf, 48 hpf, 72 hpf) was studied with this probe. The signal was strong in the early development timepoints (6 hpf-18 hpf), and it decreased at 24 hpf timepoint. The signal was decreased in the trunk of the embryos at 48 hpf and was not significant in the whole embryo at 72 hpf. The expression was ubiquitous at the embryonic stages of 6 hpf, 10 hpf, 18 hpf and 24 hpf. The signal of the RNA probe was focused in the eye and brain regions of embryos at 18 hpf and 48 hpf stages. At 18 hpf, *wdr81* expression was enriched in the optic vesicle and midbrain. At 48 hpf, *wdr81* expression was concentrated in the lens, diencephalon, midbrain and medial longitudinal fascicle (Figure 23).

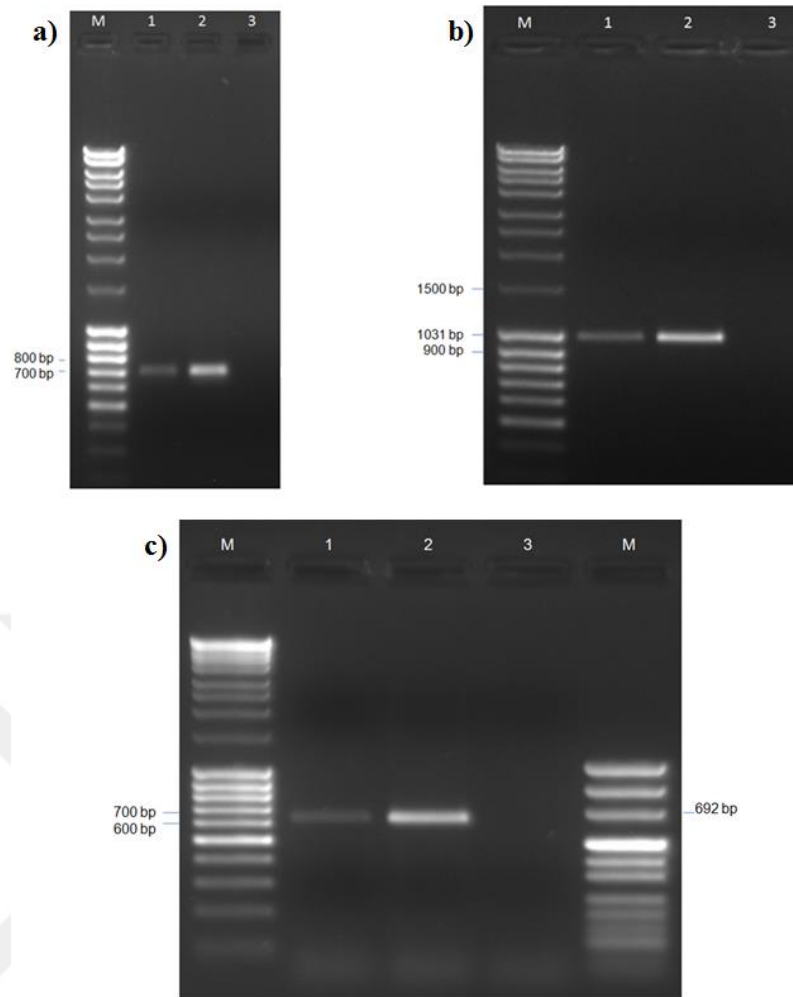


Figure 19. Agarose gel electrophoresis images of the overlapping amplicons obtained from the experiment in which 3'end of the zebrafish *wdr81* was characterized. A single DNA band was obtained from each reaction at expected sizes as a result of the PCRs with primer pairs 1 (a) and 3 (c) (Table 4), revealing that *wdr81* transcript possesses one 3'UTR. The reaction with primer pair 2 also gave a single DNA band from each reaction however it was longer than the prediction (b), which pointed out presence of an insertion. Obtaining a single DNA band from the reaction with primer pair 2 (Table 4) also showed that there is one *wdr81* transcript regarding the 3'UTR structure. In all of the gels, templates in lane 1: RACE-ready cDNA from 24 hpf embryo, lane 2: RACE-ready cDNA from adult brain. Lane 3: control reaction which did not include any templates. Lane marked as M: MassRuler Mix DNA Marker (SM0403, Thermo Scientific). Second DNA marker in (c) was pUC mix marker 8 (SM0301, Thermo Scientific) (Reproduced from Doldur-Balli et al., 2015¹³⁹).

Table 6. Variants detected in the 3'RACE products of 24 hpf embryo and adult brain *wdr81* except the insertion site which was found to be 266 bp long (Reprinted from Doldur-Balli et al., 2015¹³⁹)

c.6506 T>C
c.6707 A>T
c.6733 C>G
c.7007_7008insG
c.7015delT
c.7019_7023delACTCT
c.7103 T>C
c.7296 T>A
c.7331_7334delATAT
c.7512 A>G
c.7546 A>G
c.7770 G>T
c.7985 C>T
c.8050 A>G
c.8136 T>C
c.8252 A>G

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Plasmid1-1_insertion3'UTR      GCATTTTCTGGACAGGCCAAAACATATTGCTTGTTTTAGGAAATGATATG  50
Plasmid2-4_insertion3'UTR      GCATTTTCTGGACAGGCCGGGACATATTGCTTGTTTTAAGAAAGTGGTGTG  50
Plasmid2-6_insertion3'UTR      GCGTTTTCTGGACGGGCGGAGCATATTGCTTGTTTTAGGAAATAATATG  50
Plasmid2-3_insertion3'UTR      -CATTTTCTGGACGGGCAAGGCATATTGCTTGTTTTAAGAAAGTAATATG  49
Plasmid1-5_insertion3'UTR      GCATTTTCTGGACAAGCAGAGCATATTGCTTGTTTTGGGGAGTAATATG  50
Plasmid1-8_insertion3'UTR      GCATTTTCTGGACAGGCAGAACATATTGCTTGTTTTAGGAAAGTAATATG  50
* ***** ** ***** * * * * *

Plasmid1-1_insertion3'UTR      CCAAGTTTGGGTGGGTTTTTCTTGGAACAAGCAAAGGTGTCTGCCAATG  100
Plasmid2-4_insertion3'UTR      CCAGGTTTGGGTGGGTTTTTCTTAGAACGGGCAAGGGTATCTGCCAATG  100
Plasmid2-6_insertion3'UTR      CCGGGTTTGGGTGGGTTTTTCTTGAGACAAGCAAAGGTATCTGCCAATG  100
Plasmid2-3_insertion3'UTR      CCGAGTTTGGGTGGGTTTTTCTTAAACAAGCAAAGTATCTGCCAATG  99
Plasmid1-5_insertion3'UTR      CCAAATTTGAGTGAGTTTTTCTTGGGCAAGCAAGAATATCTGCCAATG  100
Plasmid1-8_insertion3'UTR      CCAAATTTGAGTGAGTTTTTCTTAAACAAGCAAAGATATCTGCCAATG  100
** **** * ***** * **** * *****

Plasmid1-1_insertion3'UTR      GGGTAAGCAAAATAATCTTATGTCGAAAGCAAAAACAAAATTGTTTTGCT  150
Plasmid2-4_insertion3'UTR      GGGTAAGCAAAATAATCTTATGTCGAAAGCAAAAACAAAATTGTTTTGCT  150
Plasmid2-6_insertion3'UTR      GGGTAAGCAAAATAATCTTATGTCGAAAGCAAAAACAAAATTGTTTTGCT  150
Plasmid2-3_insertion3'UTR      GGGTAAGCAAAATAATCTTATGTCGAAAGCAAAAACAAAATTGTTTTGCT  149
Plasmid1-5_insertion3'UTR      GGGTAAGCAAAATAATCTTATGTCGAAAGCAAAAACAAAATTGTTTTGCT  150
Plasmid1-8_insertion3'UTR      GGGTAAGCAAAATAATCTTATGTCGAAAGCAAAAACAAAATTATTTTGCT  150
*****

Plasmid1-1_insertion3'UTR      TGTCCCATTTGGCAGATTGTTTTGCTTGTTTTAGGAAAAACTCGCTTAAT  200
Plasmid2-4_insertion3'UTR      TGTCCCATTTGGCGGATTGTTTTGCTTGTTTTGGGGGAAACTCGCTTGAT  200
Plasmid2-6_insertion3'UTR      TATCCCATTTGGCAGATTGTTTTGCTTGTTTTAGGAGGAGCTCGCTTAAT  200
Plasmid2-3_insertion3'UTR      TATCCCATTTGGCAGATTATTTTGCTTGTTTTAAGGAAGAGCTCACTTAAT  199
Plasmid1-5_insertion3'UTR      TATCCCATTTGGCAGATTGTTTTGCTTGTTTTAAGGAAGGCTCACTTGGT  200
Plasmid1-8_insertion3'UTR      TATCCCATTTGGCAGATTATTTTGCTTGTTTTGGGGAAAAACTCACTTGGT  200
* ***** ** *****

Plasmid1-1_insertion3'UTR      TTTGGCATGTTAGTTCTTCAGGCGGGACAGTGTGTTTTGCTTGCTAGAG  250
Plasmid2-4_insertion3'UTR      TTTGGCATATTGGTTCTTCAGGCGGGACGGTGTGTTTTGCTTGCTGAGG  250
Plasmid2-6_insertion3'UTR      TTTGGCATATTAGTTCTTCAGGCGGGACGGTGTGTTTTGCTTGCTGAGG  250
Plasmid2-3_insertion3'UTR      TTTGGCATGTTGTTCTTCAGGCGGGACGGTGTGTTTTGCTTGCTGAGG  249
Plasmid1-5_insertion3'UTR      TTTGGCATGTTAGTTCTTCGAACGGGACGGTGTGTTTTGCTTGCTAGAG  250
Plasmid1-8_insertion3'UTR      TTTGGCATATTAGTTCTTCGGGCAAGACAGTGTGTTTTGCTTGCTGAGG  250
***** ** ***** * *** *****

Plasmid1-1_insertion3'UTR      GATGCTTCTTGATTG--- 266
Plasmid2-4_insertion3'UTR      GATGCTTCTTGATTGGG- 268
Plasmid2-6_insertion3'UTR      GGTGCTTCTTGATTGGG- 268
Plasmid2-3_insertion3'UTR      GATGCTTCTTGATTGGGA 268
Plasmid1-5_insertion3'UTR      GATGCTTCTTGATTGG-- 267
Plasmid1-8_insertion3'UTR      GGTGCTTCTTGATTGG-- 267
* *****

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Figure 20. Alignment of the clones of the insertion site detected in 3'UTR of zebrafish *wdr81* transcript revealed its length as 266 bp. Three plasmids per sample (24 hpf embryo and adult brain), which included the insertion site as their inserts were sequenced. Plasmids 1-1, 1-5 and 1-8 carried the insert cloned from 24 hpf embryo sample, plasmids 2-3, 2-4 and 2-6 carried the insert cloned from adult brain sample (Reprinted from Doldur-Balli et al., 2015¹³⁹).

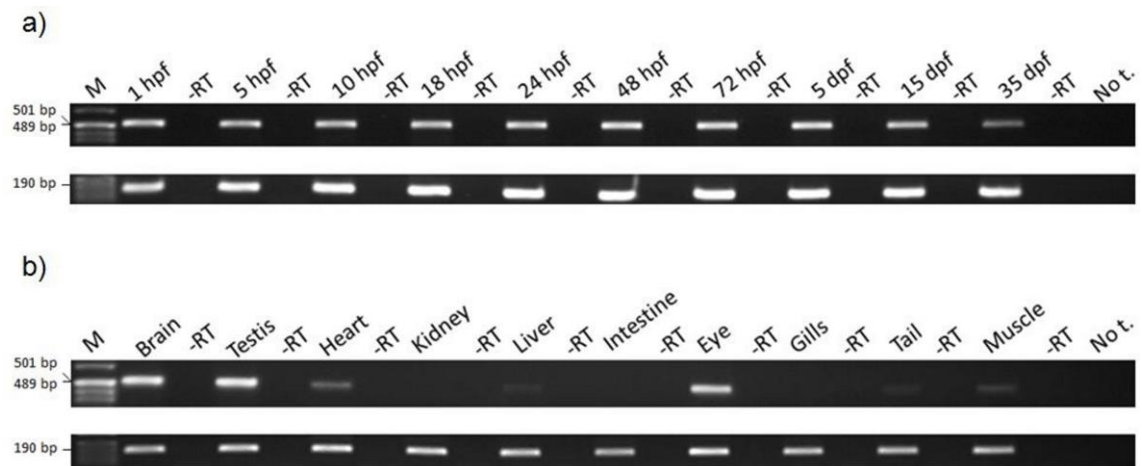


Figure 21. Agarose gel electrophoresis image of the amplicons obtained from the reactions in which presence of the insertion site was tested in samples from developmental stages and in adult tissues. a) Samples from ten developmental stages were amplified (upper gel image) and amplification of the same samples with a house keeping gene primer pair (beta-actin) confirmed the integrity of the cDNA templates (below gel image). B) Ten adult tissue samples were amplified (upper gel image) and integrity of the cDNA templates was confirmed with amplification using beta-actin primers (below gel image). The lanes in all of the gels were loaded with the amplicons from cDNA templates that they are labelled with. Lane marked as M included pUC mix DNA Marker (SM0301, Thermo Scientific). “No t.” stands for the control reaction which did not include any templates (Reprinted from Doldur-Balli et al., 2015¹³⁹).

3.5. Spatial Expression of *wdr81* and its Detection in Proliferation Zones

The head regions of WMISH specimens from 18 hpf, 48 hpf and 72 hpf stages were sectioned in order to detect the sites of expression in more detail. The intensity of the signal produced by the probe was the highest in the section from 18 hpf specimen. The signal was homogenous in the brain and the optic vesicle (Figure 24d). This might be implying that *wdr81* expression was taking place in all types of cells in these tissues.

Table 7. Intensities of the bands of PCR products from Figure 21 (*Reprinted from Doldur-Balli et al., 2015¹³⁹*).

a) Intensities of the bands of PCR products from Figure 21a.

Developmental stage	Intensities of the amplicons obtained from the insertion site	Intensities of the amplicons obtained with β -actin primers
1 hpf	8938.29	9821.86
5 hpf	7941.21	11415.03
10 hpf	8265.58	13168.54
18 hpf	8280.21	13521.12
24 hpf	8379.09	13497.54
48 hpf	9394.56	12853.64
72 hpf	9327.96	13705.19
5 dpf	9390.74	13497.32
15 dpf	7502.30	13005.18
35 dpf	4343.33	12577.55

b) Intensities of the bands of PCR products from Figure 21b.

Adult tissue	Intensities of the amplicons obtained from the insertion site	Intensities of the amplicons obtained with β -actin primers
Brain	9475.08	7483.83
Testis	10441.17	8470.90
Heart	2206.91	9003.5
Kidney	N/A	8421.62
Liver	407.82	8001.1
Intestine	N/A	6438.65
Eye	7023.97	9123.22
Gills	N/A	7566.79
Tail	415.84	7496.79
Muscle	1044.75	7563.46

Temporal Expression of *wdr81*

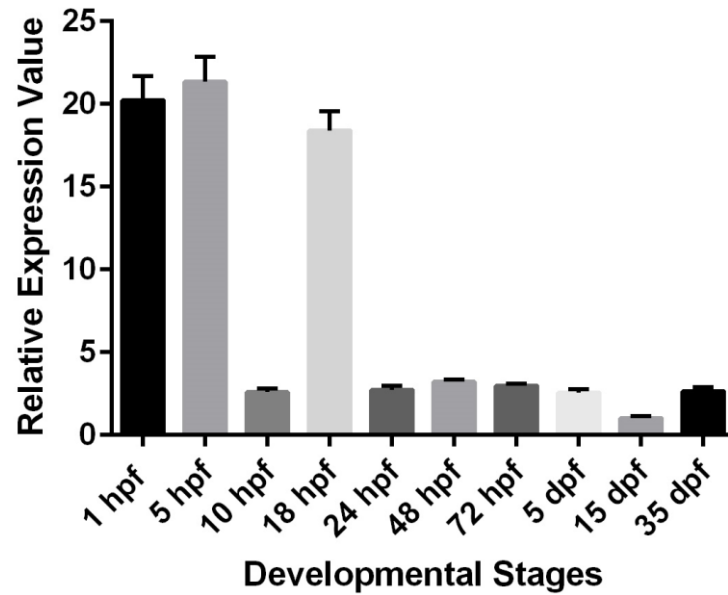


Figure 22. Relative expression of *wdr81* at ten developmental stages. The peaks in relative expression levels were detected at 1 hpf, 5 hpf and 18 hpf timepoints. Templates were cDNAs from 1 hpf embryo, 5 hpf embryo, 10 hpf embryo, 18 hpf embryo, 24 hpf embryo, 48 hpf embryo, 72 hpf larva, 5 dpf larva, 15 dpf larva, 35 dpf juvenile zebrafish. Error bars stand for +SE (Reprinted from Doldur-Balli et al., 2015¹³⁹).

The sections from 48 hpf specimen revealed expression of *wdr81* throughout the diencephalon (Di), preoptic area (Po), midbrain tegmentum (T), optic nerve, lens, nucleus of the medial longitudinal fascicle, and the retina. Observation of more condensed and distinct expression sites, along with the stained axonal fibers most probably were associated with cells, neuronal in origin (Figure 24 a-c). The intensity of the signal produced by the probe was the lowest in the sections obtained from 72 hpf specimen. The signal was observed in the midbrain tegmentum (T) and the retina (Figure 24 e-f). Relative spatial expression of *wdr81* in adult tissues was investigated using qRT-PCR. The same primer pairs used in the temporal expression study, qRT-PCR F and R (Table 5, Figure 7), were employed in quantification of expression values of ten adult tissues.

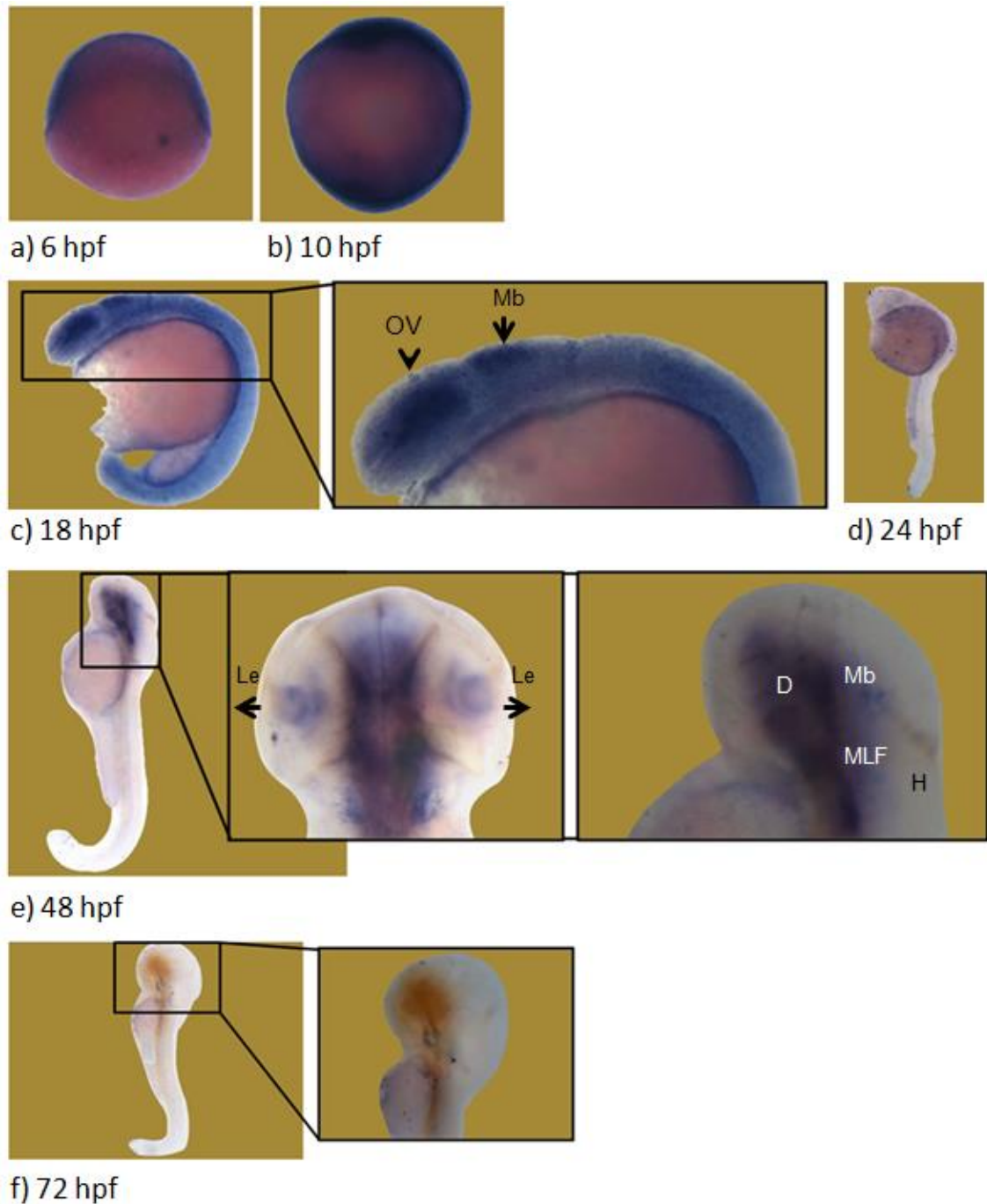


Figure 23. Spatio-temporal expression of *wdr81* during early development revealed via WMISH. The signal of the riboprobe was strong in the first 18 hours of development (a-c), then the intensity of the signal declined and continued during the other evaluated timepoints (d-f). The signal was enriched in the eye and brain at 18 hpf and 48 hpf timepoints (c,e). OV: Optic vesicle, Mb: midbrain, Le: lens, H: hindbrain, D: diencephalon, MLF: medial longitudinal fascicle (Reprinted from Doldur-Balli et al., 2015¹³⁹).

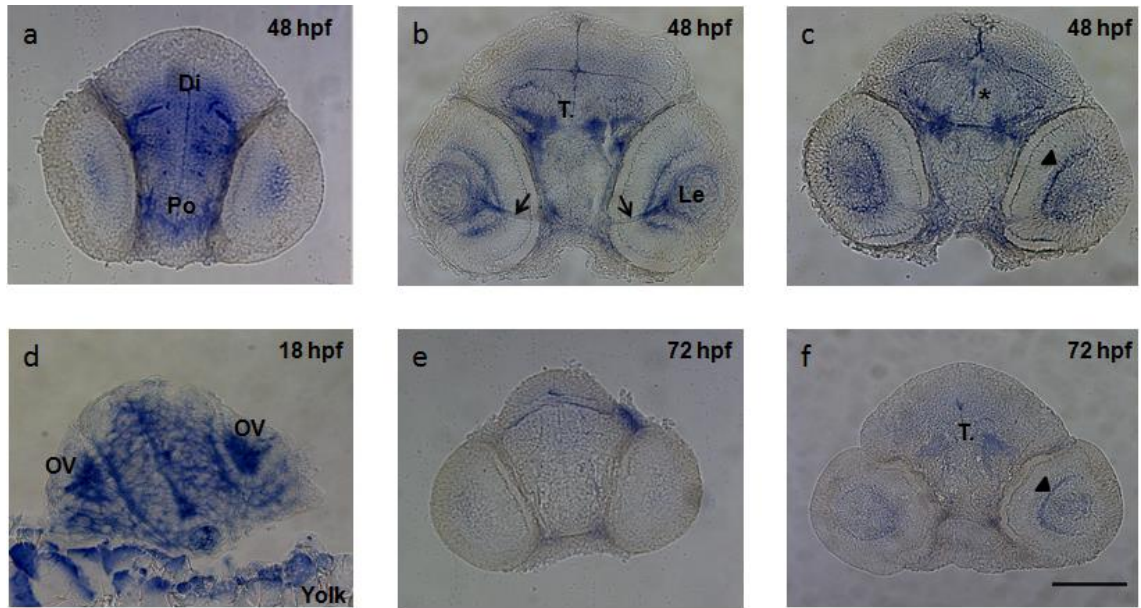


Figure 24. Transverse sections obtained from the head regions of WMISH specimens from 3 developmental stages. The signal of the riboprobe which implies *wdr81* expression was condensed in specific regions by 48 hpf (a-c), though the signal was homogenous at 18 hpf (d), and dropped down by 72 hpf (e and f). Optic nerve was indicated by arrows, the region of the nucleus of the medial longitudinal fascicle was indicated by an asterisk, and the retina was indicated by an arrowhead. Po: preoptic area; Di: diencephalon; T.: midbrain tegmentum; Le: lens; OV: optic vesicle; Yolk: yolk sac. Scale bar equals 100 micrometers (Reprinted from Doldur-Balli et al., 2015¹³⁹).

Expression of *wdr81* was detected in all of the evaluated tissues, which were brain, testis, heart, kidney, liver, intestine, eye, gills, tail and muscle. This data suggested that *wdr81* was ubiquitously expressed and still variation among the relative expression values of tissues was existing (Figure 25). Another aspect of spatial expression study in adult tissues was accomplished by applying ISH to the brain and eye. Expression of *wdr81* in these tissues was demonstrated with qRT-PCR method (Figure 25) and this data indicated the expression in whole tissues. We showed the distribution of the expression of *wdr81* on sections from two tissues by hybridizing the same riboprobe used in WMISH method (Figure 26).

Spatial Expression of *wdr81*

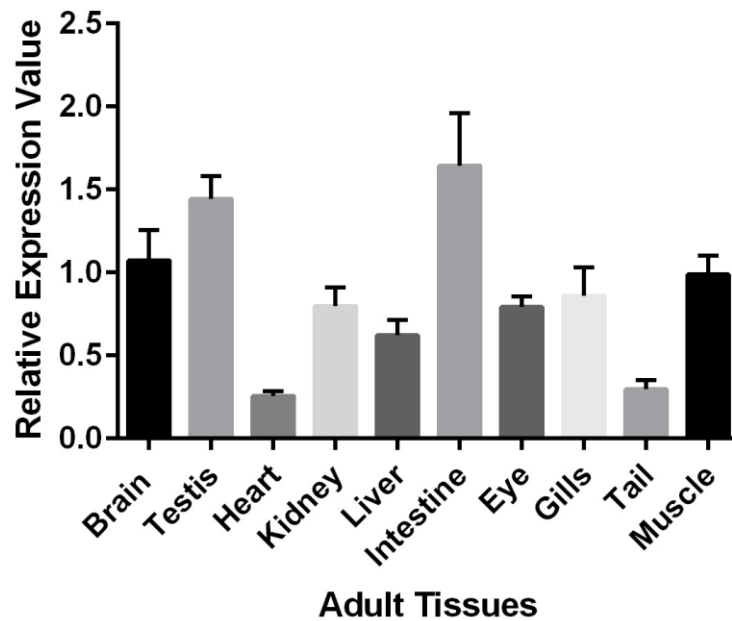


Figure 25. Relative expression of *wdr81* in ten adult tissues. Expression of *wdr81* was detected to be ubiquitous. Templates were cDNAs from brain, testis, heart, kidney, liver, intestine, eye, gills, tail and muscle. Error bars stand for +SE (Reprinted from Doldur-Balli et al., 2015¹³⁹).

Expression of *wdr81* was detected in the retinal layers, presumptive Purkinje cells of cerebellum, tectal ventricle, brain stem and optic tectum (Figure 26). The expression of *wdr81* was observed in the cells which possess a morphology like neurons in the brain areas, *lobus vagus* and optic tectum (Figure 26 f,g). Intriguingly, the areas, tectal ventricle (midline ventricle), *lobus vagus* and periventricular gray zone of optic tectum, which gave strong signals were known proliferation zones in zebrafish brain (Figure 26 c, f,g). Thereby, the data obtained from ISH experiment provided an association between *wdr81*-positive areas, which appeared to have a neuronal phenotype, and neurogenesis.

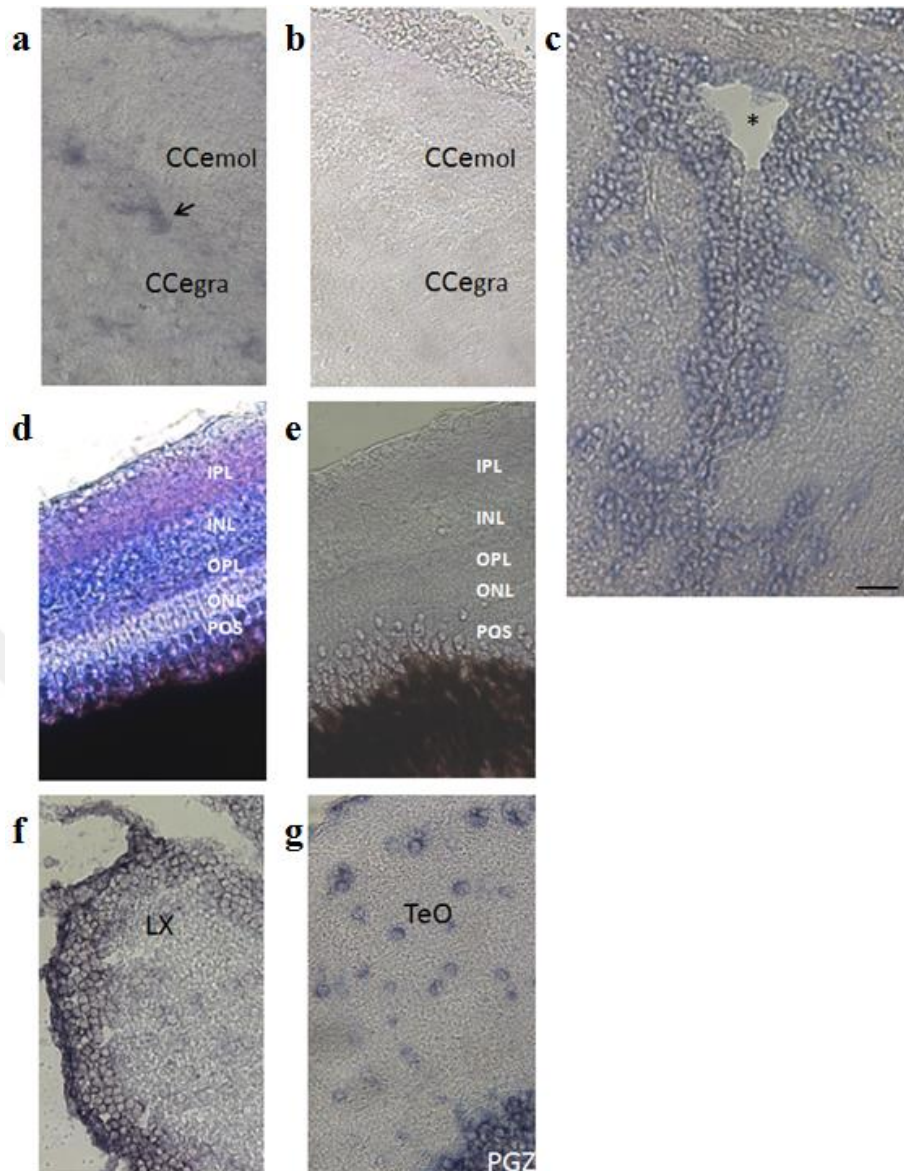


Figure 26. Distribution of the signal of riboprobe revealed the expression of *wdr81* in the adult brain areas and eye tissue. Cerebellum (a), tectal ventricle (c), retina (d), brain stem (f), and optic tectum (g) were positive for *wdr81* expression. A sense probe was used in cerebellum (b) and retina (e) sections in order to confirm that the signals from the antisense riboprobe were specific. CC_{emol}: Cerebellar molecular layer; CC_{egra}: Cerebellar granular layer; POS: photoreceptor outer segments; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; LX: lobus vagus; TeO: optic tectum; PGZ: periventricular gray zone of the optic tectum. Purkinje cell layer was indicated by an arrow and the tectal ventricle was indicated by an asterisk. Scale bar implies a length of 200 micrometers (Reprinted from Doldur-Balli et al., 2015¹³⁹).

3.6. Initial Characterization of Morphants

3.6.1. Dose Curve of *wdr81* Morpholino

Detecting whether *wdr81* was maternally supplied or not was needed before ordering the morpholino sequence since microinjection of a translation blocking morpholino may produce more severe phenotypes than that of a splice blocking morpholino¹⁴⁹. In order to fulfill this aim, cDNA samples from 1 hpf, 2 hpf, 3 hpf, 6 hpf, 12 hpf, 24 hpf and 48 hpf timepoints were amplified with a gene specific primer pair which produces an amplicon in 495 bp length. Zygotic expression in zebrafish was reported to start at 3 hpf^{147,148}. I previously showed the expression at 1 hpf timepoint (Figure 22), then cDNA samples from 2 hpf and 3 hpf timepoints were added to this experiment. The expected amplicon was obtained from all of the templates (Figure 27). This data indicated that *wdr81* was maternally supplied since there was amplification at 1 hpf, 2 hpf and 3 hpf stages. A splice blocking morpholino to knockdown *wdr81* was ordered because of two reasons: 1) *wdr81* is maternally supplied and using a translation blocking morpholino might result with a more severe phenotype, 2) lack of an efficient antibody, reacting with zebrafish samples, to detect the effect of a translation blocking morpholino on protein level. The location of the *wdr81* morpholino sequence and its target is depicted (Figure 28). When the *wdr81* splice blocking morpholino sequence was searched for any off target sequences by using NCBI Blast database¹⁵⁰, the obtained list was examined carefully and none of the hits were targeting any translation initiation sites or exon-intron junctions. WD repeat containing genes which were hit by the *wdr81* morpholino sequence, other than *wdr81*, were the genes encoding WD repeat-containing protein on Y chromosome-like isoform X1 and WD repeat-containing protein 49-like isoform X2. These hits were corresponding to intronic sequences, for this reason the risk of producing an off-target effect was evaluated to be unlikely. Tblastn database of NCBI¹³⁸ was also utilized to reveal the genes which encode proteins similar to *wdr81*. The list of genes obtained as a result of this search (Appendix E) included some genes which encode WD repeat-containing proteins. They were the genes encoding WD repeat and FYVE domain-containing protein 3 isoform X3, WD repeat and FYVE domain-containing protein 3 isoform X7, WD repeat and FYVE domain-containing protein 4

isoform X1 and WD repeat and FYVE domain-containing protein 4 isoform X2. The number of matching aminoacids was 70 with WD repeat and FYVE domain-containing protein 4 and 72 with WD repeat and FYVE domain-containing protein 3. The hits were obtained at the BEACH domain. The rest of the hits were also obtained at the BEACH domain with an exception of the gene encoding transcription initiation factor TFIID subunit 5, hitting WD40 repeats. The number of matching aminoacids in the rest of the list, except the genes encoding WD repeat-containing proteins, were maximum 90 over 2065 aminoacids. None of the results showed an overlap with neither the corresponding mutation site (residue 894) nor the morpholino target site (residues 1296-1298) in zebrafish *wdr81*. Effect of three doses of the morpholino antisense oligonucleotide sequence, which were 2 nanograms (ng), 4 ng and 8 ng, on splicing were evaluated. Similar doses of standard negative control morpholino, which did not have a target in zebrafish¹⁴⁴, were included. Uninjected control group from the same clutch were also collected. The survival rates of each experiment group were recorded (Table 8) and cDNAs from the samples were amplified in order to understand the effect of each dose of morpholino on splicing. The primer pair, which was used to understand the effect of the *wdr81* morpholino on splicing, was designed so that the amplicon would be 572 bp long if intron 2 was introduced between exon 2 and exon 3 and it would be 449 bp long if the template was a wild-type mRNA (Figure 29).

The PCR products were run on a 1% agarose gel firstly, then the rest of the reaction volumes of each sample were run on a 4% agarose gel in order to observe a better resolution. Results of the experiment showed that all of the evaluated doses efficiently worked and there was a band shift from 449 bp length to 572 bp length in the lanes where the reactions belonging to 2 ng, 4 ng and 8 ng *wdr81* morpholino injected embryos were loaded. Five hundred and seventy two bp long band had a stronger signal than the 449 bp long band in these lanes, indicating an introduction of intron 2 between exon 2 and exon 3 in the morpholino effected *wdr81* transcripts (Figure 30).

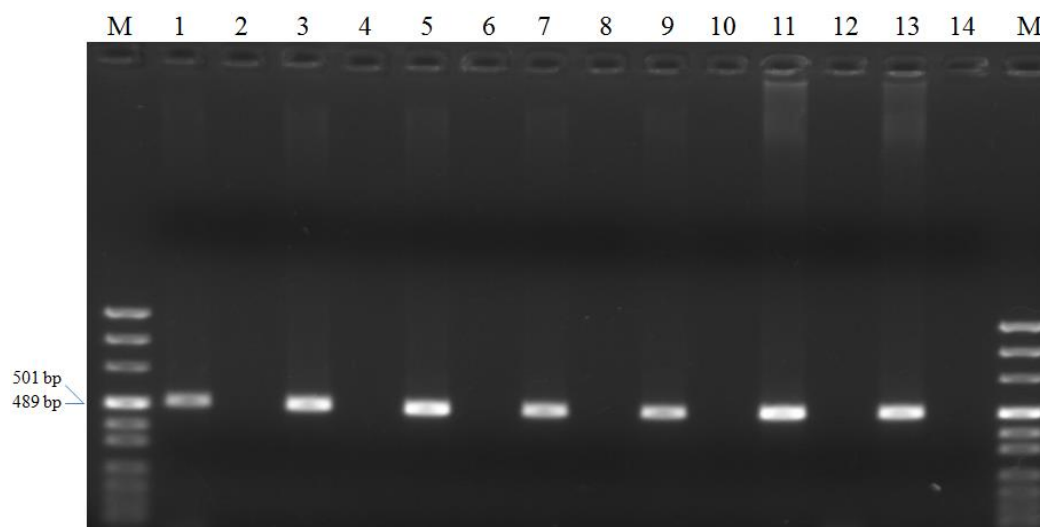


Figure 27. Agarose gel electrophoresis of the PCR products from a reaction showing *wdr81* expression between 1-48 hpf timepoints. Detecting its expression earlier than 3 hpf indicated that *wdr81* was maternally supplied. Templates in lane 1: cDNA of 1 hpf embryo, lane 2: -RT control for 1 hpf embryo, lane 3: cDNA of 2 hpf embryo, lane 4: -RT control for 2 hpf embryo, lane 5: cDNA of 3 hpf embryo, lane 6: -RT control for 3 hpf embryo, lane 7: cDNA of 6 hpf embryo, lane 8: -RT control for 6 hpf embryo, lane 9: cDNA of 12 hpf embryo, lane 10: -RT control for 12 hpf embryo, lane 11: cDNA of 24 hpf embryo, lane 12: -RT control for 24 hpf embryo, lane 13: cDNA of 48 hpf embryo, lane 14: -RT control for 48 hpf embryo. Lane indicated as M was loaded with pUC mix DNA Marker (SM0301, Thermo Scientific).

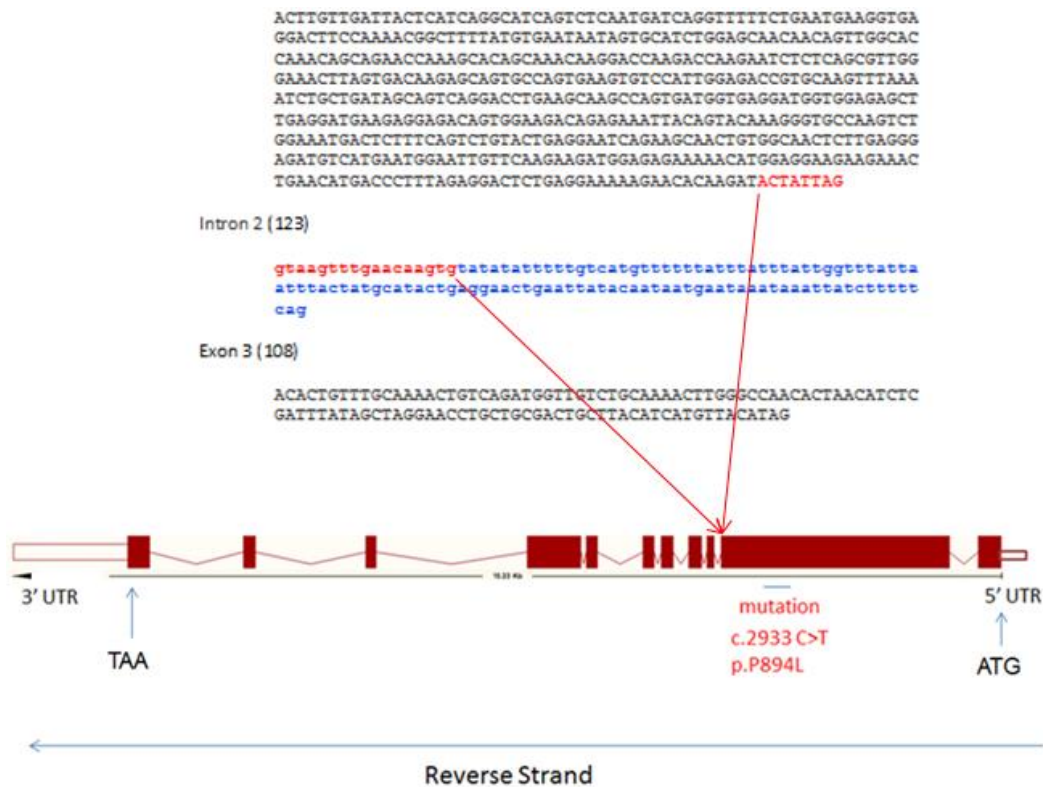


Figure 28. Illustration of the location of the target of the morpholino sequence (shown in red) which was designed to knock down *wdr81*. The splice blocking morpholino was designed to bind to the last 6 nucleotides of exon 2 and initial 17 nucleotides of intron 2.

Table 8. Survival rates of the embryos from experiment groups in the *wdr81* morpholino dose curve study.

Experiment Groups	Number of Surviving Embryos	Number of Dead Embryos	Survival Rate	Dose
<i>wdr81</i> morpholino injected group	11	40	21.57%	2 ng (data from 3 experiments were collected)
Standard negative control morpholino injected group	10	46	17.86%	
Uninjected control group	23	36	38.98%	

Experiment Groups	Number of Surviving Embryos	Number of Dead Embryos	Survival Rate	Dose
<i>wdr81</i> morpholino injected group	19	43	30.65%	4 ng (data from 3 experiments were collected)
Standard negative control morpholino injected group	31	22	58.50%	
Uninjected control group	31	29	51.66%	

Experiment Groups	Number of Surviving Embryos	Number of Dead Embryos	Survival Rate	Dose
<i>wdr81</i> morpholino injected group	13	35	27.08%	8 ng (data from 3 experiments were collected)
Standard negative control morpholino injected group	6	26	18.75%	
Uninjected control group	21	24	46.66%	

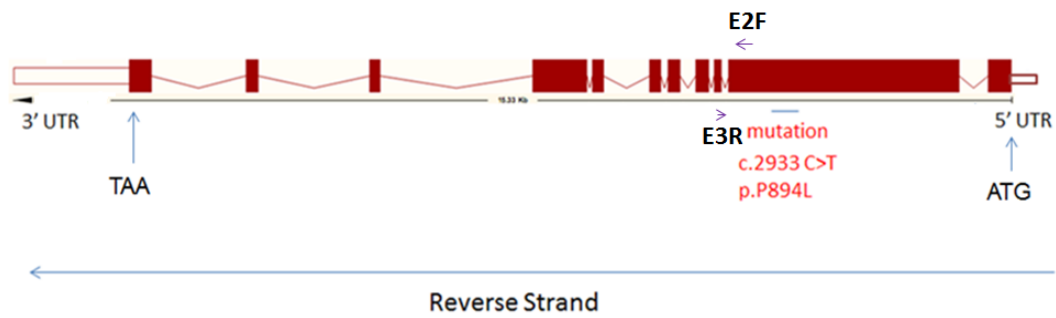


Figure 29. Illustration showing the location of the primer pair, which was designed to reveal the effect of *wdr81* morpholino on splicing. The forward primer was designed to bind exon 2 and the reverse primer was designed to bind exon 3 so that a shift in the band size would be detected as a possible outcome of the morpholino microinjection. The length of the intron 2 is 123 bp. long. The size of the band amplified from the morphant samples was expected to be 123 bp higher than that of the wild type samples.

3.6.2. Initial Results from Phenotype Characterization of *wdr81* Morphants

Since *WDR81* was suggested to be a neurodevelopmentally important gene in the literature^{12,36} and by our data, knockdown of this gene was expected to affect the central nervous system. Head size measurements were carried out in the morphant embryos firstly, in order to search for a possible widespread effect of the *wdr81* morpholino. One of the possibilities was hypothesized to be a microcephaly phenotype since significant decline in the volumes of cerebellum and corpus callosum in patients were detected¹². Head size measurements were performed by following the report of Golzio *et al.*, (2012) in parallel with the dose curve study.

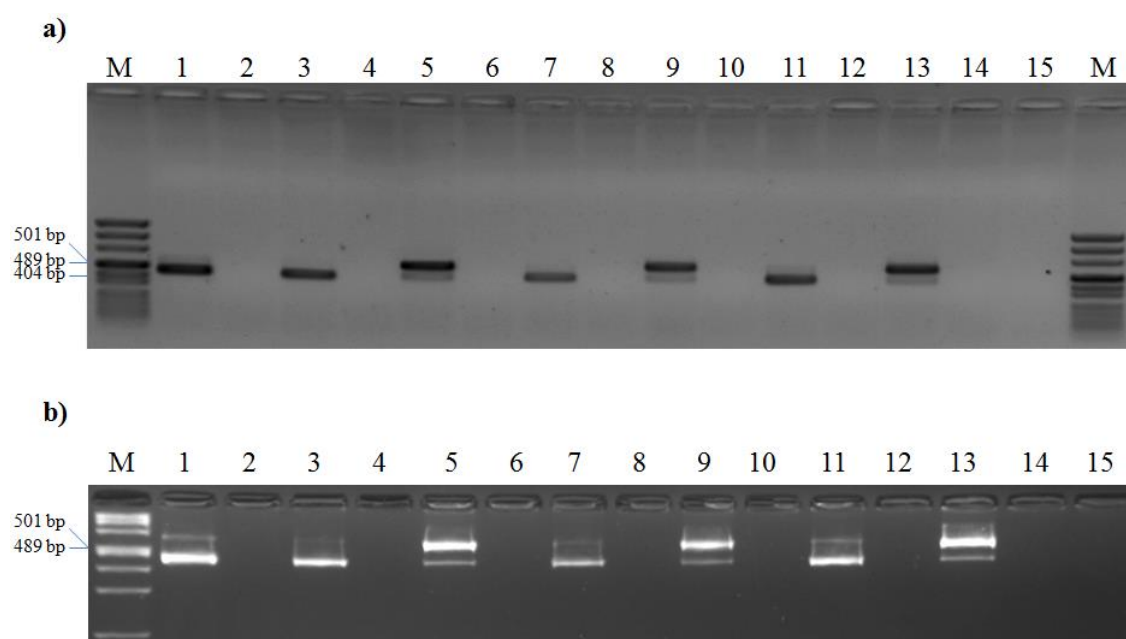


Figure 30. Agarose gel electrophoresis of the PCR products from a reaction showing the effect of three doses of *wdr81* morpholino on splicing. a) The PCR product were run on a 1% agarose gel, b) The same PCR products were run on a 4% agarose gel. Both (a) and (b) gels, templates in lane 1: cDNA of uninjected embryo, lane 2: -RT control for uninjected embryo, lane 3: cDNA of 2 ng standard negative control morpholino injected embryo, lane 4: -RT control for 2 ng standard negative control morpholino injected embryo, lane 5: cDNA of 2 ng *wdr81* morpholino injected embryo, lane 6: -RT control for 2 ng *wdr81* morpholino injected embryo, lane 7: cDNA of 4 ng standard negative control morpholino injected embryo, lane 8: -RT control for 4 ng standard negative control morpholino injected embryo, lane 9: cDNA of 4 ng *wdr81* morpholino injected embryo, lane 10: -RT control for 4 ng *wdr81* morpholino injected embryo, lane 11: cDNA of 8 ng standard negative control morpholino injected embryo, lane 12: -RT control for 8 ng standard negative control morpholino injected embryo, lane 13: cDNA of 8 ng *wdr81* morpholino injected embryo, lane 14: -RT control for 8 ng *wdr81* morpholino injected embryo, lane 15: no template negative control. Lane indicated as M was loaded with pUC mix DNA Marker (SM0301, Thermo Scientific).

Changes in the head size of embryos from experiment groups were detected with the measurement of the distance between the convex edges of the cornea of two eyes between the timepoints 4.25 and 4.5 dpf. Body lengths of the embryos were also measured to compare their development (Figure 31).



Figure 31. A representative picture (taken with a Leica MZ10F microscope) of an embryo, whose head size and body length were measured via Leica Application Suite 4.3 (LASv 4.3) software. *The body length measurements among experiment groups were not hypothesized to change significantly since a developmental delay was not expected, instead a significant change in the head size measurements among experiment groups were hypothesized based on the assumption that knockdown of *wdr81* would cause impairments in development of the nervous system.*

8 ng *wdr81* morpholino and 8 ng standard negative control morpholino were injected to embryos and an uninjected control group was separated. The survival rates of the experiment groups were recorded (Table 9). A researcher from our research group, who was blind to the experiment groups, performed the measurements, then the data was sorted according to the experiment groups. One way Anova analysis was performed in order to evaluate whether the differences between groups were statistically significant or not. As a result, 3.88% reduction in the head size of *wdr81* morpholino injected group compared to uninjected group was found to be significant with a p value of 0.027 and 2.53% reduction in the body length of negative control morpholino injected group compared to uninjected group was found to be significant with a p value of 0.043. The mean of head size measurements were 0.545 mm $\pm$ 0.042 in *wdr81* morpholino injected group; 0.560 mm $\pm$ 0.034 in negative control morpholino injected group and 0.567 mm $\pm$ 0.032 in uninjected group. The mean of body size measurements were 3.45 mm $\pm$ 0.22 in *wdr81* morpholino injected group; 3,46 mm $\pm$ 0,24 in negative control morpholino injected group and 3,55 mm $\pm$,098 in uninjected group.

Table 9. Survival rates of the embryos from experiment groups in the head size measurement study

Experiment Groups	Number of Surviving Embryos	Number of Dead Embryos	Survival Rate	Dose
<i>wdr81</i> morpholino injected group	28	77	26.66%	8 ng (data from 4 experiments were collected)
Standard negative control morpholino injected group	55	50	52.38%	
Uninjected control group	75	30	71.43%	

After testing differences in head sizes of experiment groups and after the dose curve was established, we set up another experiment design in order to evaluate changes in expression of a marker gene in the *wdr81* morphants. *gbx2* expression was compared in the three experiment groups, which were 2 ng *wdr81* morpholino injected group, 2 ng standard negative control morpholino injected group and uninjected control group. *gbx2* expression is known to be crucial for development of both midbrain and anterior hindbrain¹⁴⁶. In morpholino microinjection experiments, after the effect of the morpholino on the protein level or on splicing is shown, general approach is to check how the expression of relevant genes are affected from the knockdown of the gene of interest. Whole mount *in situ* hybridization method is generally utilized for this purpose because of the lack of a variety of antibodies in zebrafish research. This approach might be applied in order to reveal the downstream or upstream targets in the same pathway¹⁵¹, to observe how specific cell types are affected from the knockdown of the gene of interest by labelling them with probes¹⁵² or to check how expression of patterning markers such as *gbx2* are affected¹⁵³. *gbx2* was preferred in this study as a marker gene because of being a crucial gene for development of both midbrain and anterior hindbrain, where cerebellum takes place. *WDR81* was reported to be associated with CAMRQ and cerebellar hypoplasia was observed in patients¹². Knockdown of *wdr81* needed to be evaluated by taking this information into account. In addition, expression of *wdr81* was detected in the midbrain in zebrafish embryos. Testing how expression of *gbx2* was affected from the knockdown of *wdr81* was an appropriate approach because of combining both of the significant expression sites, ie. midbrain and cerebellum. Microinjection of 2 ng dose was preferred in this experiment set up because the dose curve was already established and all the tested doses were shown to be efficient. When all the doses are efficient, the common approach is to use the lowest dose of morpholino in order to avoid toxicity and higher mortality rates. The WMISH experiment was carried out under the same conditions for all three groups and their images were also taken using the same parameters. The *wdr81* morphant group appeared to be stained stronger than the control groups (Figure 32).

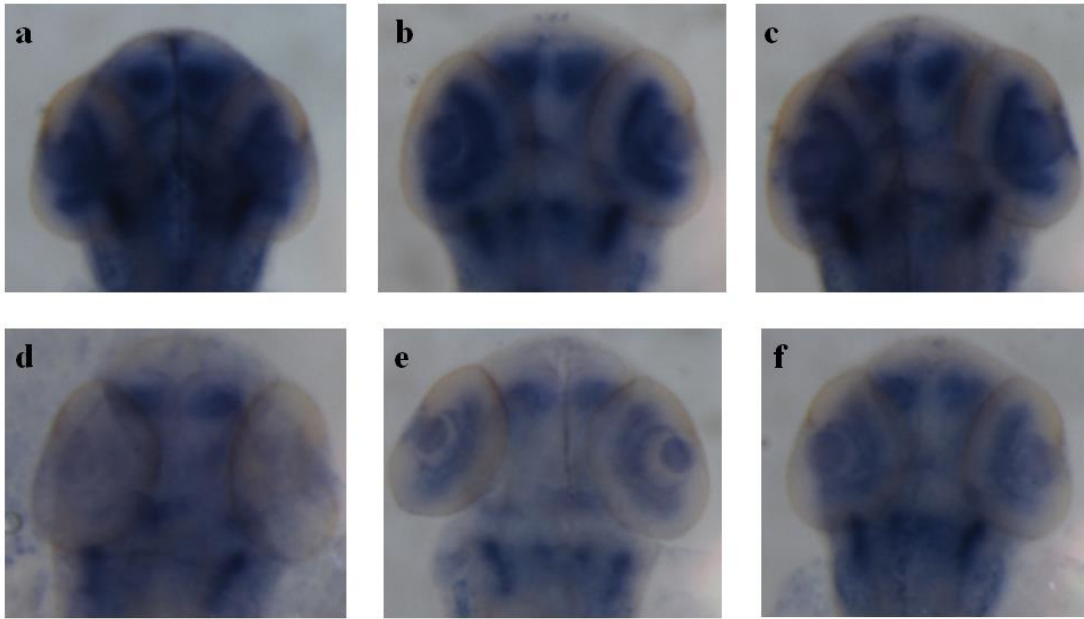


Figure 32. Comparison of *gbx2* expression among 3 experiment groups, which were 2 ng *wdr81* morpholino injected (a-c), 2 ng standard negative control morpholino injected (d,e) and uninjected (f) embryos. The intensity of the *gbx2* probe was found to be stronger in the *wdr81* morphant group (a-c) compared to the control groups. The only variable was the microinjection of *wdr81* splice blocking morpholino in the embryo group, whose representative pictures were shown in the a-c panel and the rest of the experimental conditions were identical in control groups. This result might be indicating a compensation mechanism since *gbx2* functions in the development of midbrain and anterior hindbrain and *WDR81* is associated with cerebellar hypoplasia. Also, the expression of *wdr81* in 2 dpf wild type embryo was detected the highest in the midbrain, eye region, diencephalon and MLF.

Chapter 4. Discussion

WDR81, the gene which encodes WD repeat containing protein 81 was reported to be associated with CAMRQ. Quadrupedal gait as a movement disorder was observed in the patients. The most significant abnormalities detected in the brains of the patients were cerebellar hypoplasia and volume loss in corpus callosum¹². While research on zebrafish model organism were ongoing, a study on the *Wdr81* was published, which reported a phenotype similar to CAMRQ in *nur5* mutant mouse model³⁶. In the present study, the transcript of the zebrafish *wdr81* was characterized and its expression profile during several developmental stages and in tissues of adult wild type zebrafish was investigated. In addition, morpholino studies were started in order to reveal the function of the *wdr81* and initial investigation was performed in search of a possible phenotype in *wdr81* morphants. *WDR81* was reported to be conserved among vertebrates¹². *In silico* analysis indicated that conserved domains in human and mouse *WDR81* proteins are also predicted to be present in the zebrafish orthologous protein (Figure 8, 9). This might also imply a conserved function of the protein of interest in three species. The characterization of the transcript structure and expression profile of *wdr81* was aimed in the first place since it was an uncharacterized gene in zebrafish. The information about *wdr81* in wild type zebrafish should provide a basis for future research such as genetic manipulations including morpholino experiments. Regarding morpholino studies, comprehensive information about the transcript structure and expression of *wdr81* in wild type zebrafish were necessary to design the morpholino sequence, to evaluate the effect of morpholino on splicing and to determine the critical timepoints and relevant tissues for characterization of morphants.

This study is the first to report the transcript structure and expression profile of *wdr81* in zebrafish along with attempts to characterize the *wdr81* morphants. The results of this study showed that zebrafish *wdr81* possessed one ORF and one 5'UTR structure (Figure 13, 15). The predicted sequence for 3'UTR was confirmed along with some variants (Figure 19, Table 6) and a detected insertion site in samples from ten evaluated

developmental timepoints and in several adult tissues such as brain, testis, heart, liver, eye, tail and muscle. The length of this region was determined as 266 bp and this region was not detected in rest of the adult tissues such as kidney, intestine and gills (Figure 20, 21). Detecting the insertion site in some adult tissues but not in the rest of the evaluated tissues might imply an alternative polyadenylation event. *wdr81* appeared to be maternally supplied based on the qRT-PCR study. Crucial timepoints during early development and after zygotic expression started were 5 hpf and 18 hpf because the temporal expression study revealed the peaks in *wdr81* expression at these timepoints (Figure 22). *wdr81* was also shown to be expressed in all of the evaluated adult tissues (Figure 25). In embryos, the signal of the riboprobe which showed the site of expression was stronger in the eye and brain regions at 18 hpf and 48 hpf timepoints (Figure 23). In particular, the intensity of the signal was the highest in the midbrain and optic vesicle at 18 hpf and the signal was mainly detected in lens, preoptic area, optic nerve, retina, diencephalon, midbrain, midbrain tegmentum, medial longitudinal fascicle (MLF) and nucleus of the MLF at 48 hpf. The signal, which was weaker compared to the previous developmental stages, was detected in the midbrain tegmentum and retina at 72 hpf (Figure 23, 24). The expression profile of *wdr81* in the adult brain and eye indicated that *wdr81*-positive cells had neuronal morphology and some *wdr81*-positive regions were proliferative zones (Figure 26). After establishing the expression in the wild type organism, the number and structure of the transcript, splice blocking morpholino experiments were carried out in order to search for a phenotype. These attempts were mainly including two approaches: 1) searching for a microcephaly phenotype 2) initial characterization of morphants by comparing *gbx2* expression in experiment groups. Reduction in head size was 3.88% in *wdr81* morphant group compared to uninjected control group, which was significant (p:0.027). The signal of the riboprobe, which was synthesized to detect *gbx2* expression, was stronger in *wdr81* morphant group compared to negative control and uninjected groups (Figure 32).

Existence of nine protein coding splice variants in human (Figure 10) and three protein coding splice variants in mouse (Figure 11) motivated the study to detect the number of *wdr81* transcripts in zebrafish. This set of experiments were also performed to provide an experimental validation of the transcript prediction made by Ensembl (*wdr81*-001,

ENSDART00000156621) (Figure 12), which was taken as a model for designing gene specific primers. UTR structures were characterized also because these significant regions mediate gene expression at the post-transcriptional level¹⁵⁴. RACE method was applied in order to characterize the UTR regions. Tissue specific transcription start sites^{155,156}, and 3'UTR structures in different lengths¹⁵⁶ are possible to be detected by employing this method. One ORF and one 5'UTR structure were detected as predicted (Figure 13, 15). One 3'UTR structure along with a detected insertion site appeared to exist in the tested samples which were brain and 24 hpf embryo (Figure 19). Detecting the insertion region in most of the evaluated tissues and not in kidney, intestine and gills (Figure 21b) might be explained by the fact that alternative cleavage and polyadenylation processes were identified in tissue specific regulation of protein levels encoded by most of the ubiquitously transcribed genes. This mechanism was suggested to change the proportion of 3'UTR variants and so that result in switching the targets for microRNAs and regulatory elements¹⁵⁷. When the signals of the bands from the reaction, in which the insertion site was amplified, were measured, the strongest signals were detected from brain, testis and eye (Table 7). Gene expression profiles across brain, cerebellum and testis were previously reported to be the most similar in human and mouse compared to the similarity among other tissues¹⁵⁸. The eyes, in particular the retina or neural part of it, are components of the central nervous system¹⁵⁹. A similar mechanism in 3'UTR mediated post-transcriptional regulation might take place in brain and eyes. The potential mechanisms mentioned above might have roles in producing different levels of proteins and in changing the activity of *wdr81*.

As the temporal expression study revealed that the crucial timepoints of development regarding *wdr81* expression were 5 hpf and 18 hpf (Figure 22), relevant critical anatomical developments at these timepoints were searched. At 5 hpf, presumptive brain is expected to have formed since it is known to appear between 4-4.33 hpf at sphere stage. At 18 hpf, the brain structure and optic vesicle is expected to have formed since they are known to appear between 14-16 hpf and 11.66-14 hpf at the segmentation stage, respectively¹¹². The lower levels of expression during rest of the timepoints after 18 hpf also implied that this maintained level of *wdr81* was required for development (Figure 22). WMISH results were in parallel with the qRT-PCR results in general. The

signal intensity of the riboprobe, which was designed to detect the site of *wdr81* expression, was high between 6 hpf and 18 hpf, and reduced after 18 hpf. The signal was homogenously distributed at 6 hpf, 10 hpf, 18 hpf and 24 hpf, it was condensed in the brain and eye at 48 hpf. The signal was clearly diminished at 72 hpf (Figure 23). Increased expression of *wdr81* in the brain and eye regions at 18 hpf and 48 hpf were in parallel with the previous reports from human and mouse studies^{12,35,36}. Observing the *wdr81* RNA probe signal highest in the brain and eye regions at 18 hpf and 48 hpf timepoints (Figure 23) and detecting the peaks regarding *wdr81* relative temporal expression at the early developmental stages such as 1 hpf, 5 hpf and 18 hpf (Figure 22) imply the potential significance of *wdr81* in neurodevelopment. Microarray data from the literature verified that *wdr81* was expressed in the retina of zebrafish embryo¹⁶⁰ and in brain tissues of young and old adults¹⁶¹. The potential significance of *WDR81* in neurodevelopment of human and mouse was suggested in the previous reports as well^{12,36}. Determining the critical stages of development regarding *wdr81* expression also served to designate the timepoints for characterization of *wdr81* morphants.

The spatial expression study revealed that *wdr81* was expressed in all of the evaluated adult tissues, which is also called as ubiquitous expression (Figure 25). Human and mouse *WDR81* were also detected to be expressed in all of the evaluated adult tissues^{12,36}. The levels of the expression were changing across tissues of adult zebrafish (Figure 25). The signal of the *wdr81* RNA probe was homogenously distributed at 6 hpf, 10 hpf, 18 hpf and 24 hpf timepoints, too (Figure 23). Cerebellum was not detected to be one of the brain regions with strong signal in zebrafish embryos from six developmental timepoints, which might be evaluated to be in contrast with the mouse data obtained from embryonic brain because expression of *Wdr81* was found to be increased in cerebellum¹². The expression data from juvenile mouse (P21) also showed expression in the cerebellum and retina³⁶. Nevertheless the regions detected with higher signals in embryos had connection with either cerebellum or the eye. The MLF is a group of axons, which starts from the nucleus of the MLF continues ventral to the rhombencephalic ventricle through the caudal end of the medulla oblongata, functions in coordinating conjugate eye movements^{112,162}. The diencephalon is made up of thalamus and hypothalamus. The retina originates from the diencephalon, it forms a

vesicle out from diencephalon to form the retina and is called as optic vesicle and invagination of the optic vesicle end up with the optic cups. The retina develops from the inner wall of the optic cup and the pigment epithelium develops from the outer wall of the optic cup¹⁶². The midbrain (mesencephalon) also participate in motor function and sensory functions¹⁶³. The expression of *wdr81* of the adult brain and eye tissues was observed on the sections from the cerebellum, retina, tectal ventricle, brain stem and optic tectum (Figure 26). Although strong signals were not detected from the cerebellum of the embryos, the expression of *wdr81* was detected in the retinal layers and presumptive Purkinje cells of cerebellum in adult study (Figure 26) which was consistent with the data from human and mouse studies^{12,36}. In the human study, retinal expression of *WDR81* was not mentioned¹² however neuro-ophthalmic examination of four patients from the same reported family revealed downbeat nystagmus diagnosis³⁵. This eye disorder might be indicating the expression of *WDR81* in the eye since it is an accompanying case in the patients affected from the mutation in the *WDR81* isoform 1. *Wdr81* expression was also detected in brain stem of wild type adult mouse³⁶. The common expression sites in three species are also providing a supporting evidence for conserved function of *WDR81*.

Detecting *wdr81* RNA probe in the presumptive Purkinje cells, retina, optic tectum, optic nerve and other nerve fibers (Figure 24, 26) suggested that the expression product of this gene in both embryos and adult zebrafish was located in several neuronal phenotypes. Confirmation of the neuronal phenotypes in future studies is needed to be performed using both *wdr81* RNA probe and immunostaining with neuronal markers. Some of the neuronal markers used in zebrafish research are *gad1* or *gad2* to detect GABAergic neurons¹⁶⁴, which fulfill inhibitory action, and antibodies against glutamate receptor 1, glutamate receptor 2, glutamate receptor 2/3 or glutamate receptor 4 to detect glutamatergic neurons¹⁶⁵, which fulfill excitatory action. Purkinje cells can be detected with zebrin 2¹⁶⁶ or M1¹⁶⁷ antibodies. Proliferation status of cells in zebrafish studies is possible to detect with antibodies against phosphohistone 3¹⁶⁸, proliferating cell nuclear antigen (PCNA)¹⁶⁹, histone H1¹⁷⁰ or 5-bromo-2'-deoxyuridine (BrdU)¹⁷¹. Also counterstaining with an early neuronal marker or a late neuronal marker is expected to exhibit the association of the expression of *wdr81* with the stage of the

neuronal proliferation. Hu as an early neuronal marker^{171–173} and neuronal nuclei (NeuN) as a late neuronal marker^{174,175} might be used in this type of study. Since glial cells appear at 26+ somites stage (segmentation, 22–24 hpf) in zebrafish¹¹² and *wdr81* expression was determined at the highest levels in the first 18 hpf (Figure 22, 23), this gene was not suggested to be associated with glial cells. Besides, the morphology of the cells, which were *wdr81*-positive in the adult brain sections (Figure 26a,c,f,g), were implying a neuronal morphology in nature. Neurogenesis starts at late gastrulation, around 10 hpf and proceeds during embryogenesis¹¹⁹ and synaptogenesis starts around 18 hpf in zebrafish⁸⁴. Observing the peak in expression of *wdr81* at 18 hpf (Figure 22) might also be indicating a potential role of this gene in neurogenesis, neuronal migration and survival of neurons in embryos. Furthermore, the brain regions where *wdr81* expression was detected, such as the tectal ventricle, vagal lobe, optic tectum and periventricular gray zone of the optic tectum (Figure 26) were reported as proliferative zones¹⁷⁶. Retinal neurogenesis is a persistent process during whole life of teleost fish including zebrafish¹⁷⁷. A microarray study, which demonstrated the differential expression of genes after a lesion occurred in the retina, revealed that the expression of *wdr81* increased between 5 days and 7 days post-lesion¹⁷⁸. This finding also contributes to the suggestion that *wdr81* might have a potential function relevant with neurogenesis, which might be retinal neurogenesis in this report. Detecting the expression of *wdr81* in retinal layers and other proliferative zones in the brain (Figure 26) also points out the potential role of this gene in neuronal proliferation, neuronal migration and survival of neurons in the adult zebrafish. Neurogenesis related function of *wdr81* was also predicted *in silico*^{112,179}. The pattern of the strong signals in Figure 26 c and f might indicate the relevance of the function of *wdr81* with neuronal proliferation since the signals strongly come from the proliferative zones and that in Figure 26 g might indicate the relevance of the function of *wdr81* with neuronal migration since the signals in the optic tectum appear to come from the cells spread in this layer. This set of data obtained from the expression studies provide evidence about the potential role of *wdr81*.

Gulsuner *et al.* (2011) suggested that *WDR81* might be a critical gene in neurodevelopment and motor behavior¹². Research on the *nur5* mutant mouse model,

which demonstrated a phenotype similar to CAMRQ, concluded that localization of *Wdr81* was the mitochondria of Purkinje cells and photoreceptor cells. The homozygous missense mutation, affecting the predicted MFS domain, caused loss of Purkinje cells and photoreceptor cells. Loss of Purkinje cells was suggested to take place after mitochondrial abnormalities occurred during development³⁶. *WDR81* gene was reported to be mutated in several colorectal cancer cell lines³⁷ and it was located in a metabolically important loci, which was found to be associated with serum albumin level³⁸. Serum albumin level is metabolically important because of its inverse correlation with cardiovascular and mortality risk^{39,40}. The potential function of *wdr81* might be projected based on the conserved domains. Since BEACH-domain containing proteins have roles in membrane-related events, such as vesicular transport, receptor signaling and formation of synapses⁴¹, WD40 domain-containing proteins function in cell cycle control, signal transduction, regulation of transcription, chromatin dynamics and vesicular trafficking⁴² and the MFS transporters function in transport of small molecules⁴³, these functions together might be relevant to a potential function of *wdr81* in synaptic plasticity and neuronal proliferation. A microarray study showed that the expression of *wdr81* in zebrafish was also affected by the presence of a mutation in *p100* gene, which encodes a recycling factor in spliceosome cycle. The expression of *wdr81* showed a declining trend at 3 dpf and a rising trend at 5 dpf in *egy* mutants, the strain which carries the mutation in *p100* gene, compared to the wild type siblings. The rising trend was interpreted as an indication of a compensation mechanism in the spliceosome cycle by the authors¹⁸⁰. This finding might also be emphasizing that the expression of *wdr81*, as a critical gene in neurodevelopment, is tightly bound up with the changes in cellular functions. The functions of other CAMRQ associated genes in central nervous system were defined. *VLDLR*, in interaction with reelin, acts in guiding migration of neuroblasts in the cerebral cortex and cerebellum during development^{26,27}. It also functions in coordination of Purkinje cell alignment in the cerebellum during development^{28,29} and it is expressed in synapses³². *CA8* expression in human fetal brain was detected in neuroprogenitor cells in the subventricular zone and in the neurons migrating to the cortex⁶⁹. Overexpression of *CA8* resulted in reduced cell death in neuronal cell lines and downregulation of the same gene decreased cell migration and invasion⁷¹. *Atp8a2* in coordination with *Cdc50a* functions in axon elongation of neurons and hippocampal neuronal differentiation in rats⁸⁰. Thus, CAMRQ associated genes

have roles in neuronal migration, survival and differentiation, which overlap with the roles suggested for *wdr81* in this study. Although the data accumulated so far from the literature serves some clues about the function of *WDR81* and its significance in both neurodevelopment and metabolism, exact function of this gene is not fully understood.

The morpholino experiments, which were carried out in order to develop an understanding of the function of *wdr81*, were performed with 2 ng and 8 ng *wdr81* morpholino doses. The survival and mortality rates of embryos were recorded during experiments. Based on my observations during morpholino microinjection experiments, the differences in mortality rates of zebrafish embryos were based on the wild type strain used, regular and frequent feeding with artemia along with the dry food, and the age of the fish. We had AB strain zebrafish from two sources in our zebrafish facility, University of Madison and Karlsruhe Institute of Technology. The first wild type strain to be brought to the zebrafish facility was AB strain from University of Madison and using only this strain in breeding set-up gave low rates of survival, which might be related with inbreeding. Using zebrafish pairs in combination of two sources, ie. the strain from University of Madison and the strain from Karlsruhe Institute of Technology, for breeding gave better survival rates. Especially this observation is suggested to be the explanation to the discrepancy in survival rates reported in Table 8 and 9. The microcephaly phenotype search with 8 ng morpholino resulted in 3.88% reduction in head size in the *wdr81* morphant group compared to uninjected control group ($p:0.027$), however the microcephaly phenotype in the literature was reported to exhibit a higher ratio in the reduction, being around 20% reduction¹⁴⁵. The reduction ratio in the head size should be obtained in the morphant group compared to the standard negative control morpholino injected group, not compared to the uninjected control group. Searching for a widespread change in the head size might be replaced with a strategy to search for regional changes or for changes at the molecular level because the detected abnormalities in the patients were morphological differences in the precentral gyrus and Brodmann areas BA6, BA44 and BA45, along with significant decline in the volumes of cerebellum and corpus callosum¹² instead of global changes such as microcephaly.

When *gbx2* expression among the experiment groups was compared, the signal of the *gbx2* RNA probe was observed to be stronger in *wdr81* morphant group compared to negative control and uninjected groups (Figure 32). Since *gbx2* is known to be a critical gene for development of both midbrain and anterior hindbrain¹⁴⁶, the stronger signal of the *gbx2* RNA probe in the *wdr81* morphant group could be interpreted as a possible compensation mechanism. Similar WMISH studies with other marker genes might be used to confirm this result. The changes in the structures of brain and eye might be investigated. Furthermore, the changes in the synapses, such as changes in the number, size and composition of synapses might be searched at the electron microscopy level in the morphant group compared to the control groups.



Chapter 5. Future Perspectives

The findings about the transcript structure and the expression profile of zebrafish *wdr81* serve both fundamental information about characterization of *wdr81* as a novel gene in wild type zebrafish and a basis for functional studies. In this study, morpholino studies and characterization of morphants were initiated. The search for a microcephaly phenotype in *wdr81* morphants did not give results comparable to the literature because the significant change in morphant group was not obtained compared to the negative control group and the percentage in head size reduction was below the ranges in the literature. However, the results obtained in experiments groups by comparing *gbx2* expression were promising. The signal with *gbx2* RNA probe obtained from *wdr81* morphant group was stronger than that of control groups. This might be indicating a compensation mechanism following knockdown of *wdr81*, still having obtained a change in expression suggests the method to continue with. Similar WMISH studies with other marker genes such as retinal homeobox gene 3 (*rx3*)¹⁸¹ to observe development of eye, homeobox A2b (*hoxa2b*)¹⁸² to observe development of hindbrain, orthodenticle homeobox 2 (*otx2*)^{183–185} to observe anterior neural plate formation and midbrain might be used to confirm and improve this finding. The changes in the structures of brain and eye in the morphant group might be investigated comprehensively. Moreover, electron microscopy studies might be employed to detect some subtle changes such as differences in the number, size, type and composition of synapses in the morphant group compared to the control groups.

Another approach to continue morpholino experiments might be to knock down another CAMRQ associated gene, such as *vldlr*, in parallel with knock down of *wdr81* to observe whether similar phenotypes are obtained. As the function of *vldlr* in central nervous system is known as an integrative element in the reelin pathway and in guiding neuroblast migration in the developing cerebral cortex and cerebellum^{26,27}, the potential function of *wdr81* and the possibility of two genes taking place in the same pathway might be concluded by comparing the morphant phenotypes. Double knockdown of *wdr81* and *vldlr* might be also performed to detect whether a synergistic effect on the

morphants are obtained. A knockout model might be generated to perform functional experiments on zebrafish so that how the progress of a *wdr81* lacking zebrafish embryo to adulthood would be possible to monitor. This type of a model would be stable considering the longest effect of morpholino microinjection lasts for around five days¹⁰⁰. Both WMISH with marker genes might be performed to detect the changes and relevance of *wdr81* with neuronal proliferation might be detected by using BrdU labeling, which was a method established during this study (Figure 33).

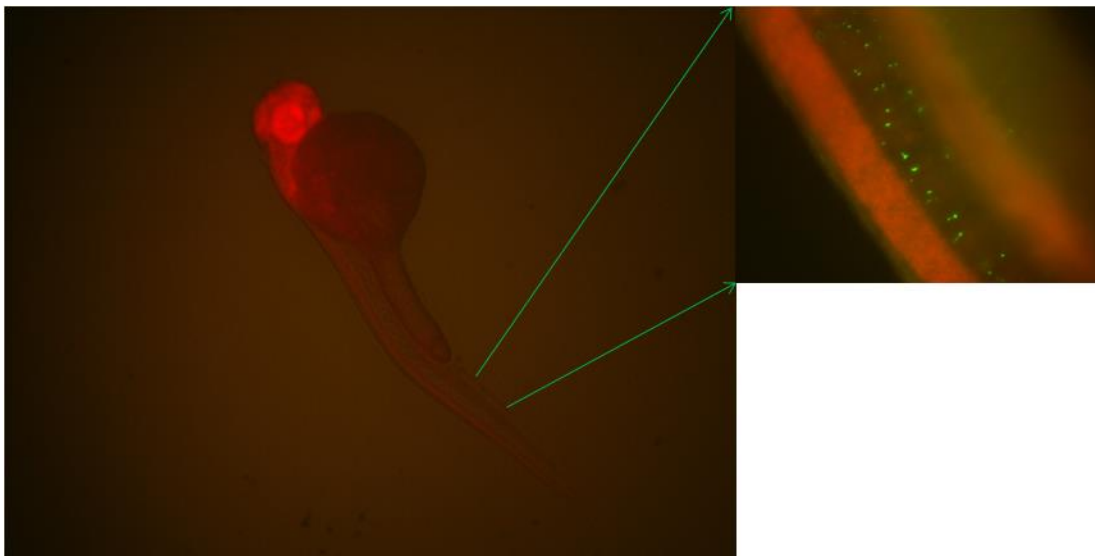


Figure 33. A twenty four hours post fertilization zebrafish embryo which was incubated with BrdU for 24 hours. BrdU label is in green and the nucleus is stained in red with 7-AAD. Establishing the BrdU labelling method in our laboratory was important regarding our capability to employ this method in neuronal proliferation experiments.

The cell culture system is an alternative method to devise a strategy for uncovering the function of *wdr81*. Primary neuron culture is an established method in the literature and neurons from zebrafish embryos might be cultivated¹⁸⁶. Three experiment groups might be prepared as a primary neuron culture overexpressing *wdr81*, a primary neuron culture in which *wdr81* is knocked down and a wildtype culture. The migration capacity of these experiment groups might be compared so that the potential function of *wdr81* in neuronal migration would be possible to test.

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Appendix A

Buffer And Solutions

Boehringer Blocking Solution (50 ml)

- Blocking reagent.....0.5 g
- FBS.....2.5 ml
- TNT buffer is added to reach the final volume, the solution is heated at 60 °C, aliquoted after cooling and the aliquotes are stored at -20 °C.

Buffer B2 for ISH (1 liter)

- 1 M Tris pH:7.5.....100 ml
- 5 M NaCl.....30 ml
- 10% Tween 20.....10 ml

Buffer B3 for ISH (1 liter)

- 1 M Tris pH:9.5.....100 ml
- 5 M NaCl.....20 ml
- 1 M MgCl₂.....50 ml

Buffer B4 for ISH (10 ml)

- BCIP (50 mg/ml).....37.5 µl
- NBT (100 mg/ml).....37.5 µl
- Levamisole (24 mg/ml).....2 drops
- Buffer B3.....9.825 ml

Danieu's Solution (100 ml)

- 2 M NaCl.....2.9 ml
- 3 M KCl.....23.3 μ l
- 100 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 400 μ l
- 100 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ 600 μ l
- 1 M Hepes pH: 7.5.....500 μ l
- pH should be between 7.1-7.3, the solution is filter sterilized and stored at 4 °C.

Denhardt's Solution (50x, 100 ml)

- Ficoll type 400.....1 g
- Polyvinylpyrrolidone (PVP).....1 g
- Bovine serum albumin (BSA) fraction V.....1g
- The solution is mixed overnight at 4 °C, filter sterilized and aliquoted, the aliquotes are stored at -20 °C.

Ethidium Bromide Solution

- Stock solution: 10 mg/ml in water
- Working solution: 30 ng/ml

E3 Medium, Stock Solution (60x, 1 liter)

- NaCl.....17.2 g
- KCl.....0.76 g
- CaCl₂.2H₂O..... 2.9 g
- MgSO₄.7H₂O.....4.9 g
- The medium is autoclaved and the stock is stored in fridge.

E3 Medium, Working Solution (1x, 1 liter)

- 60x E3 medium.....16 ml
- 0.01% Methylene blue.....3ml

Hybridization Buffer for ISH (50 ml)

- Formamide.....25 ml
- 20xSSC.....12.5 ml
- 50xDenhardt's solution.....5 ml
- Yeast RNA (20 mg/ml).....625 µl
- Salmon sperm DNA (10 mg/ml).....2.5 ml
- Heparin (5 mg/ml).....500 µl
- 0.5 M EDTA.....250 µl
- 10% Tween-20.....500 µl
- Chaps (1 mg/ml).....125 µl

Hybridization Buffer for WMISH (50 ml)

- Formamide.....25 ml
- 20xSSC.....12.5 ml
- Heparin (5 mg/ml).....500 ul
- TorulaRNA.....25 mg
- 10% Tween 20.....0.5 ml
- 1 M citric acid.....0.46 ml
- Buffer is stored at -20 °C.

Luria Bertani (LB) Agar (500 ml)

- Tryptone.....5 g
- NaCl.....5g
- Yeast Extract 2.5 g
- Agar.....7.5 g
- The solution is autoclaved.

Luria Bertani (LB) Broth (500 ml)

- Tryptone.....5 g
- NaCl.....5g
- Yeast Extract 2.5 g
- The solution is autoclaved.

NTMT buffer (60 ml, needs to be prepared freshly)

- 5 M NaCl.....1.2 ml
- 1 M MgCl₂.....3 ml
- 1M Tris pH 9.5.....6 ml
- 10% Tween 20.....600 ul

PBS (10x, 1 liter)

- NaCl.....80 g
- KCl.....2 g
- Na₂HPO₄·2H₂O.....15.2 g
- KH₂PO₄.....2 g
- The stock solution is autoclaved, pH of 1xPBS solution should be between 7.2 and 7.4.

SOB Medium (250 ml)

- Tryptone.....5 g
- Yeast Extract.....1.25 g
- NaCl.....0.146 g
- KCl.....0.047 g
- MgCl₂·6H₂O.....0.508 g
- MgSO₄·7H₂O.....0.616 g

SSC Buffer (20x, 500 ml)

- NaCl.....87.7 g
- NaCitrate 5 ½ H₂O.....63.5 g
- pH is adjusted to 7.0 and the buffer is filter sterilized.

TAE Buffer (50x, 500 ml)

- Trisma Base 121 g
- Acetic acid.....28.55 ml
- EDTA.....18.6 g

TNT buffer (500 ml)

- 1 M Tris-HCl pH: 8.0.....50 ml
- 5 M NaCl.....15 ml
- 10% Tween 20.....25ml

Transformation Buffer (400 ml)

- Pipes.....1.2096 g
- CaCl₂.2H₂O.....0.882 g
- KCl.....7.4554 g
- All components except MnCl₂.2H₂O are added and the pH is adjusted to 6.7 with KOH.
- MnCl₂.2H₂O.....3.5612 g
- After adding MnCl₂.2H₂O to the buffer, it is filter sterilized and stored at 4 °C.

Appendix B

Molecular Size Markers

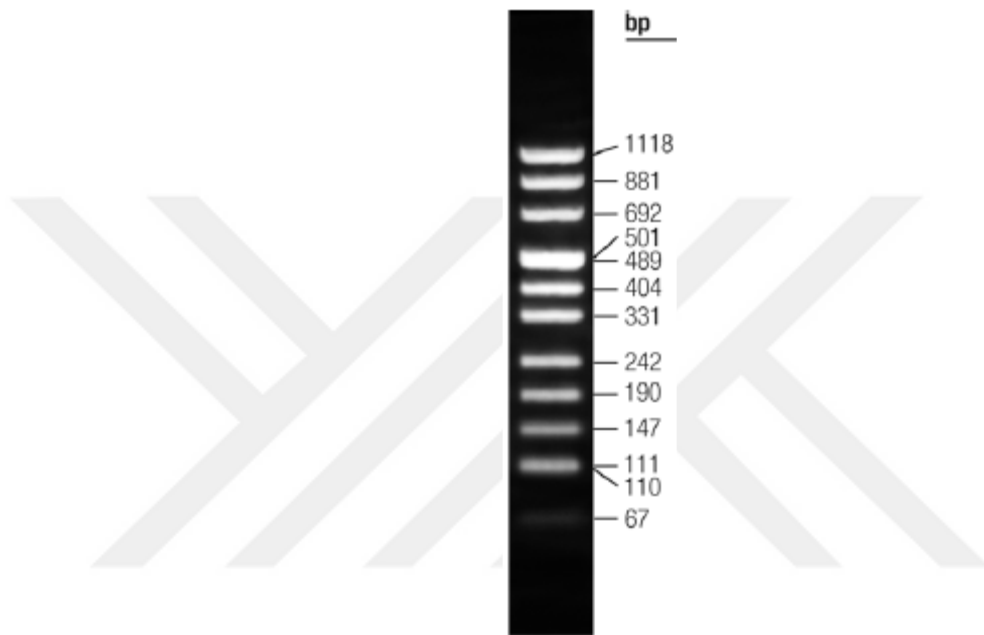


Figure B.1: pUC mix marker, 8, separated on a 1.7% agarose gel (SM0303, Fermentas).

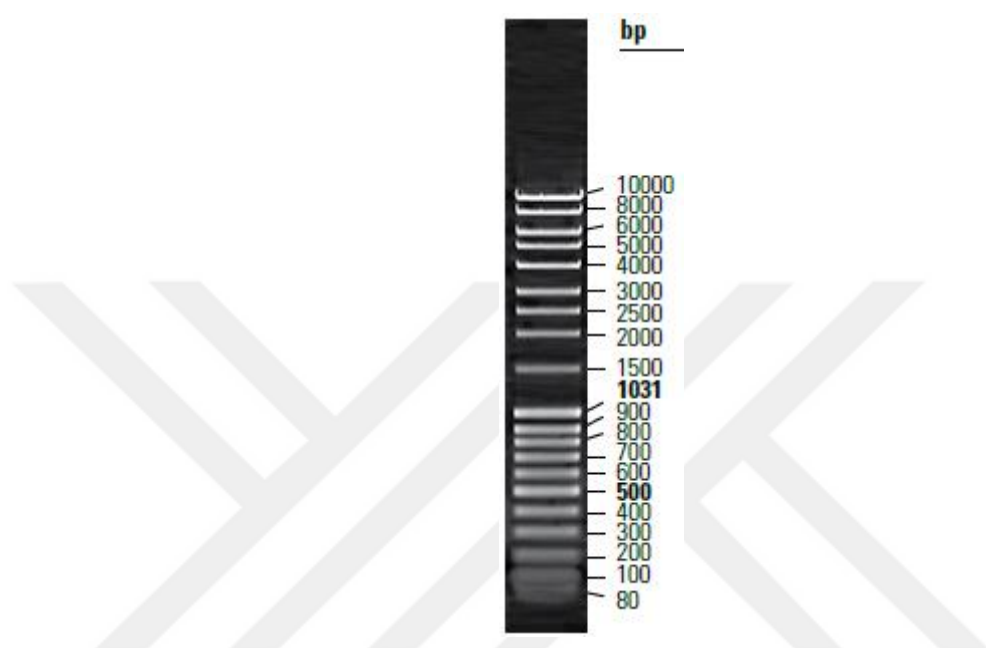


Figure B.2: MassRuler DNA ladder, seperated on a 1% agarose gel (SM0403, Thermo Scientific).

Appendix C

Cloning Vectors

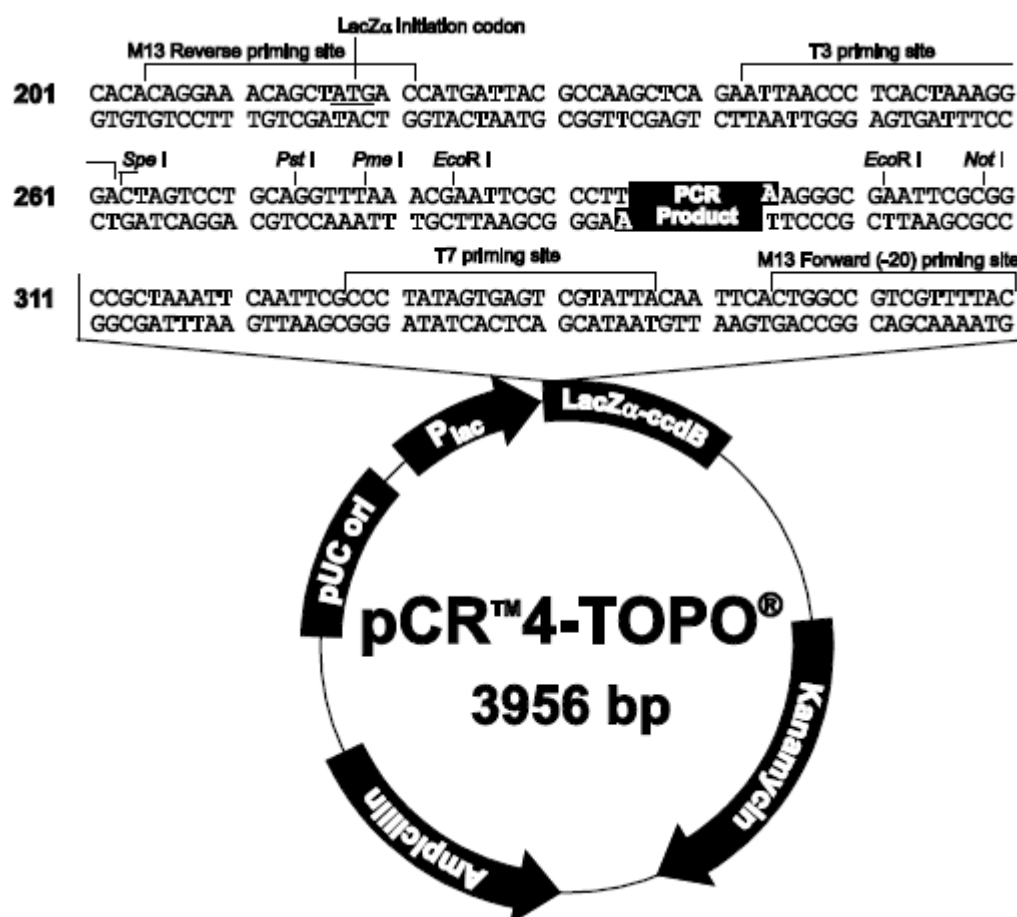


Figure C.1: pCR4-TOPO cloning vector (Invitrogen).

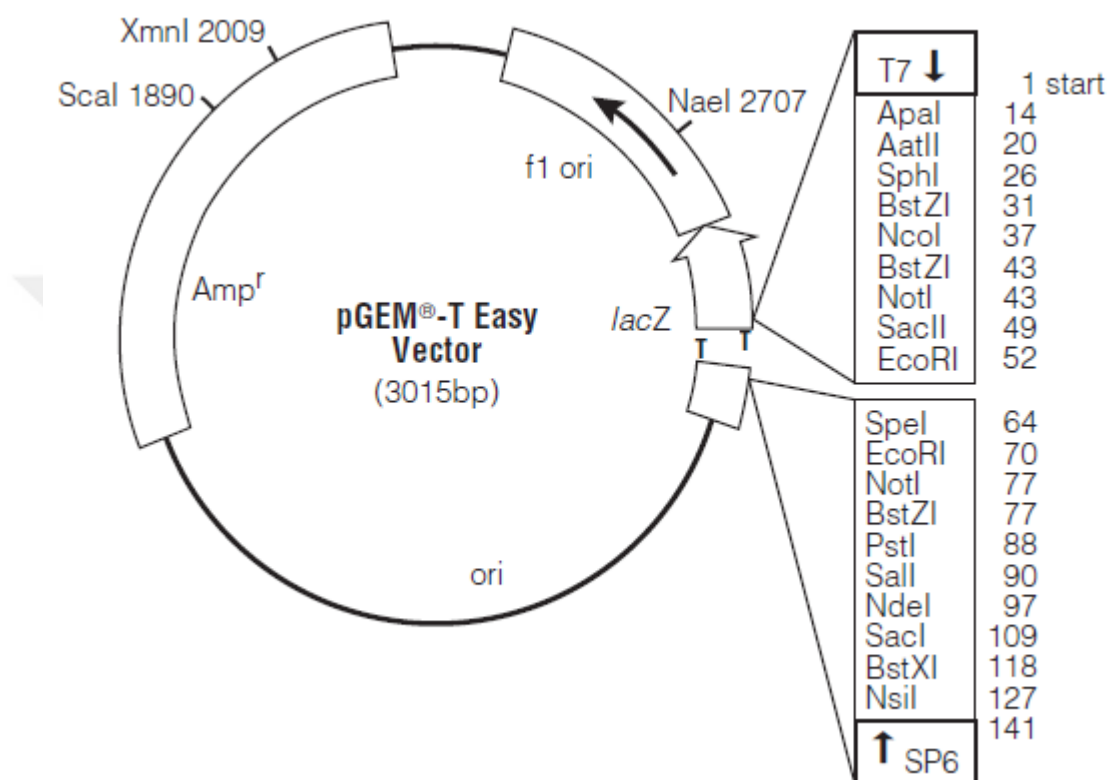


Figure C.2: pGEM-T Easy cloning vector (Promega).

Appendix D

```

gi_Danio          -----mewlapalerdlgidqrqtahsqrpnelvvlvpt
gi_Tetraodon     -----
gi_Xenopus        -----mewlleavkkelnidsrqlimaeegthvialvsq
gi_Gallus         -----maagplpgatmdallrsvekdlsinrwqlaaadgthvtalvph
gi-Taeniopygia    -----megflqsvekdlnidrrqla--perthvtafvpa
gi_Monodelphis    -----megllqsverdlidsrqlalapggthvvalvpv
gi_Mus            maqgsrrrkvvltagsegwspssgpdmeellrsverdlnidarqlalapggthvvalvst
gi_Rattus         maqgsrrrkvvltagaegcssssgpdmeellrcaerdlnidarqlalapggthvvalvst
gi_Callithrix     maqgsggregaltpaggwshsppspdmlellrsverdlidprqlalapggthvvalvpa
gi_Pongo          maqgsggregalrtpaggwshsppspdmqellrsverdlidprqlalapggthvvalvpa
gi_Homo           maqgsggregalrtpaggwshsppspdmqellrsverdlidprqlalapggthvvalvpa
gi_Pan            maqgsggregalrtpaggwshsppspdmqellrsverdlidprqlalapggthvvalvpa
gi_Oryctolagus    maqgrggrdvaltagaegwspssgpdmeellrsverdlnidarqlalapggahvvalvpa
gi_Sus            malgnrgrevaltteaegwspalspdmeellrsverdlnidarqlalapggthvvalvpa
gi_Ailuropoda     malgskgrevalthgadgswlppspdmlellqsverdlnidarqlalapggthvvalvpa
gi_Equus          malgsrgrevalttgtegwspppspdmeellqsverdlnidarqlalapggthvvalvsa

gi_Danio          rwmalrnkr--vtrcakyefsegeictllqrsqmkplsgwtrvcigglrkrklgyrfa
gi_Tetraodon     -----
gi_Xenopus        kwlasikertrvssqcpkpegfssveiqtfqlqrsvhklpagwtrieihgvrkerlaypva
gi_Gallus         kwlanlrerralpalcprpeglsvevrtflqrsvqklpagwtrveihgclkerltwplr
gi-Taeniopygia    kwlsklkerrvlpvacprpeglsvevrtflqhsvqklpagwtrveihgkrlkarlgyplr
gi_Monodelphis    rwlanlrerklppgpcprpeglsgeaetkllqrsvqklpagwnrvevhgkrlkrklayplv
gi_Mus            rwlaslrerr--lgpcpraeglgeaevrtllqrsvqrlppgwtrvevhgkrlkrklsypl-
gi_Rattus         rwlaslrerr--lgpcpraeglgeaevrtllqrsvqrlppgwtrvevhgkrlkrklsypl-
gi_Callithrix     rwlaslrdrllppgpcpraeglgeaevrtllqrsvqrlppgwtrvevhgkrlkrklsypl-
gi_Pongo          rwlaslrdrllplgpcpraeglgeaevrtllqrsvqrlpagwtrvevhgkrlkrklsypl-
gi_Homo           rwlaslrdrllplgpcpraeglgeaevrtllqrsvqrlpagwtrvevhgkrlkrklsypl-
gi_Pan            rwlaslrdrllplgpcpraeglgeaevrtllqrsvqrlpagwtrvevhgkrlkrklsypl-
gi_Oryctolagus    rwlaslrerr--lgpcpraeglgeaevrtllqrsvqrlppgwtrvevhgkrlkrklsypl-
gi_Sus            rwlaslrerrrlplgpcpraeglgeaevrtllqrsvqrlppgwtrvevhgkrlkrklsypl-
gi_Ailuropoda     rwlglslrerrrlpagpcpraeglgeaevrtllqrsvqklppgwtrvevhgkrlkrklsypl-
gi_Equus          rwlaslrerrrlppgpcpraeglgeaevrtllqrsvqrlppgwtrvevhgkrlkrklsypl-

gi_Danio          retgc--hgeglsqdsfmlmqgvsqsnfrnlwheaymthvqpyadsveqtpvlaldavr
gi_Tetraodon     -----
gi_Xenopus        lndgfle-gknidseslirfmqciavqnynmwnaartrtyvqpyesvhkvpisqaldalk
gi_Gallus         v--ppvadprgggaetlhgfmqrvasqnyrnlgwrahclyvrpyrhahappavpvdalr
gi-Taeniopygia    a--qppeqrcggttdtlhgfmqsvathnyhnlwgrahglyarpyrrpdaptpvpaldalr
gi_Monodelphis    lgsslpseehsglpetltrfmrdtaaqnyhnlwhgayhahgrpyshgnappegsaldsvr
gi_Mus            -ggvvpfeegscspetltrfmqevaaqnyrnlnwrhaytygqpyshstapsalpaldsir
gi_Rattus         -srvlpfeegscspetltrfmqevaaqnyrnlnwrhaytygqpyshstapsaipaidsir
gi_Callithrix     -ggglasedgscspetlrmfmqdvaaqnyrnlnwrhayqryaqpysrspapsaapalesvr
gi_Pongo          -ggglpfeegscspetltrfmqevaaqnyrnlnwrhaytygqpyshspapsavpaldsvr
gi_Homo           -ggglpfeegscspetltrfmqevaaqnyrnlnwrhaytygqpyshspapsavpaldsvr
gi_Pan            -ggglpfeegscspetltrfmqevaaqnyrnlnwrhaytygqpyshspapsavpaldsvr
gi_Oryctolagus    -ggglpfeegscspetltrfmqdvaaqnyrnlnwrhaytyaqpysrspapsavpalesvr
gi_Sus            -g-glpfeegscspetltrfmqdvaaqnyrnlnwrhaytygqpyshpapsavpaldsvr
gi_Ailuropoda     -ggglpfeegssspetltrfmqdvaaqnyrnlnwrhaytygqpyshnpapsavpaldlvr
gi_Equus          -ggglpfeegscspetltrfmrdvatqnyrnlnwrhaytygqpyshspapsavpaldsvr

```

Figure D.1: Multiple sequence alignment of *Danio rerio* (zebrafish) wdr81 amino acid sequence with WDR81 sequences from various species. The symbol (*) stand for the conserved residues in all WDR81 proteins, (.) and (:) symbols represent substitutions of amino acid residues.

```

gi_Danio          qalqklfcsnfistdrvspslspakekekdcplstcsapkqsteslcpnvlpaecilles
gi_Tetraodon     -----mapkicg-----vrlmantcsmrlgqsehlcpnvvpaeiglet
gi_Xenopus       lslqrvygcpfvfinkavpslspkdsss-----fvlqassssnivqaealles
gi_Gallus        aalqkaygcpilqvgrsvpsaspkg-----issvpsscpnvlqaealles
gi-Taeniopygia   aalhraygcpvlqvgrnvagmspakes-----vakavplcpnvlqaealles
gi_Monodelphis  qalqkvfgcpflqvgrgaqlspareaps-----apragtvcpnllraealies
gi_Mus           qalqrvygctflpvgesipclsnvrdgpc-----psrgspacpsllraealles
gi_Rattus        qalqrvygctflpvgesmqclsnvrdgp-----srgssacpsllraealles
gi_Callithrix    qalqrvygcsflpvgeatqcpsharegpc-----pprggpacpsllraealles
gi_Pongo         qalqrvygcsflpvgettqcpsyaregpc-----pprgslacpsllraealles
gi_Homo          qalqrvygcsflpvgettqcpsyaregpc-----pprgspacpsllraealles
gi_Pan           qalqrvygcsflpvgettqcpsyareapc-----pprgspacpsllraealles
gi_Oryctolagus   qalqrvygcpflpvgeatqgpcscardgpc-----lprgsptcpsllraealles
gi_Sus           qalqrvygcpflpvgettqcpsyardgsc-----pprgcpaspsllraealles
gi_Ailuropoda    qalqrvygcsflpvgettqcpsyardgpc-----pprgslscpsllraealles
gi_Equus         qalqrvygcsflpvgeatqcpsyardgpc-----pprgspacpsllraealles
                  .
                  . .: ** *::

gi_Danio          eevlyvvfpytqytvhdvityspaklansnakilfilyqliamrechaslclcgelsll
gi_Tetraodon     aemlyvilpytqyslhdivsfspaklanshakvlfilyqliallqachsaglsclcgelsll
gi_Xenopus       vsilyiilpyiqyslydvvtfspaklanshakvlfilylnvlqalkachqgglssgaltlr
gi_Gallus        aemlyiipyvvyqclhdivtfspakltnshakilfllfhvqlamrachqaglacgafslr
gi-Taeniopygia   admlyviyppvyqclhdivtfspakltnshakilfllfhvqlamrachqaglacgafslr
gi_Monodelphis  pemlyviyphvyqclhdivtfspakltnshakilfllfhvqlamdachrqglacgalslc
gi_Mus           pemlyvvhpyvqfslhdvvtfspakltnsqakvlfllfrvlramdachrqglacgalslh
gi_Rattus        pemlyvvhpyvqfslhdvvtfspakltnsqakvlfllfrvlramdachrqglacgalslh
gi_Callithrix    pemlyvvhphlpfslhdvvtfspakltnsqakvlfllfrvlramdachrqglacgalslh
gi_Pongo         pemlyvvhpyvqfslhdvvtfspakltnsqakvlfllfrvlramdachrqglacgalslh
gi_Homo          pemlyvvhpyvqfslhdvvtfspakltnsqakvlfllfrvlramdachrqglacgalsly
gi_Pan           pemlyvvhpyvqfslhdvvtfspakltnsqakvlfllfrvlramdachrqglacgalsly
gi_Oryctolagus   pemlyvihpyvqfslhdvvtfspakltnsqakvlfllfrvlrameachrqglacgalslh
gi_Sus           pgmlyvvhpyvqfslhdvvtfspakltnsqakvlfllfrvlramdachrqglscgplslh
gi_Ailuropoda    pemlyvvhpdvqfslhdvvtfspakltnsqakvlfllfrvlramdachrqglacgaltlh
gi_Equus         pemlyvvhpyvqfslhdvvtfspakltnsqakvlfllfrvlramdachrqglacgalslh
                  :*: * : :*:*:*****:*.*:*:*:*:*: * : ** * * .*:
                  :*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:

gi_Danio          diavdeqlcsrlkislahyekfkeyrdavpyalqnkvpmstvskdn-hnngvsgqlcrnc
gi_Tetraodon     dvavdeqlcarlklnlvhyeelgdgerdgnvtgicqmpkgvqtqnevlwscqedkrlcqdc
gi_Xenopus       sylvdeklcsqlrpnfidyereckstkven-----inteqlrtvgltsc
gi_Gallus        dvavdeklcsrlrvnfrfeyegprkeetn-aeadlervn-----eqsgagmrqdmarcytc
gi-Taeniopygia   dvavdeklcsrlrvnfrfeyegpggeeeenlkggldres-----eqs--cvqrqevtcsac
gi_Monodelphis  hvaideklcsqlqldlsayaheeeeikpmsnileaes-----qcapesrkgeglmypic
gi_Mus           hiavdeklcselrldlsayempsedengegseek-ngt-----giksekegegrtecptc
gi_Rattus        hiavdeklcselrldlsayempsedengevseek-drt-----gvksekdgegrpgcpc
gi_Callithrix    hiavdeklcsqlrldlsayerpeedesqealvar-gea-----grkseeragqpppppc
gi_Pongo         hiavdeklcselrldlsayerpeedekneeapvar-dea-----gitsqeeqggrrpgqptg
gi_Homo          hiavdeklcselrldlsayerpeedeneeapvar-dea-----givsqeeqgggrrpgqptg
gi_Pan           hiavdeklcselrldlsayerpeeneneeapvar-dea-----giasqeeqgggrrpgqptg
gi_Oryctolagus   hiavdeklcselrldlsayerpeddehqgspgvr-dga-----ggesgeegprgaacptr
gi_Sus           hiavdeklcselrldlrayerpkedeneespaar-ngv-----gaepgeegrrpgcpc
gi_Ailuropoda    hiavdeklcselrldlsayerpqedeneeipvar-nga-----gigpgeegrrpgcpc
gi_Equus         hiavdeklcselrldlsayerpkedekgetpvtr-dka-----gikpgeegggrrpcptc
                  : :*:*:*:*:*: * : ** .

gi_Danio          qdelkslvldwvngqvsnfqylmelnrlagrrregdpnyhvpwvvdftvpygrfrdlrk
gi_Tetraodon     fgelkslvldwvhgrvsnfycylmelnrlagrrrggdpnyhvpwvvdftvpfgkfrdlrr
gi_Xenopus       nnelkqilidwihgrisenfydylmynklagrrregdpnyhvpwvvdfttkngkfrdlrk
gi_Gallus        qqelrldvlqvwvhgrvsnfydylmhlslagrrmgdpnyhvpwvvdfttkngkfrdlrk
gi-Taeniopygia   qkdlrldvlqvwvhgqvsnfqylmrlslagrrmgdpnyhvpwvvdfttkngkfrdlrk
gi_Monodelphis  qeelrslvldwvhgrvsnfhylmqlnlwlagrrrgdpnyhvpwvvdfttengrfrdlrk
gi_Mus           qkelrglvldwvhgrisenfhylmqlnlagrrrggdpnyhvpwvvdfttppgrfrdlrk
gi_Rattus        qkelrglvldwvhgrvsnfyylmqlnlagrrrggdpnyhvpwvvdfttppgrfrdlrk
gi_Callithrix    qeelrglvldwvhgrisenfhylmqlnlagrrrggdpnyhvpwvvdfttphgrfrdlrk
gi_Pongo         qeelrslvldwvhgrisenfhylmqlnlagrrrggdpnyhvpwvvdfttphgrfrdlrk
gi_Homo          qeelrslvldwvhgrisenfhylmqlnlagrrrggdpnyhvpwvvdfttphgrfrdlrk
gi_Pan           qeelrslvldwvhgrisenfhylmqlnlagrrrggdpnyhvpwvvdfttphgrfrdlrk
gi_Oryctolagus   qeelrgfvldwvhgglsnfhylmqlnlagrrrggdpnyhvpwvvdftstphgrfrdlrk
gi_Sus           qeelrglvldwvhgrisenfhylmqlnlagrrrggdpnyhvpwvvdfttphgrfrdlrk
gi_Ailuropoda    qeelrglvldwvhgrisenfhylmqlnlagrrrggdpnyhvpwvvdfttphgrfrdlrk
gi_Equus         qeelrglvldwvhgrisenfhylmqlnlagrrrggdpnyhvpwvvdfttphgrfrdlrk
                  * :*:*:*:*: **: ** * * ***** :*:*:*:*:

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Figure D.1: continued


```

gi_Danio      aldtllqvhkhclktetiimhpggplflfkydpiceglpppnwqllspivsplpfpeyf
gi_Tetraodon aletllqigkhtktt--vetpdgqrpllfkynpicdglpppnpyqlnlntisppfpsyf
gi_Xenopus    mlesllqlhtpeeellkd-ggedtgallfefphiskglpppcpslllspicsiipfpayf
gi_Gallus     vlhillqlsvpveklkt-rlgkdtvqlfeykpsqglpppcptqllspfsiipfptyf
gi-Taeniopygia vlhvllqlsvpveklks-rlgkgavqlfeyepisqglpppcptqllspfsiivpfptyf
gi_Monodelphis vletllqlsepmgspltk-rqg--agrflflyrpiswglpppspaqllspysvvtfpsyf
gi_Mus        vldtllqlsgpkspmvs-k-gk--ldplfeyrpvsqglpppspaqllspfsiivpfptyf
gi_Rattus     vldtllqlsgpkspvlvk-k-gk--ldplfeyrpvsqglpppspaqllspfsiivpfptyf
gi_Callithrix vldtllqlsgpegplgae-r-gk--ldrlfeyrpvsqglpppcpgqllspfsiivpfptyf
gi_Pongo      vldtllqlsgpkvpmgae-r-gk--ldqlfeyrpvsqglpppcpsqllspfsiivpfptyf
gi_Homo       vldtllqlsgpkvpmgae-r-gk--ldqlfeyrpvsqglpppcpsqllspfsiivpfptyf
gi_Pan        vldtllqlsgpegptvag-r-gg--laplfeyrpvsqglpppcpaqllspfsiivpfptyf
gi_Oryctolagus vldtllqlsgpespvvag-r-gg--ldplfeyrpisqglpppcpaqllspfsiivpfptyf
gi_Sus        vldtllqlsgpegpvvag-r-gk--laplfeyrpvsqglpppcpaqllspfsiivpfptyf
gi_Ailuropoda vldmllqlsgsegpvvar-r-gn--ldplfeyrpvsqglpppcpaqllspfsiivpfptyf
gi_Equus      *. **:::                ** : .: ***** * *. * . ** : *

gi_Danio      ptlhkffisyhskmesinniqgrdivfnlwqqletllkgdittegleillpfvlslmsee
gi_Tetraodon  aglhrffisyhakrsttcsvqgrdvfvhlwqqletllrshitaegleillpfilalmiee
gi_Xenopus    pnlhkfiltyqsrrt-edesqgrelvfqlwqqidgil-ceinpegleillpfvlslmtee
gi_Gallus     palhkffiftyqakki-edegqgrelvfqlwqqlegil-seitpegleillpfvlslmsee
gi-Taeniopygia palhkffiftyqakki-edegqgrelvfqlwqqlegil-seitpegleillpfvlslmsee
gi_Monodelphis pslykfilllyqtrkv-edevqgrelvfqlwqqldgil-qeitpegleillpfvlslmsee
gi_Mus        palhkfilllyqarrv-edevqgrelafalwqqlgavl-nditpegleillpfvlslmsee
gi_Rattus     palhkfilllyqarrv-edevqgrelvfalwqqlgavl-neitpegleillpfvlslmsee
gi_Callithrix palhkfilllyqtrrv-eadeaggrelevftlwqqlgavl-sditpegleillpfvlslmsee
gi_Pongo      palhkfilllyqarrv-eadeaggrelevfalwqqlgavl-kditpegleillpfvlslmaee
gi_Homo       palhkfilllyqarrv-eadeaggrelevfalwqqlgavl-kditpegleillpfvlslmsee
gi_Pan        palhkfilllyqarrv-edkaaggrelevfalwqqlgavl-kditpegleillpfvlslmsee
gi_Oryctolagus palhkfilllyqarrv-eadeaggrelevfalwqqlgavl-sditpegleillpfvlslmsee
gi_Sus        palhkfilllyqakrv-eadeaggrelevfalwqqlgavl-sditpegleillpfvlslmsee
gi_Ailuropoda palhkfilllyqarrv-eadeaggrelevfalwqqlgavl-sditpegleillpfvlslmsee
gi_Equus      palhkfilllyqarcv-eadeaggrelevfalwqqlgavl-sditpegleillpfvlslmsee
               *::*: *::: . *:::.* ***** :* *. *****:*.** *

gi_Danio      stavyaawylfepvsrvlpgprnaskylikplvgvyenprclrgrfylytdcfvqlivrl
gi_Tetraodon  stavyaawylfepisrvlpgprnankyllkplinvyenpncler-fylytdcfilqlivrl
gi_Xenopus    stavyaawylfepvakalgpknankyllkpligayespshlgrfylytdcfvaqlivrl
gi_Gallus     ntavyaawylfepiakslgpknankyllkpligayetpcsrhgrfylytdcfvaqlivrl
gi-Taeniopygia ntavyaawylfepiakslgpknankyllkpligayempcsrhgrfylytdcfvaqlivrl
gi_Monodelphis htavytawylfepiaralgpknankyllkpligayenpchlhgrfylytdcfvaqlmvrl
gi_Mus        htavytawylfepvakalgpknankyllkpligayespcrlhgrfylytdcfvaqlvrvl
gi_Rattus     htavytawylfepvakalgpknankyllkpligayenpcrlhgrfylytdcfvaqlvrvl
gi_Callithrix htavytawylfepvakalgpknankyllkpligayespcqlhgrfylytdcfvaqlmvrl
gi_Pongo      htavytawylfepvakalgpknankyllkpligayespcqlhgrfylytdcfvaqlmvrl
gi_Homo       htavytawylfepvakalgpknankyllkpligayespcqlhgrfylytdcfvaqlmvrl
gi_Pan        htavytawylfepvakalgpknankyllkpligayespcqlhgrfylytdcfvaqlmvrl
gi_Oryctolagus htavyaawylfepvakalgpknankyllkpligayespcqlhgrfylytdcfvaqlmvrl
gi_Sus        htavytawylfepvakalgpknankyllkpligayespcrlhgrfylytdcfvaqlivrl
gi_Ailuropoda htavytawylfepvakalgpknankyllkpligayespcrlhgrfylytdcfvaqlmvrl
gi_Equus      htavytawylfepvakalgpknankyllkpligayespcrlhgrfylytdcfvaqlmvrl
               *****::: *::*: *****: .** * . *****: **::**

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Figure D.1: continued. The mutation site in human patients, proline at residue 856, is conserved among all of the species of vertebrates, which were included to this alignment.

```

gi_Danio          glqvflssllphvlqvmgtgfescntaagtewegmkvlrgaagaldeeeeeyecddrrssn
gi_Tetraodon     glqafllssllphvlqvitgfescipglsgt--ckglt dg--tmhleeeedfrcdesrhss
gi_Xenopus       glqafllsnllnhvlqiliglletsgee-----rkllsggae---deesg--g-d-----
gi_Gallus        glqsfllnllphilqilvgiessree-----skslgaae---ddesg--g-g-----
gi-Taeniopygia   glqpflnllphilqilvgiessree-----sksfllgtae---ddesg--g-e-----
gi_Monodelphis  glqpflshllphvlqvlagvetsqek-----tkclggtte---deeigegd-e-----
gi_Mus           glqafllthllphvlqvlagveasqee-----gkglvgtte---deeselpv-s-----
gi_Rattus        glqafllhllphvlqvlagveasqee-----gkglvgtte---deenelpv-p-----
gi_Callithrix    glqafllthllphvlqvlagteasqee-----tkdlvaiae---deesglpg-a-----
gi_Pongo         glqafllthllphvlqvlagaeasqee-----skdlagaae---eesglpg-a-----
gi_Homo          glqafllthllphvlqvlagaeasqee-----skdlagaae---eesglpg-a-----
gi_Pan           glqafllthllphvlqvlagaeasqee-----skdlagaae---eesglpr-a-----
gi_Oryctolagus   glqafllvllphvlqvlagveasqee-----skglagaae---deevalpg-p-----
gi_Sus           glqafllvllphilqvlagveasqee-----skglvgaae---deesglpg-a-----
gi_Ailuropoda    glqafllshllphvlqvlagveasqee-----skglvgaae---dedgelag-a-----
gi_Equus         glqafllvllphvlqvlagveasqee-----skglagaae---deesglpg-a-----
*** *:  ** *:***:  * *:.          * :          :::

gi_Danio          atssgkvvgggsgggsggvvgvdqglvdyssgisln dqvflnegedfqngfyvnnsasga
gi_Tetraodon     gsvsgtmg--gsggaaggvagvgdtglsnypsgisln dqvflsdaedfqnefyvnnsaagk
gi_Xenopus       spvscnfg--earqtsidqs--asshell dytsgvt fhdqgylaenedfqgtlyvsdpl---
gi_Gallus        spmscmfmg--eeikmdvehs--staldlldytsgv sfhdqaylpesedfqsglyvgesl---
gi-Taeniopygia   spvscvfg--eeikmdvehs--saaldlldytsgv sfhdqaylpesedfqsglyvgesl---
gi_Monodelphis  gqgpcafg--eaiqmegdpq--ssglelldytsgv sfhdqtdlpesedfqaglyvsgsp---
gi_Mus           gpgscafg--eeiqmdgqpaassglglpdyrsgv sfhdqadlpdtedfqaglyvaesp---
gi_Rattus        gpgscafg--eeiqmaggqpaassglglpdyrsgv sfhdqadlpdtedfqaglyvaesp---
gi_Callithrix    gpgscafg--eeipmdgeppassglglpdytsgv sfhdqadlpdtedfqaglyvtesp---
gi_Pongo         gpgscafg--eeipmdgeppassglglpdytsgv sfhdqadlpdtedfqaglyvtesp---
gi_Homo          gpgscafg--eeipmdgeppassglglpdytsgv sfhdqadlpdtedfqaglyvtesp---
gi_Pan           gpgscafg--eeipmdgeppassglglpdytsgv sfhdqadlpdtedfqaglyvtesp---
gi_Oryctolagus   gpgscafg--eeiqmddeepat saglglpdytsgv sfhdqadlpdtedfqgglvagsp---
gi_Sus           gpsscafg--eemqmdgeptassglglpdytsgv sfhdqadlpdtedfqaglyvaesp---
gi_Ailuropoda    rpsscafr--eeipmdgepaassglglpdytsgv sfhdqaylpdtedfqaglyvaesp---
gi_Equus         rasscafg--eeiqmdgepaassglglpdytsgv sfhdqadlpdtedfqaglyvaesp---
.               . * : * * : : . * * * : * : * * * : * : * *

gi_Danio          ttvgtkqnnqstankdqdqesls vglksdkssasevsigd-----raslksad
gi_Tetraodon     -----epnqnsackdqdqesls vglksdkssstelslgd-----gdsmdraslksad
gi_Xenopus       -----qpqepesls lgrlsdkssasevslgdekvt d--sdslikegslksgd
gi_Gallus        -----qpqepesls lgrlsdkssasevslgedrpd--gdsqkdksslksvd
gi-Taeniopygia   -----qpqepesls lgrlsdkssasevslgedrpd--gdsqkdksslksmd
gi_Monodelphis  -----qpqepesls lgrlsdkssstsdaslgedrpgdeigl tr-----dk
gi_Mus           -----qpqeaavsl glqlsksstseasqgeerggdggapadknsvksgd
gi_Rattus        -----qpqeaavsl glqlsksstseasqgeerggdggapvdknsvksgd
gi_Callithrix    -----qpqeaavsl glrlsksstsets lgeerapddgaapmdksslrsgd
gi_Pongo         -----qpqeaavsl glrlsksstsets lgeerapdeggapvdksslqsgd
gi_Homo          -----qpqeaavsl glrlsksstsets lgeerapdeggapvdksslrsgd
gi_Pan           -----qpqeaavsl glrlsksstsets lgeerapdeggapvdksslrsgd
gi_Oryctolagus   -----qeaals lgrlsdkssstsdts lgeeragdegcapvdksslrsgd
gi_Sus           -----qpqeaavsl glrlsksstsets lgeervadeggapvdksslrsgd
gi_Ailuropoda    -----qpqeaavsl glrlsksstsets lgeeraaeggapvdksslrsgd
gi_Equus         -----qpqeaavsl glrlsksstseas lgeera--degapvdksslksgd
* : * : * : * : * : * : * : * : * : * : * : * : * : * : * :

gi_Danio          ssqdlkqasdgedggeledeeetvedr-----ei--tv--qr vpslmtlsvcteesea
gi_Tetraodon     ssqdlkqasegeeggeldeeevpetse-----geeg--tdgtvascleltlsg---vtqt
gi_Xenopus       ssqdlkqseeseeeee dr-----epspgl sel t lsvntdvs v-
gi_Gallus        ssqdlkqsedseeeee-ereeeerddta-----vdaelv gadvgvsvdagtsv-
gi-Taeniopygia   ssqdlkhllgeeeeeeeeheede hedav-----vdpeltvv-----vdaagasv-
gi_Monodelphis  ssqdlkqsegseeeeeeeedeevvgggdvgeeeeeeeedeqdstpavseftls-dpgvst-
gi_Mus           ssqdlkqsegseee---eeee-----gcvvleed---qedevtgt sel t l s--dtlmsm-
gi_Rattus        ssqdlkqsegseee---eeee-----gcvvleee---eqdevtgt sel t l s--dtlmsm-
gi_Callithrix    ssqdlkqsegseee---eeed-----gcvlleeeg--eqeevt gasel t l s--dtvlsm-
gi_Pongo         ssqdlkqsegseee---eeed-----scmvleeeeg--eqeevt gasel t l s--dtvlsm-
gi_Homo          ssqdlkqsegseee---eeed-----scvleeeeg--eqeevt gasel t l s--dtvlsm-
gi_Pan           ssqdlkqsegseee---eeed-----scvleeeeg--eqeevt gapel t l s--dtvlsm-
gi_Oryctolagus   ssqdlkqsegsedee-eeee-----gcvvleeeegv--eqdevtgt sel t l s--dtvlsm-
gi_Sus           ssqdlkqsegseee---eeed-----gcvvlee--geeeqdevpeasel t l s--dtvlsm-
gi_Ailuropoda    ssqdlkqsegseeeeeegeee-----gcvvleeeegdgeqdeitraselalp--dtvlsm-
gi_Equus         ssqdlkqsegseee---eeee-----gcvvleeeeggeqdevtgt sel t l p--dpvlsm-
*****:      *:                               :

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Figure D.1: continued

```

gi_Danio          tvatlegdvmngivqedgeknmeeeeetehdpledseekehkilldtvcktvrwlsaklgp
gi_Tetraodon     svttlegeftnnlmtmetekgdvgaqeehdpsgdseekehrilqdtvcktvrwlsatlgp
gi_Xenopus       -----dtvlasesr--edeeeeeqlnnqteekeqkilldtackmvrwlsaklgp
gi_Gallus        -----dvtladd-gsepedgegeelpdhsddkeqtilldtackmvrwlsaklgp
gi-Taeniopygia   -----dvtladd-ssepedgegeelpdhsddkeqtilldtackmvrwlsaklgp
gi_Monodelphis   -----dvtlpndggn---geeeedisqsevkeqkilldtacktvrwlsaklgp
gi_Mus           -----etvvapgddgr-dreeeeeplteqteqkeqkilldtackmvrwlsaklgp
gi_Rattus        -----etvvapgddgr-dreeeeeplpeqteqkeqkilldtackmvrwlsaklgp
gi_Callithrix    -----eavvagssgg-dgegqeeplpeqsegkeqkilldtackmvrwlsaklgp
gi_Pongo        -----etvvagsggg-dgeeeeeplpeqsegkeqki-----
gi_Homo          -----etvvagsggg-dgeeeeealpeqsegkeqkilldtackmvrwlsaklgp
gi_Pan           -----etvvasgsgg-dgeeeeealpeqsegkeqkilldtackmvrwlsaklgp
gi_Oryctolagus   -----etvvagdggg-dgeeeeepltgqsegkehrilldtackmvrwlsaklgp
gi_Sus           -----dtvvaggggaddgeeeeeplteqsegkeqkilldtackmvrwlsaklgp
gi_Ailuropoda    -----dtvvargget-ggeedteplteqsegkeqkilldtackmvrwlsaklgp
gi_Equus         -----dtvvagggga-dgeeeaeplteqsegkeqqilldtackmvrwlaaklgp
.                .                .:: **: *

gi_Danio          tltsrfiarnllrlltscyigldkhqfmlsvneens-lecvgsvyekppvvgdqtarpvl
gi_Tetraodon     tvtsryvarnllrlltncyigpanhqlvtpvseeklesagmsnvyekktvvgdqtarpvl
gi_Xenopus       tvtsryiarnllrlltscyvgpqrqqfvanmeens--pls----vkkpvcgdqvsakpvl
gi_Gallus        tvtsrfiarnllrlltscyigpqrqqfvpnsdeng--plstgnyqkrpvlgdqvsakpvl
gi-Taeniopygia   tvtsrfiarnllrlltscyigltrqqfvpssdens--plstgnyqkrpvlgdqvsakpvl
gi_Monodelphis   tvtsryvarnllrlltscyvgatrqqfmgsssdts--plssgnyqkrpvlgdvasvpvl
gi_Mus           tvasrhvarnllrlltscyvgpqrqqftvssddtp--plnagniyqkrpvlgdivsgpvl
gi_Rattus        tvasrhvarnllrlltscyvgpqrqqftvscddsp--plnagniyqkrpvlgdivsgpvl
gi_Callithrix    tvasrhvarnllrlltscyvgpqrqqftassgdspl--slsagniyqkrpvlgdivsgpvl
gi_Pongo        -----
gi_Homo          tvasrhvarnllrlltscyvgpqrqqftvssgesp--plsagniyqkrpvlgdivsgpvl
gi_Pan           tvasrhvarnllrlltscyvgpqrqqftvssgesp--plsagniyqkrpvlgdivsgpvl
gi_Oryctolagus   tvasrhvarnllrlltscyvgpqrqqftvssgesp--plsagniyqrrpvlgdvvsapvl
gi_Sus           tvasrhvarnllrlltscyvgpqrqqftasngesp--plsagniyqkrpvlgdivsgpvl
gi_Ailuropoda    tvasryvarnllrlltscyvgpqrqqftvssgesp--plsagsiyqkrpvlgdivsgpvl
gi_Equus         tvasrhvarnllrlltscyvgpqrqqftmssgesp--plsagniyqkrpvlgdivsgpvl

gi_Danio          ecliyiahlygepvltyqylpyigyilvspss---crlntrkeagllgavvltqkiivfl
gi_Tetraodon     dcliyvaqlygepvltyqylpyigyilvspss---qrlntrkeasllgaialtrkivvfl
gi_Xenopus       dclmylahlygepvltyqylpyinyilvaptsg---crlnsrkeasllaavltqkiivyl
gi_Gallus        acllhvaylygepvltyqylpyisylvapgsagggrlnsrkeagllaavltqkiivcl
gi-Taeniopygia   acllhvaylygepvltyqylpyisylvapgagsggvrlnsrkeagllaavltqkiivcl
gi_Monodelphis   gclmhiaclygepvltyqylpyisylvapgggggsrlnsrkeagllaavltqkiivyl
gi_Mus           scllhiaylygepvltyqylpyisylvapgsnspnrsrlnsrkeagllaavltqkiivyl
gi_Rattus        scllhiaylygepvltyqylpyisylvapgsnsspsrlnsrkeagllaavltqkiivyl
gi_Callithrix    scllhlahlygepvltyqylpyisylvapgsasggsrlnsrkeagllaavltqkiivyl
gi_Pongo        -----
gi_Homo          scllhiarlygepvltyqylpyisylvapgsasggsrlnsrkeagllaavltqkiivyl
gi_Pan           scllhiarlygepvltyqylpyisylvapgsasggsrlnsrkeagllaavltqkiivyl
gi_Oryctolagus   scllhiaylygepvltyqylpyisylvapgsasggsrlnsrkeagllaavtlaqkitvyl
gi_Sus           scllhhvahlygepvltyqylpyisylvapgstsggsrlnsrkeagllaavltqriivyl
gi_Ailuropoda    scllhiaylygepvltyqylpyisylvapgstsggsrlnsrkeagllaavltqkiivyl
gi_Equus         scllhiaylygepvltyqylpyisylvapgstsggsrlnsrkeagllaavltqkiivyl

gi_Danio          sdttlmdmlmkingevllplldlltstkmgfpgsgvqtrsavclktlslmaliclrigrem
gi_Tetraodon     sdttlmdmlmkingdvllplldllttpcmgfpvgvhttrtvclktlslialiclrigrem
gi_Xenopus       sdttlmdilpkingdvllpvlsltsatvgfpgsgaqaralcvkcslslialiclrigrem
gi_Gallus        sdttlmdilpkisgevllpvlglftslsvgfpgsgaqarvvlciktisliialiclrigrem
gi-Taeniopygia   sdttlmdilpkisgevllpvlglftspavgfpgsgaqarvvlcvktisliialiclrigrem
gi_Monodelphis   sdttlmdilprishevllpvlssltslvtgfpfgaqartvlcvktisliialiclrigrem
gi_Mus           sdttlmdilprishevllpvlglftsfvtgfpfgaqartvlcvktisliialiclrigrem
gi_Rattus        sdttlmdilprishevllpvlslftsfvtgfpfgaqartvlcvktisliialiclrigrem
gi_Callithrix    sdttlmdilprishevllpvlslftslvtgfpfgaqartilcvktisliialiclrigrem
gi_Pongo        -----
gi_Homo          sdttlmdilprishevllpvlslftslvtgfpfgaqartilcvktisliialiclrigrem
gi_Pan           sdttlmdilprishevllpvlslftslvtgfpfgaqartilcvktisliialiclrigrem
gi_Oryctolagus   sdttlmdilprlshevllpvlslftslvtgfpfgaqartvlcvktisliialiclrigrem
gi_Sus           sdttlmdilprishevllpvlslftslvtgfpfgaqartvlcvktisliialiclrigrem
gi_Ailuropoda    sdttlmdilprishevllpvlslftslvtgfpfgaqartvlcvktisligliclrigrem
gi_Equus         sdttlmdilprishevllpvlslftslvtgfpfgaqartvlcmktisliialiclrigrem

```

Figure D.1: continued

gi_Danio	vqqhmaetlsrffqvfsllqflqnqigsapr----revaectyldlripdgaeltielgv
gi_Tetraodon	vqqhmadtldrqqffavfslinslqpqvlhahvthadlivadvttvvdvctsdnsfvtyselgv
gi_Xenopus	vqqhlgdtlriffsnftlmqelhegglvmen----pdslepvvqhvqckdgstlpvdlsl
gi_Gallus	vqqhlsdtvrnffgafslqlqldqgltaes----lsgcevpmvevplregkllaldpav
gi-Taeniopygia	vqqhlsdtvrnffgafslqlqldqgltaes----lsscompvmevplsdgkllaldpsv
gi_Monodelphis	vqqhlsetvatffqvfslvhelqcqalrpd-----pspneghvlvfpfsdgqqravdpal
gi_Mus	vqqhlsepvatffqvfsfshlhelrqddlpldp----kgctegqlpeatfsdgqrrpvdptl
gi_Rattus	vqqhlsepvatffqvfsfshlhelrqddlpldl----kgctegqlpeatfsdgqrrpvdptl
gi_Callithrix	vqqhlsepvaaffqvfsqlhelrqddlklp----tgrsegqlpevvfsdgqqrpdptl
gi_Pongo	-----
gi_Homo	vqqhlsepvatffqvfsqlhelrqddlklp----agrgegqlpqvvfsdgqqrpdvpal
gi_Pan	vqqhlsepvatffqvfsqlhelrqddlklp----agrgegqlpqvvfsdgqqrsvdpal
gi_Oryctolagus	vqqhlsepvatffqvfsqlhelrhqdlkwds----agrsegqlpeaafsdgqqrpdvpal
gi_Sus	vqqhlsepvatffqvfsrlhldlqhgdwkles----mgrsegqlpevafsdgqlrlvdptv
gi_Ailuropoda	vqqhlsepvatffqvfsqlhelrhqdlklp----tgrsegqlpevafsdgqqrpdptl
gi_Equus	vqqhlsepvatffqvfsqlhelrhqdlklp----vsrsegqlpevafsdgqqrpdptl
gi_Danio	leelqavfnpemayasyipfycligdsgrklvtnhelvwslasqsyheraspgspesnpv
gi_Tetraodon	leelrcvfnpemayasyvpfccligdmairklvpnheliwqltqsyqlsvsqrgpecnpa
gi_Xenopus	ldelekvyanaemayatypfsccligdg-ighvipnhdliwklmtvyqeaavipkdiesspa
gi_Gallus	l1elqkvfnpemayityipfsccllgdv-irtvvpnhslveklaslhlennvpknqlqv-vg
gi-Taeniopygia	lvelqkvfnpemayityipfsccllgdv-irtvvpnhslveklaslhlennvpknqxxg-vg
gi_Monodelphis	ldelqkvftlemaymtypfsccllgdv-ikivpnghglvgqqlagqylesspvg-qdpmn
gi_Mus	leelqkvftlemaytiyvpfsccllgdi-irkiipnhelvgelaglylesmsps--rnpas
gi_Rattus	leelqkvftlemaytiyvpfsccllgdi-irkiipnhelvgelaglylesmspsl--rnpas
gi_Callithrix	ldelekvtlqmaytiyvpfsccllgdi-iwkiipnhelvgelaglylesispss--hspas
gi_Pongo	-----
gi_Homo	ldelqkvftlemaytiyvpfsccllgdi-irkiipnhelvgelaalylesispss--rnpas
gi_Pan	ldelqkvftlemaytiyvpfsccllgdi-irkiipnhelvgelaalylesispss--rnpas
gi_Oryctolagus	ldelqkvftlemaytiyvpfsccllgdi-irkiipnhelvgelaglylesislss--qnpas
gi_Sus	ldelqkvftlemaytiyvpfsccllgdi-irkiipnhelvgqglaglylesispss--rspas
gi_Ailuropoda	ldelqkvftlemaytiyvpfsccllgdi-irkiipnhelvgelaglylesvspss--rspas
gi_Equus	ldelqkvftlemaytiyvpfsccllgdi-irkiipnhelvgelaglylksispns--rspas
gi_Danio	ggqgrasav----glspsmgrqmsrspfpapsststplggdilpesgtfgshlvgnriqvt
gi_Tetraodon	stsrvevsapsaavssgfprrvgcsafpaptfs---stgnlipesgtfgshlvgnriqvs
gi_Xenopus	plssgsr-----igssf-----yiaeddgsgtfgsvmvgnriqvp
gi_Gallus	leqapsma-----gsdqdm-rgteaf-----sspqedshsgtfgsvlvgnriqvp
gi-Taeniopygia	leqtpsvv-----gsdqdp-rgaepf-----sspqedthsgtfgsvlvgnriqvp
gi_Monodelphis	l-apmpsa-----speqdp-qg---r-----gvnhddgnsgtfgsvlvgnriqip
gi_Mus	meptmasa-----gpewdp-qs---g-----sclqddghsgtfgsvlvgnriqip
gi_Rattus	mepvtpsa-----gpewnp-qs---g-----sclqddghsgtfgsvlvgnriqip
gi_Callithrix	veptvpsm-----gpewdp-qg---g-----rcpqddghsgtfgsvlvgnriqip
gi_Pongo	-----
gi_Homo	veptmpgt-----gpewdp-hg---g-----gcpqddghsgtfgsvlvgnriqip
gi_Pan	veptvpst-----gpewdp-qg---g-----gcpqddghsgtfgsvlvgnriqip
gi_Oryctolagus	veptvpss-----saerdp-qg---g-----gcpqddshsgtfgsvlvgnriqip
gi_Sus	leptapst-----spewdp-qg---g-----scpqddghsgtfgsvlvgnriqip
gi_Ailuropoda	veptvpst-----gpewdp-qs---g-----gcprrddghsgtfgsvlvgnriqip
gi_Equus	veptvpst-----pggwds-qg---g-----gcpqddghsgtfgsvlvgnriqip
gi_Danio	rdteacgspnlssletwthgrpygsnappmslaltalssagps--fshssyswvmgptpe
gi_Tetraodon	pgadydsnpnlslssswgrsah-----tptiittassftggsagtaslssswvtgftte
gi_Xenopus	kdiqypmsrs-----sswgrsah-----t-cttpvmetfqsd
gi_Gallus	vdtqreglgllrl-----sagtd---gfipsssse
gi-Taeniopygia	vdtqreglgllrl-----sagad---gfapsssse
gi_Monodelphis	vdtqpesspgplgp-----ssgvc-gsvgglcemgge
gi_Mus	d-sppqspgplgs-----lsgvg-s-sggl-snrne
gi_Rattus	d-sppqssgplgs-----isgvg-s--ggl-ssrne
gi_Callithrix	adsqpesspgplgp-----isga-----gsgse
gi_Pongo	-----
gi_Homo	ndsrlenpgplgp-----isgvg-g--ggl-gsgsd
gi_Pan	ndsrlenpgplgp-----isgvg-g--ggl-gsgsd
gi_Oryctolagus	sdpqpesspgplgp-----isgvg-s--ggl-gagae
gi_Sus	gdsqpesspgplgp-----isgvg-g--ggv-gslse
gi_Ailuropoda	ddsqpesspgllgp-----ipgvg-s--ggl-sse-e
gi_Equus	ddshdpdsgplgp-----isgvg-s--ggl-gsene

Figure D.1: continued

gi_Danio	dsalkqdlprssrslqgnwlaywqyeiglnqqdshfhfhqirlqsfhsgtakclapla
gi_Tetraodon	dsalkqelprsgsrslqgnwlaywqyeiglnqqdphfhfhqirlqsfhsgttkclaplt
gi_Xenopus	ensfkqdlqknaaglcgnwlaywqyeigvsqqephfcfhqiklqsfvghsgaikcvkpls
gi_Gallus	entlkhdprsthvclcnwlaywqyeigvsqhdprfhfhqiklqsfsghsgaikcvapls
gi-Taeniopygia	esalkhdprsthmlcgnwlaywqyeigvsqhdprfhfhqiklqsfsghsgaikcvaplg
gi_Monodelphis	enslkrdlprcahglsgnwlaywqyeigvsqhdahfhfhqirlqsfhsgavkcvaplg
gi_Mus	dnalkrelprsaahglsgnwlaywqyeigvsqqdahfhfhqirlqsfpghtgavkcvvaals
gi_Rattus	dnalkrelprsaahglsgnwlaywqyeigvsqqdahfhfhqirlqsfpghtgavkcvvaals
gi_Callithrix	dnalkqelprsaahglsgnwlaywqyeigvsqqdahfhfhqirlqsfpghsavkcvapls
gi_Pongo	-----
gi_Homo	dnalkqelprsvhglsgnwlaywqyeigvsqqdahfhfhqirlqsfpghsavkcvapls
gi_Pan	dnalkqelprsvhglsgnwlaywqyeigvsqqdahfhfhqirlqsfpghsavkcvapls
gi_Oryctolagus	dnalkrelprsaahglsgnwlaywqyeigvsqqdahfhfhqirlqsfpghsavkcvvaals
gi_Sus	dnalkrelprsaahglsgnwlaywqyeigvsqqdahfhfhqirlqsfpghsavkcvvpls
gi_Ailuropoda	dnalkrelprsaahglsgnwlaywqyeigvsqqdahfhfhqirlqsfpghsavkcvapls
gi_Equus	dnalkrelprsaahglsgnwlaywqyeigvsqqdahfhfhqirlqsfpghsavkcvapls
gi_Danio	gedyflsgskdktvrlwplnyhgdgtrepeprltytehrksifyvvgqlealqevvscdgt
gi_Tetraodon	gedyflsgskdktvklwplnhhgdgtkevepkltysdhrksvfyvgqleasqevvscdgt
gi_Xenopus	gedffmsgskdktvrlwplnygdgtrdlearhiyaehkrsvfyvgqlesvqqvlscdga
gi_Gallus	sedfflsgskdktvrlwplnygdgtsevpprfttysehkksvfyvsgleasqhvvscdgt
gi-Taeniopygia	sedfflsgskdktvrlwplnygdgtsevpprfttyaehkksvfyvsgleapqhvvscdgt
gi_Monodelphis	nedfflsgskdrtvrlwplnycgdgtsetvprlvysqhrksifyvsgleasqqvvscdgt
gi_Mus	sedfflsgskdrtvrlwplnygdgtsetasrliyaqhrksvfyvgqleapqyvscdga
gi_Rattus	sedfflsgskdrtvrlwplnygdgtsetaprliyaqhrksvfyvgqleapqyvscdga
gi_Callithrix	gedfflsgskdrtvrlwplnygdgtcetaprlvytqhrksvffvqdqlealqhvscdga
gi_Pongo	-----
gi_Homo	sedfflsgskdrtvrlwplnygdgtsetaprlvytqhrksvffvvgqleapqhvvscdga
gi_Pan	gedfflsgskdrtvrlwplnygdgtsetaprlvytqhrksvffvvgqleapqhvvscdga
gi_Oryctolagus	sedfflsgskdrtvrlwplnygdgtsetaprlvytqhrksvfyvgqleapqyvscdga
gi_Sus	sedfflsgskdrtvrlwplnysgdgtretaprlvytqhrksvffvvgqleapqcvscdga
gi_Ailuropoda	sedfflsgskdrtvrlwplnysgdgtgetaprlvyaqhrksvffvvgqleapqyvscdga
gi_Equus	sedfflsgskdrtvrlwplnysgdgtsetaprliyaqhrksvffvvgqleapqcvscdga
gi_Danio	vhlwdqftgknircnepldgknpitavttmpapchsvvfasadsvlrfdiprkpglqhef
gi_Tetraodon	vhlwdqytgkqirsyeavdgkspitavstmpgphchsvvfgsadsvlrfdiprkpglqhef
gi_Xenopus	vhiwdqytgksirtyeaadsklpitavatmppyctvsgsadsilrfdilrkpglqhef
gi_Gallus	vhiwdqftgklirtfdeldskpitavttmpppyhsisvasadsvlrfdihrkpglqhef
gi-Taeniopygia	vhiwdqftgklirtfdeldskvpitavttmpppyhsisvasadsvlrfdihrkpglqhef
gi_Monodelphis	vhwdpftgktvrtveawdsrvpltavavmsaphtsitlasadstlrfvdrckpglqhef
gi_Mus	vhwdpftgktlrvtdpsdsrvpltavavmpaphtsitmassdstlrfvdrckpglqhef
gi_Rattus	vhwdpftgktlrvtdpsdsrvpltavavmpaphtsitmassdstlrfvdrckpglqhef
gi_Callithrix	vhwdpftgktlrveldsdrvpltavavmpaphtsitmassdstlrfvdrckpglqhef
gi_Pongo	-----
gi_Homo	vhwdpftgktlrvtepldsrvpltavavmpaphtsitmassdstlrfvdrckpglqhef
gi_Pan	vhwdpftgktlrvtepldsrvpltavavmpaphtsitmassdstlrfvdrckpglqhef
gi_Oryctolagus	vhwdpftgktlrvtepsdsrvpltavavmpaphtsitmassdstlrfvdrckpglqhef
gi_Sus	vhiwdpftgktlrvtepsdsrvpltavavmpaphtsitmassdstlrfvdrckpglqhef
gi_Ailuropoda	vhwdpftgktlrvmpwdsrvpltavavmpaphtsitmassdstlrfvdrckpglqhef
gi_Equus	vhwdpftgktlrvtepadsvrpltavavmpaphtsitmassdstlrfvdrckpglqhef
gi_Danio	rlaysnlsaglirclavspgggrtiaagfstgfvllldartglvlrgwpahegdilqmkaa
gi_Tetraodon	rlaynnvgaglirylavspgrtvaagfssgfvllldartglilrgwpahegdilqmkaa
gi_Xenopus	kla-snv naglirclavspngrsvvagfssgfvllldtrtglvlrgwpahegdilqmkaa
gi_Gallus	rla-ggvnaglirclavspngrsvmagfssgfvllldtrtglimrgwpahegdilqikaa
gi-Taeniopygia	rla-sgvsaglirclavspsgrsvvagfssgfvllldtrtglimrgwpahegdilqikaa
gi_Monodelphis	rlg-gglnaglvrslavspsgrsvvagfssgfmvllldtrtgililrgwpahegdilqikav
gi_Mus	rlg-gglnpglvrslavspsgrsvvagfssgfmvllldtrtgilvlrgwpahegdilqikav
gi_Rattus	rlg-gglnpglvrslavspsgrsvvagfssgfmvllldtrtgilvlrgwpahegdilqikav
gi_Callithrix	rlg-sglntglvrslavspsgrsivagfssgfmvllldtrtgilvlrgwpahegdilqikav
gi_Pongo	-----
gi_Homo	rlg-gglnpglvrslavspsgrsvvagfssgfmvllldtrtgilvlrgwpahegdilqikav
gi_Pan	rlg-gglnpglvrslavspsgrsvvagfssgfmvllldtrtgilvlrgwpahegdilqikav
gi_Oryctolagus	rlg-gglnpglvrslavspsgrsvvagfssgfmvllldtrtgililrgwpahegdilqikav
gi_Sus	rls-gglnpglvrslavspsgrsvvagfssgfmvllldtrtgilvlrgwpahegdilqikav
gi_Ailuropoda	rlg-gglnpglvrslavspsgrsvvagfssgfmvllldtrtgilvlrgwpahegdilqikav
gi_Equus	rlg-gglnpglvrslavspsgrsvvagfssgfmvllldtrtgilvlrgwpahegdilqikav

Figure D.1: continued

gi_Danio	egnlvssssdhtltvwkdvehkplhqyrtpsdpiahfdlygaeivagtvankigvysil
gi_Tetraodon	egnlvissssdyltltvwkeleqkplrkyksqsdpihafdlygseivtvtvankigvysma
gi_Xenopus	dgnivvssstdhsaltvwkeleqkplhqfksnsdpiahfdlygneivtvtvankigiymsl
gi_Gallus	egnvliissssdhsmtvwkeleqkplhhyksasepihafdlygnevvtgtvankigvysml
gi-Taeniopygia	egnvliissssdhsaltvwkeleqkplhhyksasepihafdlygnevvtgtvankigvysml
gi_Monodelphis	egsilissssdhsaltiwkeldqkpthhykspsdpihtfdlygsevvgtvankigvcsmm
gi_Mus	egsvliissssdhsaltvwkeleqkpthhyksasdpihtfdlygsevvgtvankigvcsll
gi_Rattus	egsvivssssdhsltvwkeleqkpthhyksasdpihtfdlygsevvgtvankigvcsll
gi_Callithrix	egsvlvssssdhsltvwkeleqkpthhyksasdpihtfdlygsevvgtvsnkigvcsll
gi_Pongo	-----
gi_Homo	egsvlvssssdhsltvwkeleqkpthhyksasdpihtfdlygsevvgtvsnkigvcsll
gi_Pan	egsvlvssssdhsltvwkeleqkpthhyksasdpihtfdlygsevvgtvsnkigvcsll
gi_Oryctolagus	egsvlvssssdhsltvwkeleqkpthhyksasdpihtfdlygsevvgtvankigvcsll
gi_Sus	egsllvssssdhsltvwkeleqkpthhyksasdpihtfdlygsevvgtvankigvcsll
gi_Ailuropoda	egsvlvssssdhsltvwkeleqkpthhyksasdpihtfdlygsevvgtvankigvcsll
gi_Equus	egsvlvssssdhsltvwkeleqkpthhyksasdpihtfdlygsevvgtvankigvcsll
gi_Danio	dstaslagstklstenfrgtltslsvlptkrllllgsdngairlla
gi_Tetraodon	disvspvsctklssenfrgtltslavlptkrllllgsdngairlla
gi_Xenopus	dslalpnsttklssenfrgtltlnlavlptkrllllgsdngvvrlla
gi_Gallus	essalpvsttklssenfrgtltslavlpmkchllllgsdngvirlla
gi-Taeniopygia	essalptsttklssenfrgtltslavlptkchllllgsdngiirlla
gi_Monodelphis	epps--qattklssenfrgtltslavlptkrhllllgsdngairlla
gi_Mus	epps--qattklssenfrgtltslallptkrhllllgsdngiirlla
gi_Rattus	epps--qattklssenfrgtltslallptkrhllllgsdngiirlla
gi_Callithrix	epps--qattklssenfrgtltslallptkrhllllgsdngvirlla
gi_Pongo	-----
gi_Homo	epps--qattklssenfrgtltslallptkrhllllgsdngvirlla
gi_Pan	epps--qattklssenfrgtltslallptkrhllllgsdngvirlla
gi_Oryctolagus	epps--qattklssenfrgtltslallptkrhllllgsdnglirlla
gi_Sus	epps--qattklssenfrgtltslallptkrhllllgsdngvirlla
gi_Ailuropoda	epps--qattklssenfrgtltslallptkrhllllgsdngvvrlla
gi_Equus	epps--qattklssenfrgtltslallptkrhllllgsdngvirlla

Figure D.1: continued.

Appendix E

Tblastn Search for *wdr81*

Table E.1: Tblastn Scores of Zebrafish *wdr81*

Sequences producing significant alignments:		(Bits)	Value
ref NC_007126.6	Danio rerio strain Tuebingen chromosome 15,	2262	0.0
ref NC_007112.6	Danio rerio strain Tuebingen chromosome 1,	49.3	0.001
ref NC_007121.6	Danio rerio strain Tuebingen chromosome 10,	49.3	0.002
ref NC_007117.6	Danio rerio strain Tuebingen chromosome 6,	42.4	0.18
ref NC_007116.6	Danio rerio strain Tuebingen chromosome 5,	48.1	0.004
ref NC_007118.6	Danio rerio strain Tuebingen chromosome 7,	49.3	0.001
ref NC_007124.6	Danio rerio strain Tuebingen chromosome 13,	60.8	4e-07
ref NC_007127.6	Danio rerio strain Tuebingen chromosome 16,	47.8	0.004
ref NC_007134.6	Danio rerio strain Tuebingen chromosome 23,	37.4	5.3

Table E.2: List of the Hits Obtained As A Result of Tblastn Search of Zebrafish *wdr81*

Features	
ref NC_007126.6	WD-repeat containing protein 81, neurobeachin isoform X2 and neurobeachin isoform X6
ref NC_007112.6	lipopolysaccharide-responsive and beige-like anchor protein isoform and transcription initiation factor TFIID subunit 5
ref NC_007121.6	neurobeachin a isoform X1 and neurobeachin a isoform X4
ref NC_007117.6	neurobeachin-like protein 1
ref NC_007116.6	WD repeat and FYVE domain-containing protein 3 isoform X3 and WD repeat and FYVE domain-containing protein 3 isoform X7
ref NC_007118.6	Protein FAN
ref NC_007124.6	lysosomal-trafficking regulator isoform X1, lysosomal-trafficking regulator isoform X2, WD repeat- and FYVE domain-containing protein 4 isoform X1, WD repeat- and FYVE domain-containing protein 4 isoform X2
ref NC_007127.6	neurobeachin-like protein 2 isoform X5 and neurobeachin-like protein 2 isoform X3
ref NC_007134.6	57258 bp at 5' side: keratin and 515440 bp at 3' side: keratin, type II cytoskeletal 8