

T.C.
DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL BIOMEDICINE
AND GENOME INSTITUTE

**INVESTIGATION OF THE ROLE OF
NEUROTROPHIN RECEPTOR ASSOCIATED
DEATH DOMAIN PROTEIN IN REGULATION OF
WNT/B-CATENIN SIGNALING USING THE
EMBRYONIC AND CRISPR/CAS9 KNOCKOUT
MODELS OF ZEBRAFISH**

ÖZGE ÇARK

MOLECULAR BIOLOGY AND GENETICS

MASTER DISSERTATION

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T.C.
DOKUZ EYLÜL ÜNİVERSİTESİ

İZMİR ULUSLARARASI BİYOTİP VE GENOM ENSTİTÜSÜ



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ABBREVIATIONS

NRADD	: Neurotrophin receptor-associated death domain
TNFR	: Tumor necrosis factor receptor
DD	: Dead domain
P75NTR	: p75 neurotrophin receptor
CRISPR	: Clustered Regularly Interspaced Short Palindromic Repeats
FZDs	: Frizzled receptors
GPCRs	: G-protein coupled receptors
CRD	: Cysteine rich domain
LRP5/6	: Low-density-lipoprotein-related protein5/6
Trk	: Tyrosinase kinase
ROR	: Receptor Tyr kinase-like orphan receptor
RYK	: Receptor Tyr kinase
DVL	: Dishevelled
PCP	: Planar Cell Polarity
GSK3	: Glycogen synthase kinase 3
APC	: Adenomatosis polyposis coli
CKIα	: Casein kinase I α
PP2a	: Protein phosphatase 2A
TCF/LEF	: T-cell factor/lymphoid enhancing factor
WIF	: Wnt inhibitory factor
Rnf43	: Ring finger 43
Znrf3	: Zinc and ring finger 3
NGF	: Nerve growth factor
BDNF	: Brain-derived neurotrophic factor
NT-3	: Neurotrophin 3
NT-4/5	: Neurotrophin 4/5
PI3K	: Phosphatidylinositol 3-kinase
Akt	: Protein Kinase B
Erk	: Extracellular signal-regulated kinase
PLCγ	: phospholipase C- γ
EC	: Extracellular region
TM	: Transmembrane
IC	: Intracellular region
NRH2	: Neurotrophin receptor homolog 2
PLAIDD	: p75-like apoptosis-inducing death domain
NFκB	: Nuclear factor- κ B
DRG	: Dorsal Root Ganglion
FADD	: Fas-associated death domain adaptor
FasL	: Fas ligand
ENU	: Ethylnitrosourea
ZFIN	: The Zebrafish Information Network
DSB	: Double stranded break
NHEJ	: Nonhomologous end-joining
HDR	: Homology-directed repair
gRNA	: Guide RNA
dpf	: Day-post fertilization
hpf	: Hour-post fertilization

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ABSTRACT

Wnt/ β -catenin signaling pathway plays essential roles in many biological processes including cell proliferation, migration, differentiation and apoptosis during development and adulthood. Many regulatory molecules of the pathway were revealed by researchers over the years. Yet, the whole mechanism is a puzzle with missing pieces, waiting to be solved. In this study, we identified *neurotrophin receptor-associated death domain (nradd)*, a homolog of *p75 neurotrophin receptor (p75NTR)*, as a negative feedback regulator of Wnt/ β -catenin signaling in zebrafish embryos. We revealed that *nradd* overexpression significantly suppresses Wnt8-mediated/ β -catenin signaling which controls mesoderm and neuroectoderm patterning during gastrulation. Also, its overexpression suppresses the expression of Wnt-target genes: *Lymphoid Enhancer Binding Factor 1(Lef1)*, *Sp5 transcription factor-like (Sp5l)*, and *conductin (Axin2)* and can rescue the effect of high Wnt8-mediated/ β -catenin signaling activity, loss of forebrain and eyes. In addition, we investigated *nradd* F0 knockout model generated by CRISPR/Cas9 gene-editing. To observe *nradd* loss-of-function efficiently in F0 embryos, we injected Cas9 protein with 4 guide RNAs targeting *nradd* gene to one-celled embryos. Our approach is significantly suppressed *nradd* expression in F0 crispants. In crispants, Wnt-target genes' expressions are significantly upregulated, supporting the negative regulator role of Nradd in Wnt/ β -catenin signaling. Moreover, we also analyzed the apoptosis level in crispants to evaluate the defined apoptotic function of *nradd* by previous studies. Interestingly, we observed an increased apoptosis level in crispant embryos at 24 hours-post-fertilization (hpf), confirmed by

increased expression level of pro-apoptotic genes and increased immunoreactivity of cleaved-caspase-3 antibody. In summary, Nradd acts as a negative feedback modifier of Wnt/ β -catenin signaling and a regulator of cell death during embryonic development. As the pathway modifiers serve as attractive drug targets especially in targeted therapies for cancer, further characterization of Nradd at the molecular level will significantly contribute to both elucidations of as yet unknown pathway mechanisms and the discovery of novel drugs targeting the pathway.

Keywords: Nradd; p75 neurotrophin receptor; Wnt/ β -catenin signaling; apoptosis; CRISPR/Cas9.



NÖROTROFİN RESEPTÖRÜ İLE İLİŞKİLİ ÖLÜM BÖLGESİ PROTEİNİNİN WNT/ β - KATENİN SİNYAL İLETİMİNİN DÜZENLENMESİNDEKİ ROLÜNÜN ZEBRABALIĞI EMBRİYONİK MODELİ VE CRISPR/CAS9 KNOCKOUT MODELİ İLE ARAŞTIRILMASI

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ÖZET

Wnt/ β -katenin sinyal yolu, gelişim ve yetişkinlik döneminde hücre proliferasyonu, göçü, farklılaşması ve apoptoz gibi birçok biyolojik süreçte önemli roller oynar. Yıllar boyunca araştırmacılar tarafından yolun birçok düzenleyici molekülü ortaya çıkarılmasına rağmen tüm mekanizma hala kayıp parçaları olan ve çözölmeyi bekleyen bir yapboz gibidir. Bu çalışmada, bir p75NTR homoloğu olan *nörotrofin reseptörü ile ilişkili ölüm bölgesi (nradd)* geninin, zebra balığı embriyolarında Wnt / β -kateninkatenin sinyal yolağının negatif bir geri bildirim düzenleyicisi olduğunu belirledik. Zebra balığı embriyolarında *nradd*'ın aşırı ifade edilmesinin gastrulasyon sırasında Wnt8 aracılı /-katenin sinyal iletimi tarafından kontrol edilen ventrolateral mezoderm hücre kaderlerini ve nöroektodermal hücre kaderlerini önemli ölçüde baskıladığını ortaya çıkardık. Ayrıca, *nradd* aşırı ifadesi *Lymphoid Enhancer Binding Factor 1(Lef1)*, *Sp5 transcription factor-like (Sp5l)*, and *conductin (Axin2)* gibi Wnt hedef genlerinin ifadesini bastırıp, aşırı yüksek Wnt8 aracılı / β -katenin sinyal aktivitesi sonucu embriyolarda oluşan, ön beyin ve gözlerin kaybı gibi etkileri kurtarmıştır. Ek olarak, CRISPR/Cas9 gen düzenleme sistemi ile oluşturulan *nradd* F0 fonksiyon kaybı modelini araştırdık. F0 embriyolarında *nradd* fonksiyon kaybını verimli bir şekilde gözlemlemek için, tek hücreli embriyolara *nradd* genini hedefleyen 4 kılavuz RNA içeren Cas9 proteinini enjekte ettik. Yaklaşımımız, F0 crispanlarda *nradd* ifadesini önemli ölçüde bastırmıştır. Crispanlarda, Wnt-hedef genlerin ifadeleri, önemli ölçüde artması, Wnt / β -katenin sinyallemede *Nradd*'ın negatif düzenleyici rolünü desteklemiştir. Ayrıca, önceki çalışmalarda *Nradd*'ın tanımlanmış apoptotik işlevini değerlendirmek için crispanlarda apoptoz seviyesi de analiz ettik. İlginç bir şekilde 24 saatlik crispan embriyolarda, pro-apoptotik genlerin artan ekspresyon seviyesi ve bölünmüş kaspaz-3 antikorunun artan

imm noreaktivitesi ile teyit edilen artan bir apoptoz seviyesi g zlemledik.  zetle, Nradd'ın Wnt / -katenin sinyal yolađının negatif bir geri bildirim d zenleyicisi olduđunu ve embriyonik gelişim sırasında h cre  l m n n bir d zenleyicisi olarak hareket ettiđini ortaya  ıkardık. Yolak d zenleyicileri,  zellikle kanser i in hedeflenen tedavilerde ila  hedefleri olarak hizmet ettiđinden, molek ler d zeyde Nradd'ın daha fazla karakterizasyonu, hem hen z bilinmeyen yolak mekanizmalarının aydınlatılmasına hem de yolu hedefleyen yeni ila ların keşfedilmesine  nemli  l  de katkıda bulunacaktır.

Anahtar Kelimeler: Nradd; p75 n rotrofin resept r ; Wnt/  -katenin sinyal yolađı; apoptoz; CRISPR/Cas9.



INTRODUCTION

The Wnt/ β -catenin signaling pathway is an evolutionarily conserved signal transduction cascade that controls the organization and sustainability of tissue architecture both during embryogenesis and adult life. Hypo- or hyper-activation of the Wnt/ β -catenin signaling caused by genetic or epigenetic changes have also been associated with multiple growth-related and neurological pathologies, and cancer. Since the discovery of the signaling pathway 40 years ago, many questions regarding the main mechanism of Wnt signaling have been answered to contribute therapeutic approaches for such pathologies, yet there are still many unanswered questions about its regulation and regulators. Therefore, the identification and characterization of such regulators still take an important place in understanding the whole mechanism of the signaling.

Death receptors, a subset of the tumor necrosis factor receptor (TNFR) superfamily, are found in many cell types where they possess apoptotic and non-apoptotic roles varying on cell context. All members harbor the death domain (DD) protein-interaction motif in their cytoplasmic portions. DDs interact with other DDs found in adaptor proteins, leading to a common scaffold for death receptor-induced caspase activation. However, death receptors not only activate cytotoxic signaling pathways associated with death but also function in cell differentiation and proliferation, chemokine production, and inflammatory responses. Strikingly, the p75 neurotrophin receptor (P75NTR) is one of the members which can control both programmed cell death and cell survival.

Nradd, a homolog of P75NTR, is a membrane receptor that starts to function from early embryonic development to adulthood. Previous studies demonstrated that Nradd can induce programmed cell death by interacting with P75NTR and sortilin. However, there was no literature information regarding its role in Wnt/ β -catenin signaling until the research paper of Ozalp *et al* which includes some of the results of this thesis. The results of the transcriptomic analysis performed in embryos with high Wnt activity pointed out *nradd* as a potential Wnt-target gene. Therefore, in this study, we aimed to

investigate the role of Nradd in the regulation of Wnt/ β -catenin signaling and apoptosis by using the embryonic and CRISPR/Cas9 knockout models of zebrafish.

1.1 Wnt/ β -catenin Signaling Pathway

Wnt/ β -catenin signaling pathway plays essential roles in many biological processes including cell proliferation, migration, differentiation, apoptosis and organogenesis during development and adult homeostasis. Due to these essential roles, the disorganization of Wnt signaling pathway causes various human diseases including cancer, neurodegenerative diseases, and developmental defects (Nusse and Clevers 2017a; Steinhart and Angers 2018).

The main starters of the signaling are Wnt proteins. The first Wnt protein known as *wingless* (*wg*) was identified in the landmark 1980 by Nüsslein-Volhard and Wieschaus during a genetic screen for body pattern defects (e.g: without wings) in *Drosophila melanogaster* (Nüsslein-volhard and Wieschaus 1980; Sharma and Chopra 1976). After a short time, in 1982, it was revealed that *wg* is highly conserved among multiple species with the discovery of *int-1* (*mouse mammary tumor virus integration site 1*) protein which is the vertebrate homolog of *wg* (Nusse and Varmus 1982). Thus, the Wnt name has emerged from the name combination of *wg* and its vertebrate homolog *int-1* (Nusse, 1991). Currently, there are multiple identified Wnt genes in any animal genome—19 in mammals, 7 in *Drosophila melanogaster*, and 5 in *Caenorhabditis elegans*, supporting evolutionarily conserved functions of Wnt signaling among animals (Nusse and Clevers 2017b; Pond, Doubrovinski, and Thorne 2020).

Wnt proteins are cysteine-rich glycoproteins modified by a lipid attachment an acyl group termed palmitoleic acid. This lipid modification generates a motif for binding to primary Wnt receptors, Frizzled receptors (FZDs) on the cell surface, and increases the hydrophobicity of Wnt protein, enabling to tether it to cell membranes. FZDs are seven-transmembrane-span protein (like G-protein coupled receptors (GPCRs)) with an extracellular N-terminal cysteine-rich domain (CRD) (Schulte and Bryja 2007). Wnt ligands can bind to the CRD of FZD with multiple combinations of co-receptors for mediating Wnt signaling like the low-density-lipoprotein-related protein5/6 (LRP5/6) or

receptor Tyr kinase-like orphan receptor (ROR) or receptor Tyr kinase (RYK) (He et al. 2004; Ng et al. 2019).

Upon binding of Wnt to the receptor complexes (FZD and co-receptors), the signal is received by cytoplasmic phosphoprotein Dishevelled (DVL) through three different highly conserved protein domains: an amino-terminal DIX domain (DVL and Axin), a central PDZ domain (postsynaptic density-95, discs-large and zonula occludens-1) and a carboxy-terminal DEP domain (Wallingford and Habas 2005). Depending on which of the three domains is activated by the receptor complexes, Wnt signals can initiate three different intracellular downstream pathways resulting in different outcomes in biological processes (Ng et al. 2019; Komiya and Habas 2008).

The Wnt-induced signal transduction pathways can be categorized into the canonical or β -catenin-dependent pathway and the non-canonical or β -catenin-independent pathways which include Planar Cell Polarity (PCP) pathway and the Wnt/Ca²⁺ pathway.

The best characterized of these is the canonical Wnt pathway that controls the accumulation and translocation of transcriptional co-activator β -catenin into the nucleus (Komiya and Habas 2008). In the absence of Wnt ligand, β -catenin is degraded by a cytosolic destruction complex composed of glycogen synthase kinase 3 (GSK3), adenomatosis polyposis coli (APC), Axin, casein kinase 1 α (CK1 α), protein phosphatase 2A (PP2a), and E3 ubiquitin ligase. Firstly, CK1 α and GSK3 phosphorylate β -catenin in the cytoplasm to expose the β -catenin TrCP ubiquitin target site for ubiquitination. Subsequently, E3 ubiquitin ligase ubiquitinates β -catenin and leads to proteasomal degradation (Stamos and Weis 2013; MacDonald, Tamai, and He 2009; Pond, Doubrovinski, and Thorne 2020). With the binding of a Wnt ligand to the FZD - LRP 5/6 receptor complex, series of events that disrupt the destruction complex is triggered starting with the activation of DVL through the DIX and PDZ domains. Following, both LRP5/6 and DVL deactivate members of the destruction complex by binding to first Axin, then GSK3, APC, CK1 α hence prevent the degradation and lead to the accumulation of β -catenin which is now free to translocate to the nucleus for activation of the target genes (Nusse and Clevers 2017; Pond, Doubrovinski, and Thorne 2020).

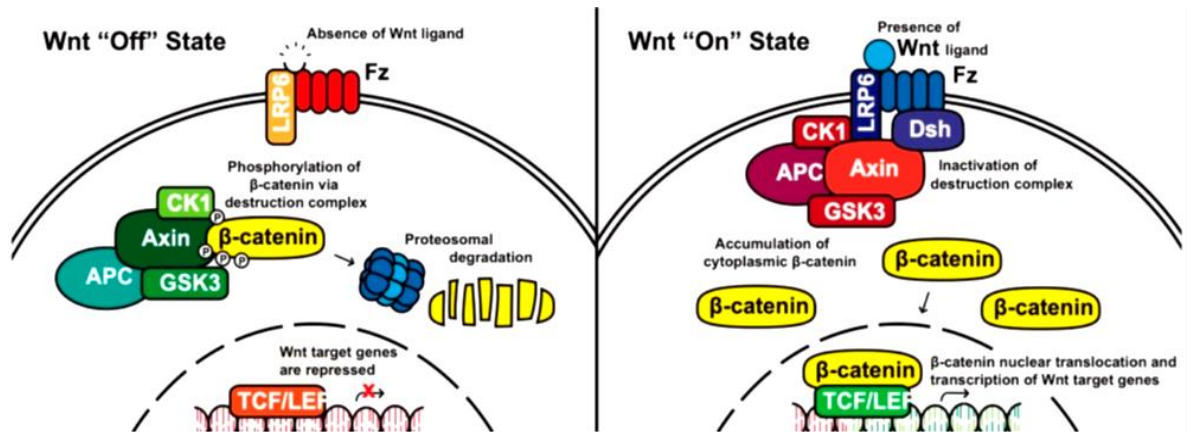


Figure 1: The mechanism of the canonical Wnt pathway in the presence or absence of Wnt ligand. APC, adenomatous polyposis coli; CK1, casein kinase 1; GSK3, glycogen synthase kinase 3; TCF/LEF, T-cell factor/lymphoid enhancing factor; LRP6, lipoprotein receptor-related protein 6; FZD, Frizzled; Dsh, Disheveled (Ng et al. 2019).

In the nucleus, β-catenin engages with TCF/LEF transcription factors which are bound to DNA through the motif 50-AGATCAAAGG-30 found in the promoter of target genes to transiently convert TCF into a transcriptional activator. β-catenin is not only interacted with TCF but also binds to chromatin modifiers such as CBP and Brg-1 and Parafibromin/Hyrax, homologs of yeast Cdc73 through its C-terminal domain, for transcriptional activation. In the absence of β-catenin, TCFs form a complex with Groucho proteins which repress transcription of Wnt target genes by mediating Histone deacetylation (Nusse and Clevers 2017; Komiya and Habas 2008).

1.1.1 The Regulation of Wnt/β-catenin Signaling

Many mechanisms exist to regulate Wnt signaling negatively or positively at the ligand, receptor, intracellular and nuclear levels. Wnt signaling can be inhibited through the cleavage of N terminus of Wnts by Tiki, a protease or removing palmitoleate moiety of Wnts by Notum, a carboxylesterase at ligand level (Kakugawa et al. 2015; Steinhart and Angers 2018). Moreover, Wnt ligands can be sequestered through interaction with proteins that prevent their interaction with the receptor complex, such as Wnt inhibitory factor (WIF) or secreted frizzled-related proteins which show homology with the Frizzled CRD (Hsieh et al. 1999).

As one of negative-feedback loops of the pathway, Dickkopf-1, a Wnt-β-catenin target gene, binds to LRP5/6 and acts as a competitor of Wnts, leading to sequestration of LRP5/6 (González-Sancho et al. 2005). In addition, transmembrane E3-ubiquitin-

ligases, ring finger 43 (Rnf43) and (zinc and ring finger 3) Znf3 which are Wnt- β -catenin target genes, leads to ubiquitin-dependent Frizzled endocytosis and proteasomal degradation, hence decrease receptor levels at the plasma membrane (Hao et al. 2012).

One of the best-characterized negative feedback regulations of β -catenin signaling in the cytoplasm is achieved by Axin2. Axin proteins, Axin1 and Axin2 are equivalent in terms of their functions in the pathway. However, Axin2 is a Wnt target gene so its expression is regulated by β -catenin signaling in contrast to its paralog Axin1 which is the member of the destruction complex. The increase in the level of Axin2 which is induced by Wnt signaling activity supports destruction complex formation and β -catenin degradation thereby form a negative feedback loop to balance the pathway activity (Chia and Costantini 2005; Steinhart and Angers 2018; Goentoro and Kirschner 2009).

Also, there are some positive regulation mechanisms in the pathway. Recently, it was revealed that the cell surface protein Leucine-rich repeat-containing G-protein coupled receptor 5 which is also a Wnt target gene, and R-spondin proteins complex amplify Wnt signaling activity by blocking the activity of Rnf43 and Znf3 which downregulate Frizzled at the cell surface (Steinhart and Angers 2018; Hao et al. 2012).

1.2 Neurotrophins and Their Receptors

Neurotrophins, a group of trophic growth factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5), control various biological processes regarding cell survival, development, and apoptosis (Hempstead 2014; Meldolesi 2018)

Neurotrophins, like most growth factors, are synthesized as precursors called pro-neurotrophins that consist of an N-terminal prodomain and a C-terminal mature domain. After translation, the N-terminal prodomain can undergo proteolytic cleavage by furin and proconvertase in the Golgi or in secretory vesicles to form mature neurotrophins (Meldolesi 2018). Mature neurotrophins operate through activating tyrosine kinases (Trk) A, B, C receptors, and p75NTR which is the member of the Tumor Necrosis Factor Receptors superfamily (TNFR) (Meldolesi 2018; Hempstead 2014). While Trk family members work in a ligand-specific manner: NGF is the

preferential ligand for TrkA, BDNF and NT-4 for TrkB, and NT-3 for TrkC, p75NTR have an affinity to all mature neurotrophins and exceptionally to all precursor versions, proneurotrophins. (Ichim, Tauszig-Delamasure, and Mehlen 2012).

Activation of Trk receptors by mature neurotrophins can activate several signaling pathways like (phosphatidylinositol 3-kinase) PI3K-Akt (Protein Kinase B), extracellular signal-regulated kinase (Erk) and phospholipase C- γ (PLC γ) pathways which regulate a large spectrum of processes including the proliferation and survival of neurons, axonal and dendritic growth, cytoskeleton remodeling, synapse formation and maintenance (Ichim, Tauszig-Delamasure, and Mehlen 2012; Meeker and Williams 2015). However, P75NTR exhibits differential roles resulting in opposing actions in cells depending on the ligand type, and with this feature, is the most studied neurotrophin receptor.

1.3 Nradd: The Homolog of p75NTR

p75NTR is composed of a four-cysteine-rich extracellular region (EC), a transmembrane (TM) domain, and a relatively short intracellular region (IC). EC binds to neurotrophins with low affinity and their respective precursors with high affinity. The intracellular portion of the receptor contains a death domain that can induce pro-apoptotic pathways via interactions with other effector proteins (Frankowski et al. 2002). While pro-neurotrophin binding to P75NTR mostly results in the activation of pro-apoptotic pathways through sortilin interaction, the binding of mature neurotrophins to P75NTR leads to biological fates parallel to ones activated by Trk receptors like cell survival and growth by enhancing neurotrophin binding to Trk receptors. Yet, some studies showed that also mature neurotrophin binding can induce cell death in certain situations (Meeker and Williams 2015).

The Neurotrophin receptor associate death domain gene (NRADD, also termed as neurotrophin receptor homolog 2, NRH2 and p75-like apoptosis-inducing death domain, PLAIDD) is a homolog of p75NTR that show high similarity in its transmembrane and intracellular domains to p75NTR (Frankowski et al. 2002; Wang et al. 2003; Kim and Hempstead 2009). However, it contains a unique truncated N-terminal extracellular domain that cannot serve as a ligand-binding site alone, unlike other members of the TNFR family including p75NTR. A high level of *NRADD*

expression is seen starting from early development but gradually downregulated in adult tissues. In subpopulations of cells in the spinal cord, neonatal retina, dorsal root ganglion, *NRADD* expression is seen together with p75NTR expression. It has been reported that pro-neurotrophin-induced apoptosis is driven by the interaction between NRADD, p75NTR, and sortilin in these tissues. It was shown that Nradd is a trafficking switch to regulate sortilin localization which affect pro-apoptotic fate decisions (Kim and Hempstead 2009).

The results of *NRADD* over-expression experiments in cell lines and primary neurons suggested that *NRADD* may possess an apoptosis-inducing role (Frankowski et al., 2002; Wang et al., 2003), likely through the activation of caspase-8, the extrinsic apoptotic pathway. Moreover, it has been reported that overexpression of NRADD-IC-domain mimics p75NTR-triggered Nuclear factor- κ B (NF κ B) signaling. Acute *NRADD* knockdown cause a decrease in the levels of total and plasma membrane sortilin in cortical and Dorsal Root Ganglion (DRG) sensory neurons, again supporting NRADD's role in pro-neurotrophin signaling (Kim and Hempstead 2009). On the other hand, NRADD can also enhance NGF signaling which controls cell survival and differentiation, by increasing NGF binding affinity to TrkA. Interestingly, Sung *et al* reported that transgenic mice overexpressing *NRADD* showed a reduced level of apoptosis (Sung et al. 2013; Vilar et al. 2014). They showed that NRADD forms a complex with Fas-associated death domain adaptor (FADD) and inhibits FADD's role in Fas ligand (FasL)-induced caspase activation / cell death. In addition, they observed that transgenic mice over-expressing *NRADD* exhibit a significant improvement in neuronal survival and functional recovery in response to nerve injury (Sung et al. 2013). Thus, there are conflicting results about the role of NRADD in both apoptosis and cell survival.

A recent work regarding NRADD protein reported that the complete *NRADD* knockout (KO) mice are viable and fertile with no observable malformations or defects. However, the proportion of DRG sensory neurons expressing p75NTR showed an increase, but their total number remained same in *NRADD* KO mice (Unsain et al. 2016).

1.4 Zebrafish (*Danio rerio*) as a Model Organism

The widespread acceptance of zebrafish as a model is mostly attributed to George Streisinger who initiated one of the first genetic screens of zebrafish by intentionally mutating offsprings for obtaining notable phenotypes (forward genetics)(Chakrabarti et al. 1983; Walker and Streisinger 1983). Following years, Christiane Nusslein-Volhard and Wolfgang Driver enlarged genetic screening of zebrafish by describing over 4000 mutant lines obtained using chemical ethylnitrosourea (ENU) mutagenesis in 1996 (Haffter and Nüsslein-Volhard 1996). This study was greatly contributed to the sequencing of the zebrafish genome which began in 2001 at the Sanger Institute and completed in 2013 (Howe et al. 2013). The zebrafish genome is composed of about 26,000 genes over 1.4 billion base pairs on 25 chromosome pairs (Daga, Thode, and Amores 1996; Howe et al. 2013). Approximately 70% of zebrafish genes have at least one obvious human orthologue (Howe et al. 2013).

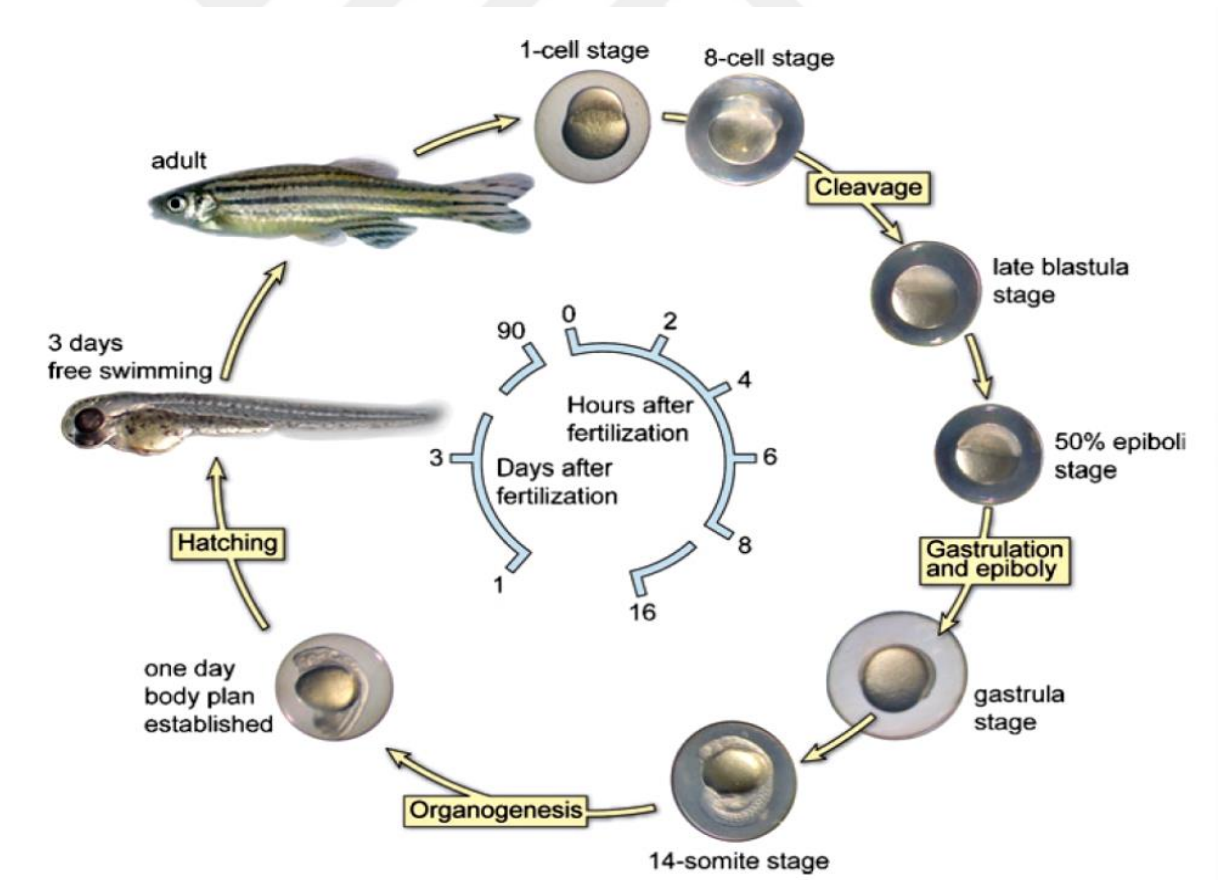


Figure 2: A schematic representation of the embryonic developmental stages of zebrafish (Willemsen et al. 2011).

Zebrafish is rapidly developing vertebrate with a relatively short generation time. Offsprings can reach sexual maturity after approximately 3 to 4 months and their average life span is between 2 and 4 years. They are relatively easy to maintain and breed in a laboratory. Zebrafish embryos with its rapid external development are tolerant to experimental manipulations like microinjection, cell transplantation, and gene-editing (Dahm and Geisler 2006). Their natural transparency until 24 hour-postfertilization enables us to observe early developmental stages (gastrulation, somitogenesis, and the beginning of organogenesis) without any other experimental procedure (Meyers 2018). In addition, the transparency of embryos can be lengthened to later stages with the usage of some chemicals inhibiting pigment formation without affecting development (Choi et al. 2007).

A large number of offsprings (approximately 200-300 eggs) can be obtained from mating a single female and male pair under optimal conditions. This feature makes zebrafish an advantageous vertebrate model organism for population genetics. The size of zebrafish embryos is approximately 1 mm in diameter/length. Therefore, relatively small embryos and larvae can fit into wells of standard 96-well plates, allowing genetic mapping approaches and high-throughput screening of small compound libraries (Dahm and Geisler 2006; Meyers 2018). Another important factor that makes working with zebrafish easier is The Zebrafish Information Network (ZFIN). This network provides almost all information about zebrafish including genomic information, gene expression data, available constructs, transgenic lines, experimental procedures, and laboratories working with zebrafish, etc, and can be found online at <https://zfin.org> (Sprague et al. 2006)

1.4.1 Genetic Tools and Methods for Studying Gene Function in the Zebrafish

The presence of many optimized protocols and tools for genetic manipulation of zebrafish embryos makes the zebrafish a well-suited model organism to investigate the function of a particular gene of interest (Meyers 2018).

1.4.1.1 Random Mutagenesis

One of the most widely used techniques in the early 1990s is mutagenesis screening methods based on the random induction of mutations and investigation of

individuals with mutant phenotypes. Several mutagenesis strategies have been established for the zebrafish including protocols for chemical mutagenesis using ENU as a mutagen, radiation mutagenesis with mainly gamma rays, and insertional mutagenesis using retroviruses or transposable elements (Haffter et al. 1996; Sivasubbu et al. 2007; Fritz et al. 1996).

1.4.1.2 Gene Knock Down

Another widely used method is morpholino knock-down used for silencing the expression of a particular gene. The protocol covers the sequence-specific translational inhibition (degradation of mRNA) using synthetic antisense oligonucleotides targeting the messenger ribonucleic acid (mRNA) of that particular gene. The simplicity of oligonucleotide design and procedure made morpholino-knock-down one of the broadly used techniques to investigate the function of various genes (Nasevicius and Ekker 2000). However, as the new genetic manipulation methods emerging, many of the phenotypes obtained by morpholinos were unable to be replicated by these gene-editing strategies. This situation can be explained by off-target effects or different compensation mechanisms between translational inhibition and loss-of-function mutations in the genome (Rossi et al. 2015). Therefore, discussions on the reliability of morpholino usage for gene loss-of-function studies still continue in academia (Meyers 2018).

1.4.1.3 Transgenesis

Transgenesis methods that are used to generate organisms with novel properties are also easily performed in zebrafish. To insert transgene sequences into the zebrafish genome, there are many established strategies including Tol2 and Sleeping Beauty transposon system (Davidson 2003; Kawakami 2007; Kwan et al. 2007). The presence of such applicable systems facilitated the generation of stable transgenic lines expressing fluorescent proteins, cre-recombinases, Gal4-UAS system, genes under inducible promoters by temperature, light, and chemicals. These transposon systems have also been employed to set enhancer traps in zebrafish to uncover novel and useful regulatory elements (Balciunas et al. 2004; Parinov et al. 2004)

1.4.2 Genome-Editing Strategies

Targeted genome-editing in zebrafish can be achieved by several highly versatile technologies. Before the discovery of clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) technology, zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs) were mostly used for introducing sequence-specific modifications into the genome. Both ZFNs and TALENs utilize modifiable chimeric proteins with (deoxyribonucleic acid) DNA binding domains specific to a gene of interest (target gene) and a nuclease domain to drive a double-stranded break (DSB) at the target DNA. This break provokes double-strand DNA break repair mechanisms such as nonhomologous end-joining (NHEJ) leading to random base insertions or deletions which result in gene knockout. As another option, if there is a donor template with homology to the targeted chromosomal site, additional gene insertion or base-editing can be achieved by homology-directed repair (HDR). Even though these two methods are reliable in genome editing, their procedure requires laborious steps like the design, production, and validation of the chimeric proteins to achieve robust gene-targeting (Doyon et al. 2008; Bedell et al. 2012; Dahlem et al. 2012; Gaj et al. 2016)

1.4.2.1 From Bacterial Defense Mechanism to Genome Editing Technology: CRISPR/Cas9 system

On the other hand, the CRISPR/Cas9 system simplified the genome editing procedure far more than ZFNs and TALENs. The CRISPR/Cas9 system, which is originally a bacterial defense mechanism for foreign DNAs of invading viruses and plasmids is transformed into a powerful, efficient, and rapid genome editing technology by Charpentier, Doudna, and co-workers in 2012 (Sorek, Lawrence, and Wiedenheft 2013; Jinek et al. 2012; Wiedenheft, Sternberg, and Doudna 2012). In the original defense mechanism, short fragments of invader DNA are introduced into the CRISPR locus on the bacteria genome and transcribed into CRISPR RNA (crRNA) which then form a complex with trans-activating crRNA (tracrRNA). Following, the crRNA:tracrRNA duplex directs Cas9 nuclease for sequence-specific degradation of foreign DNA (Gaj et al. 2016). The pioneers of CRISPR/Cas9 genome editing technology inspired by this sequence-specific nuclease-targeting strategy in bacteria

and this inspiration was awarded the 2020 Nobel Prize in Chemistry only 8 years after its discovery (Ledford and Callaway 2020).

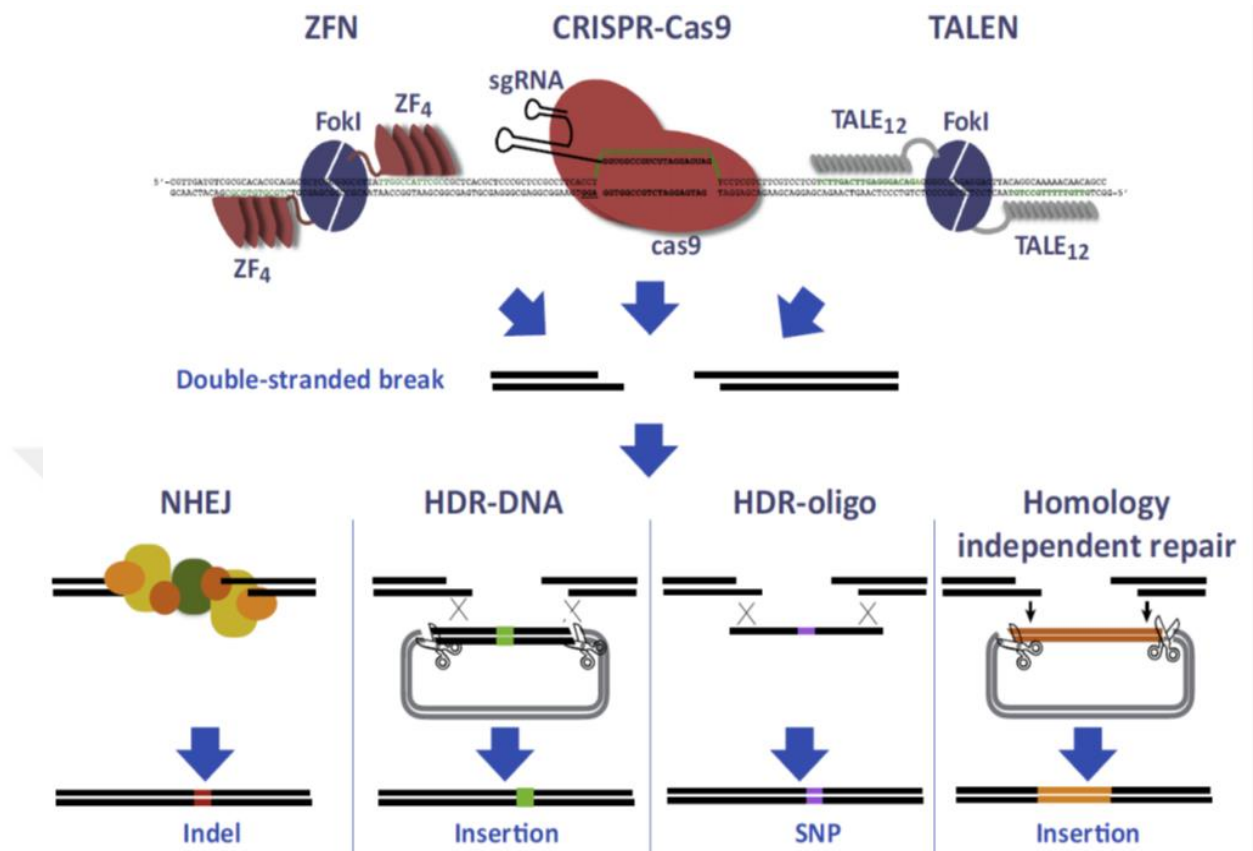


Figure 3: Gene-editing Mechanism Using Zinc-Finger Nuclease (ZFN), Transcription Activator-Like Effector Nuclease (TALEN), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–Cas9 Endonucleases (M. Li et al. 2016).

1.4.2.2 The Mechanism of CRISPR/Cas9 editing

Cas9 protein has a distinguished domain that attaches to a short DNA sequence named the protospacer adjacent motif (PAM). After Cas9 protein forms a complex with the crRNA:tracrRNA duplex or its combined version single guide RNA (sgRNA), it is specifically directed to target DNA strand via base pairing between the 20-nucleotide spacer sequence within the crRNA-tracrRNA duplex or sgRNA and the targeted DNA strand. Accomplished base pairing of spacer to target DNA along with PAM-site recognition triggers two endonuclease domains of Cas9, leading to a DSB in the target DNA. As mentioned before, DSB activates repair mechanisms NHEJ and HDR which allows manipulations on the genome (M. Li et al. 2016).

Over the following years, many research groups contributed to CRISPR /Cas9 technology with various approaches aiming to modify gene function or gene expression, to insert DNA fragments, and even to label nucleic acids fluorescently. One of the first improvements was combining the crRNA-tracrRNA duplex into single guide RNA (sgRNA) which is fully functional to increase the efficiency of the process (Jinek et al. 2012). Following, Cas9 protein has been undergone several modifications. One of the first modifications is the addition of nuclear localization signal promoting translocation of Cas9 to the nucleus, allowing genome editing in eukaryotes. This was followed by many modifications which aim to increase the specificity and efficiency of Cas9 protein and decrease the toxicity of the process (M. Li et al. 2016). Since one of the decisive factors in the Cas9 specificity is guide RNA which is responsible for directing Cas9 to specific genomic loci, ideal guide RNA design is very important for successful genome editing experiments. There are many web-based tools developed for guide RNA design specific to target sequences. Even, some of them specialized for several model organisms and each of them has different features and advantages (Liu et al. 2019).

1.4.2.3 CRISPR-Cas9-mediated gene knockout in Zebrafish

The process of gene knock-out in zebrafish is not laborious as it is in other vertebrate model organisms. Simply, guide RNAs along with in vitro-synthesized mRNA encoding a nuclear-localized Cas9 or with Cas9 protein with a nuclear localization sequence are directly injected into one-celled zebrafish embryo (Meyers 2018; Sorlien, Witucki, and Ogas 2018) . The injection results in highly mosaic F0 embryos. The mosaic F0 adult zebrafish are either incrossed to another zebrafish with the same gene targeted or outcrossed to wild-type lines. The incross of offsprings result in trans-heterozygous offspring (TF1), while the outcross ends up with heterozygous individuals (F1) that can be raised and incrossed to obtain homozygous mutants termed as F2 (Sorlien, Witucki, and Ogas 2018; M. Li et al. 2016; Moreno-Mateos et al. 2015). Generally, mutant phenotypes are studied in the F2 generation which takes around 9-10 months to obtain. However, several gene-targeting strategies have been developed to obtain mutant phenotypes in F0 zebrafish, speeding up the mutant characterization process (Hoshijima et al. 2019; Kroll et al. 2021; Wu et al. 2018).

1.4.2.4 A simple and effective F0 knockout method

To reveal the functional role of a certain gene necessitates identification and characterization of the phenotype associated with the complete loss of gene function. However, the key limiting factor is the space and time required to breed animals possessing a homozygous mutation in the gene of interest. Recently, to overcome this problem, a simple CRISPR-Cas9 strategy that delivers rapid, consistent biallelic gene disruption in F0 zebrafish embryos was developed by several groups. The protocol utilizes a mixture of guide RNAs (3 or 4), each of them targeting different sites within the gene of interest and this provides high and efficient mutagenesis. This F0 knockout method mostly succeeds to convert > 90% of injected F0 embryos into biallelic knockouts showing homozygous null phenotypes with penetrance close to standard F2 homozygous knockouts and shortens the experimental time required for loss of function analysis in zebrafish from months to one week (Hoshijima et al. 2019; Kroll et al. 2021; Wu et al. 2018).

1.4.2.5 The validation of guide RNA efficiency

The validation of guide RNA efficiency is crucial for the F0 Knockout method. To identify possible insertions or deletions induced by the injection of guide RNA mix, there are several genotyping methods. In zebrafish, the most common and traditional genotyping method is based on loci-specific amplification by polymerase chain reaction (PCR) followed by digestion of amplicons harboring specific restriction sites at the targeted site by specific enzymes, or direct sequencing. These methods require a considerable amount of genomic DNA for the reaction and laborious and time-consuming steps to complete the screen. Recently, a new method that uses fluorescent PCR or high-resolution melting curve (HRM) analysis arose and eases the genotyping process and saves time. Simply, HRM analysis is based on the high-resolution monitoring of the denaturation process of a double-stranded fluorescently labeled amplicon. This technique compares melting curves of mutant amplicons with wild-type amplicon, and it can determine CRISPR-induced micro-indels and even class-4 single nucleotide polymorphisms (SNPs) in F0 embryos rapidly and cost-effectively (Samarut, Lissouba, and Drapeau 2016).

In a nutshell, in this study, we generated F0 *nradd* knockout model to investigate whether the loss of *nradd* function affects Wnt/ β -catenin signaling and apoptosis using novel methods.

MATERIALS & METHODS

2.1 Zebrafish Handling and Maintenance

All zebrafish studies were conducted with the approval of IBG-HADYEK in DEU-IBG Institute, Izmir. Zebrafish were maintained in the IBG aquarium system in standard conditions and kept on a light-dark cycle of 12 hours in light and 12 hours in dark at 28°C. AB strains were used as WT zebrafish. Embryos from natural spawnings are maintained in E3 medium until 5 days-post-fertilization (dpf) then transferred to the aquarium system as described (Meyers 2018). Developmental staging was done based on Kimmel et al (Kimmel et al. 1995). Embryos were microinjected at the one-cell stage and analyzed between 4 hours and 5 days post-fertilization. In all experiments, microinjections were performed on embryos from multiple simultaneously produced clutches.

2.2 Transgenic Fish Lines

Transgenic zebrafish lines Tg(hsp70l:wnt8a-GFP)^{w34} and Tg(hsp70l:Mmu.Axin1-YFP)^{w35} were outcrossed to AB strain (WT). Embryos were heat shocked in hot E3 medium at 38°C for 1 hour in determined stages (gastrulation, somitogenesis and organogenesis). Transgenic embryos were identified under fluorescent microscope as described previously (Kagermeier-Schenk et al. 2011; Weidinger et al. 2005; Ozalp et al. 2021). Animal experiments were approved by the Animal Experiments Local Ethics Committee of Izmir International Biomedicine and Genome Institute (IBG-AELEC) on 26.07.2017 with the protocol number 12/2017.

2.3 RNA Extraction, cDNA Synthesis and Quantitative PCR

RNA was extracted from whole embryos in Qiazol (Qiagen) and purified (Qiagen-miRNeasy Micro Kit). Thirty embryos were pooled for each experimental group. All samples were converted to cDNA using ProtoScript II First Strand cDNA Synthesis Kit (New England BioLabs, MA, United States) according to the manufacturer's instructions. Firstly, 1 µg of template RNA and random primer mix was mixed up to 8

μl in a sterile RNase-free microfuge tube and denatured for 5 minutes at 65°C. After spinning briefly, the tube was put promptly on ice and 10 μl ProtoScript II Reaction Mix (2X) and 2 μl of ProtoScript II Enzyme Mix (10X) were added (Table 1). Then, cDNA synthesis reactions were kept at 25°C for 5 minutes, followed by incubating at 42°C for one hour. The reaction enzyme was inactivated at 80°C for 5 minutes. cDNA products were stored at -20°C until qPCR experiments. For qPCR experiments, cDNA mixes were diluted to 1:30

Table 1: cDNA synthesis reaction.

COMPONENT	VOLUME
<i>Total RNA</i>	1–6 μl (up to 1 μg)
<i>Random Primer (60 μM)</i>	2 μl
<i>Nuclease-free H₂O</i>	to a total volume of 8 μl
<i>ProtoScript II Reaction Mix (2X)</i>	10 μl
<i>ProtoScript II Enzyme Mix (10X)</i>	2 μl

Zebrafish *rpl13a* (ribosomal protein L13a) was used as the reference gene for normalization to determine the relative gene expression levels. Quantitative PCR (qPCR) was performed in triplicate using GoTaq qPCR Master Mix (Promega, Madison, WI, United States) at an Applied Biosystems 7500 Fast Real-Time PCR machine (Foster City, CA, United States). The data were analyzed using the GraphPad Prism 8 software (Graphpad Software Inc., CA, United States). The values are the mean ± SEM (standard deviation of mean) of three samples. The following primers were used for qPCR:

zebrafish *sp5l* forward 5'-GCTTCACGCAGGTGTGGAT-3'

zebrafish *sp5l* reverse 5'-TTCTGGAGATGAGCTGGGAGT-3'

zebrafish *axin2* forward 5'-TAGTTTTGCCCCTGCCACG-3'

zebrafish *axin2* reverse 5'TCCCAGCTTGTAAGGAGGAATG-3'

zebrafish *cdx4* forward 5'CCAGAGAAAATCAGAGCTGGCA-3'

zebrafish *cdx4* reverse 5'TTGCACCGAGCCTCCACTATT-3'

zebrafish *nradd* forward 5'AAGTGTCTCAGCCATGCCAAGA-3'

zebrafish *nradd* reverse 5'AGGAATACCGATGGGGCAATG-3'

zebrafish *rpl13a* forward 5'-TCTGGAGGACTGTAAGAGGTATGC-3'

zebrafish *rpl13a* reverse 5'-AGACGCACAATCTTGAGAGCAG-3'

Capped Sense mRNA Synthesis, Microinjection

Capped sense RNAs of WT *nradd* and *nradd-GFP*, *Wnt8* and *GFP* (control RNA) were synthesized with Sp6 mMessage mMachine Kit (Thermo Fisher Scientific, Waltham, MA, USA). Firstly, 5 µg of each plasmid (containing coding sequences of *nradd*, *nradd-GFP*, *Wnt8*, *GFP*) is linearized in 5-hour digestion using XbaI restriction enzyme (Thermo Fisher Scientific, Waltham, MA, USA) and its commercial reaction buffer. After that, linearized plasmids were purified using NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL). Capped sense RNAs were in vitro transcribed with 2-hour incubation at 37°C with the following transcription mix (Table 2). Then, mRNAs were purified using RNA Clean and Concentrator-25 (Zymo Research).

Table 2: Capped sense mRNA synthesis reaction.

Component	Volume/Amount
Linear Plasmid Template	0.1–1 µg
10X Reaction Buffer	2 µl
2X NTP/CAP	10 µl
Enzyme mix	2 µl
Nuclease-free water	up to 20 µl total

For whole-mount in situ hybridization (WMISH), 150-250 pg mRNA was injected into one-cell zebrafish embryos and embryos were either fixed at the indicated stages (30%, 60%, 80, % 100% epiboly, 3,5,10-somite, or 24 hpf) in 4% PFA overnight or pooled for RNA extraction for next experimental procedures.

2.4 RNA Probe Synthesis

For the preparation of the plasmid DNA template, 5 µg of each plasmid (containing probe sequences of *nradd*, *gooseoid*, *hoxb1b*, *krox20*, *foxd1a*, and *her*) is linearized in 5-hour digestion using the appropriate restriction enzyme and its

commercial reaction buffer. After that, linearized plasmids were purified using NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL).

The antisense RNA probes were in vitro synthesized with 2-hour incubation at 37°C with the following transcription mix (Table 3) and commercial protocol (T7 RNA Polymerase, EP0111, Thermo Fisher Scientific).

Table 3 : RNA probe synthesis reaction for *In situ* hybridization.

Component	Volume/Amount
<i>Linear Plasmid Template</i>	1µg
<i>Transcription buffer</i>	4µl
<i>NTP-DIG-RNA</i>	2µl
<i>RNAse inhibitor</i>	1µl
<i>T7 RNA polymerase</i>	1µl
<i>Nuclease-free water</i>	up to 20 µl total

Then, the antisense RNA probes were purified using RNA Clean and Concentrator-25 (Zymo Research).

2.5 Whole-Mount *In situ* Hybridization

WMISH was performed with *nradd*, *goosecoid*, *hoxb1b*, *krox20*, *foxd1a* and *her5* antisense RNA probes (Thisse and Thisse 2008) After fixation step, embryos were dechorionated in 1X PBS manually by using sharp forceps and put into 2 ml tubes. Then, embryos were washed (dehydrated) with following solutions respectively: 25% methanol-75% PBT for 5 minutes, then 50% methanol-50% PBT for 5 minutes, then 75% methanol-25% PBT for 5 minutes and finally 100% methanol for 5 minutes. After dehydration step, embryos were re-hydrated by washing with following solutions respectively: 75% methanol-25% PBT for 5 minutes, then 50% methanol-50% PBT for 5 minutes, then 25% methanol-75% PBT for 5 minutes and finally 100% PBT for 5 minutes 4 times. After washing steps, permeabilization of embryos was achieved by incubation of 24 hours-embryos in solution of 10 µg of Proteinase K /ml in PBT for 5 minutes. After that, embryos were rinsed 2 times in PBT to stop permeabilization. Then, embryos were refixed in 4% PFA for 20 min at room temperature. After that, embryos were washed in PBT for 5 minutes at 5 times. At the pre-hybridization step, embryos

were incubated 0,6-0,7 mL hybridization buffer for three hours at 67 °C. After that, the hybridization mix was removed. After removing hybridization buffer, 200 ul of pre-heated hybridization mix containing DIG labeled RNA probe (200 ng of *nradd*, *goosecoid*, *krox20*, *foxg1a*, and *her4* RNA probes) was added into tubes and incubated overnight at 67 °C. Firstly, probes were recovered for next re-use and then embryos were washed several times according to following steps to remove hybridization/probe mix.

Wash Steps: in Hybridization buffer at 67 °C for 20 minutes

in 50 %SSCT 2X + 50% formamide at °C for 20 minutes at 3 times

in SSCT 2X at 67 °C for 20 min at 2 times

in SSCT 0,2X at 67 °C for 30 min at 4 times

in PBT at room temperature for 5 min.

After washing steps, embryos were incubated in blocking buffer for 1 hour with shaking. After removing blocking buffer, embryos were incubated in 200 ul of antibody solution (anti-DIG-AP (Roche) antiserum 1:4000 in PBT +2 mg/ ml BSA.) at 4 °C overnight. After that, antibody was recovered for next re-use, and embryos were washed in PBT for 20 minutes at 6 times. Then embryos were washed with staining buffer NTMT for 5 minutes at 3 times. During last wash, embryos were transferred to 24-well plate. After that, the staining solution was prepared (3,5 µL of NBT and 3,5 µL of BCIP per 1 ml of NTMT). 500 ul of staining solution was added to each well. Embryos were left to incubation at room temperature without light. After the incubation, to stop reaction, firstly embryos were rinsed by 2X PBT and 1X stop solution. After that, embryos were kept in 80% glycerol and 20% stop solution at 4 °C. Embryos were visualized under a stereomicroscope.

Table 4: Required Solution Contents for Whole-Mount In situ Hybridization.

Solution	Content
10x PBS	80.0g NaCl 2.0g KCl 14.4g Na ₂ HPO ₄ 2.4g KH ₂ PO ₄ Up to 1000 ml distilled water
1X PBST	1X PBS, 0,1% Tween 20
4% PFA	8g PFA 0.5ml 2M NaOH 20 ml 10X Up to 200 ml
20x SSC	175.3 g NaCl 88.2 g sodium citrate Up to 1000 ml distilled water Use citric acid to adjust the pH to 5
SSCT	1X SSC, 0,1% Tween 20
NBT	50 mg Nitro Blue Tetrazolium dissolved in 0.7 ml de N,N dimethylformamide anhydrous and 0.3 ml of water (store at -20°C)
BCIP	50 mg of 5-Bromo 4-Chloro 3-indolyl Phosphate dissolved in 1 ml of N,N-dimethylformamide anhydrous (store at -20°C)
NTMT	2ml 1M Tris-HCl pH9.5 1ml MgCl ₂ 1M 2ml NaCl 1M 100µl 20%Tween20 Up to 20 ml (prepared fresh each time)
Staining Solution	3.5µl BCIP and 3.5µl NBT to 1ml NTMT
Hybridization Buffer	50% formamide 5 x SSC 50 µg/ml Heparin 500 µg/ml tRNA 0,1% Tween 20
Stop Solution	5ml 0.5M fosfat tamponu pH: 5.8 0.1ml 0.5M EDTA 0.5ml 20%Tween20

2.6 Guide RNA Targeting Strategy

The coding sequence of zebrafish *nradd* and *tyrosinase* was divided into five equal segments and 4 sgRNAs were designed to target the first four segments in accordance with the strategy described in the paper of Wu et al.

2.7 sgRNA Synthesis

Oligonucleotide templates for target-specific forward gRNA primers containing a T7 promoter and the common reverse primer encoding the standard chimeric scaffold gRNA were obtained from Oligomer A.Ş. The sequences used are listed in Table 5. Oligonucleotide templates were dissolved in distilled water as a 100 mM stock solution. Ten microliters each of the forward and reverse oligonucleotides were mixed with 180 µL distilled water and annealed by following an annealing PCR protocol (Table 6) (Phusion High-Fidelity DNA Polymerase, New England Biolabs). The annealed double-stranded oligonucleotide templates were purified and recovered in distilled water following the column purification protocol by NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL). sgRNAs were in vitro transcribed from the annealed double-stranded oligonucleotide templates by using HiScribe™ T7 Quick High Yield RNA Synthesis Kit (New England Biolabs) (Table). RNA was purified using RNA Clean and Concentrator-25 (Zymo Research).

Table 5: Sequences of template primers for guide RNA synthesis.

Primer Name	Primer Sequence	Spacer Sequence
<i>nradd_Target_1</i>	TAATACGACTCACTATAGGCATGTGTGCGGCGGGCA CGTTTTAGAGCTAGAAATAGC	AGCATGTGTGCGGCGGGCAC
<i>nradd_Target_2</i>	TAATACGACTCACTATAGGCCAGGCCTGTGGACTA CGTTTTAGAGCTAGAAATAGC	GTCCCAGGCCTGTGGACTAC
<i>nradd_Target_3</i>	TAATACGACTCACTATAGGGCTCCAGAGCCTGGTGC TGTTTTAGAGCTAGAAATAGC	GGGCTCCAGAGCCTGGTGCT
<i>nradd_Target_4</i>	TAATACGACTCACTATAGGTCTGGTGAAGGCCCGGG TGTTTTAGAGCTAGAAATAGC	GCTCTGGTGAAGGCCCGGGT
<i>tyr_Target_1</i>	TAATACGACTCACTATAGGCGCACACAGAGCCGTCG CGTTTTAGAGCTAGAAATAGC	GCCGCACACAGAGCCGTCGC
<i>tyr_Target_2</i>	TAATACGACTCACTATAGGATGCATTATTACGTGTCC GTTTTAGAGCTAGAAATAGC	GGATGCATTATTACGTGTCC
<i>tyr_Target_3</i>	TAATACGACTCACTATAGGCTCTACATCGGCGGATGT GTTTTAGAGCTAGAAATAGC	GACTCTACATCGGCGGATGT
<i>tyr_Target_4</i>	TAATACGACTCACTATAGGATCCGTCGTTGTGTCCGA GTTTTAGAGCTAGAAATAGC	GTATCCGTCGTTGTGTCCGA

<i>scr_control_1</i>	TAATACGACTCACTATAGGCAGGCAAAGAATCCCTG CCGTTTTAGAGCTAGAAATAGC	no target
<i>scr_control_2</i>	TAATACGACTCACTATAGGTACAGTGGACCTCGGTGT CGTTTTAGAGCTAGAAATAGC	no target
<i>scr_control_3</i>	TAATACGACTCACTATAGGCTTCATACAATAGACGAT GGTTTTAGAGCTAGAAATAGC	no target
<i>scr_control_4</i>	TAATACGACTCACTATAGGTCGTTTTGCAGTAGGATC GGTTTTAGAGCTAGAAATAGC	no target
<i>the common reverse primer</i>	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTTG CTATTCTAGCTCTAAAC	

Table 6: Annealing PCR reaction contents.

Annealing PCR

<i>5X Phusion HF Buffer</i>	<i>10 µL</i>
<i>10 mM dNTPs</i>	<i>1 µL</i>
<i>Primer Mix</i>	<i>5 µL</i>
<i>Phusion DNA polymerase</i>	<i>0,5 µL</i>
<i>Nuclease-free Water</i>	<i>33,5 µL</i>

Table 7: SgRNA Synthesis reaction contents.

SgRNA Synthesis

<i>Annealed Template DNA</i>	<i>75 ng to 1000 ng</i>
<i>NTP Buffer Mix</i>	<i>10 µL</i>
<i>T7 RNA Polymerase</i>	<i>2 µL</i>
<i>Nuclease-free Water</i>	<i>Up to 30 µL</i>

2.8 Cas9/sgRNA complex preparation

Guide-it Recombinant Cas9 protein (Cat. No. 632641) was mixed 1:1 (1000ng/1000ng) with sgRNA solution to generate Cas9/sgRNA complexes. 0.05% phenol red dye was added for visualization of injections. When sgRNAs were mixed, from 1 to 4 guides were combined such that the total sgRNA concentration remained 1000 ng/ul, so the concentration of each guide RNA was 250 ng/ul. The Cas9 protein sgRNA mixture was incubated at room temperature for 5 minutes and then maintained

on ice during microinjection. Microinjection was performed by injecting 0.5-1 nL of the mixture into the cytoplasm of one-cell stage zebrafish embryos.

2.9 Genomic DNA Extraction

Embryos injected with Cas9 protein/sgRNA (F0) at 3dpf were anesthetized in tricaine methanesulfonate at a final concentration of 160 mg/L. After that, they were either pooled or contained individually in 1,5 ml tubes. 50 µL DNA extraction buffer (10 mM Tris HCl pH: 8.0, 50 mM KCl, 0,3 % Tween 20, 1mM EDTA) was added to each tube, and samples were incubated at 98 °C for 10 minutes to inactivate cytoplasmic nucleases. After that, 3 µL of Proteinase K solution (5mg/mL) was added to samples and incubated at 55 °C overnight. To inactivate Proteinase K, samples were incubated at 98 °C for 10 minutes. Following, samples were centrifuged at 13,000 rpm for 5 minutes at room temperature. Supernatants were transferred to a new tube containing 50 µL of distilled water, 10 µL of 3M sodium acetate, and 180 µL of isopropanol and vortexed to mix. Samples kept on ice for 10 minutes were centrifuged again at 13,000 rpm for 15 minutes at 4 °C. After that, supernatants were discarded, and the white pellet at the bottom of the tube was washed with cold 70% ethanol, followed by, tubes were left to air dry for removing ethanol completely. As final, white pellets containing genomic DNA were resuspended in 20 µL of distilled water.

2.10 High Resolution Melting Curve Analysis

HRM primers were designed to amplify genomic DNAs (90 bp to 150 bp) containing targeted sequences by gRNAs using NCBI Primer Blast software (Table 8). The PCR reactions were made with 10 µL of master mix for HRM analysis (Forget-Me-Not™ EvaGreen® qPCR Master Mix), 0.5 µL of each primer (10 µM), and 20 ng of genomic DNA and water up to 20 µL. The PCR was performed in a LightCycler 480 Instrument II (Roche) using white 96 well plates. Two-step Evagreen PCR reaction protocol was 95 °C for 2 minutes, then 45 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds, followed by 95 °C for 30 seconds, 6t3 °C for 60 seconds, the temperature was increased by 0.02 °C/s until 95 °C for 10 seconds, then cooling at 40 °C. Curves were analyzed using the Roche LightCycler 480 software version 1.5.1.62 (Samarut, Lissouba, and Drapeau 2016).

Table 8: Sequences of HRM primers.

<i>Primer Name</i>	<i>Sequence</i>
<i>Nradd Target 1 (exon 2) Forward HRM Primer</i>	CTTTGGCAAGACGTGCACC
<i>Nradd Target 1 (exon 2) Reverse HRM Primer</i>	GTCTTCCTGGCCACAGCT
<i>Nradd Target 2 (exon 3) Forward HRM Primer</i>	GCAGGAAGTGTGGGCCAG
<i>Nradd Target 2 (exon 3) Reverse HRM Primer</i>	AGGTCCCTGGTTTACATAGCTC
<i>Nradd Target 3 (exon 4) Forward HRM Primer</i>	TGATGGTCCTCGTGAAACTCC
<i>Nradd Target 3 (exon 4) Reverse HRM Primer</i>	GTTGTTGTTTCCACCTTGGTCC
<i>Nradd Target 4 (exon 5) Forward HRM Primer</i>	GCTGGAAATCATGTCAGCAGAAG
<i>Nradd Target 4 (exon 5) Reverse HRM Primer</i>	CTGTGCAGTTTCTCTCCTTCCC

2.11 Whole-mount Immunofluorescence Staining

Embryos were transferred to 1.5 ml tubes and fixed with 4% PFA at 4°C overnight. To remove PFA, they were washed 2 times with PBST (1x PBS, 0.1% Tween-20). After the fixation and wash step, embryos were dechorionated manually by using sharp forceps. Embryos were incubated in ice-cold methanol at -20 °C for 24 hours. Methanol was removed and embryos were washed twice with 1 mL PDT (1X PBST, 0.3% Triton-X, 1% DMSO) for 30 min by rocking gently at RT. Next, embryos were incubated with 500 uL blocking buffer (1X PBST, 10% heat-inactivated fetal bovine serum, 2% bovine serum albumin) at RT for 1 hour and incubated with rabbit anti-cleaved caspase 3 antibody (1:400; Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. The next day, embryos were washed twice with 1X PDT, incubated with rhodamine (TRITC)-AffiniPure donkey anti-rabbit IgG (1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 hours at RT, re-washed with 1X PDT, mounted in 50% glycerol in PBS, and imaged using a Zeiss LSM 880 confocal microscope.

2.12 Chemicals and Devices

Following table shows the chemicals used in this study.

Table 9: Chemicals used in experiments.

Chemicals	Catalog Number	Brand
NaCl	SOD001.1	Bioshop
Agar	VM623914347	Merck
Tris	0826_1KG	Amresco
Ultra-pure agarose	16500500	Thermo Fisher
EDTA	EDT001.500	Bioshop
Potassium chloride	K45117936405	Merck
Na ₂ HPO ₄	0404_1KG	Amresco
KH ₂ PO ₄	PPM666.500	Bioshop
Hydrogen chloride	295426	Sigma
Sodium hydroxide	BIO78998435	Merck
Citric acid	CIT001.500	Bioshop
Tween20	0777-1L	Amresco
Paraformaldehyde (PFA)	30525-89-4	Merck
Magnesium chloride	0288-500G	Amresco
BCIP	0885-500MG	Amresco
NBT	0329-1G	Amresco
Formamide	0606-100ML	Amresco
Heparin	24590	SERVA
tRNA	15401029	Thermo Fisher
Phenol red 0.5% solution	P0290-100ML	Sigma-Aldrich
Chloroform	1.02431.2500	Merck
2-propanol	K45187334402	Merck
Ethanol Absolute (2,5L)	1.00986.2500	Merck
Methanol	32213-2,5L	Sigma
Glycerol	0854-1L	Amresco

Following table shows devices used in this study.

Table 10: Devices used in experiments.

Devices	Brand
Basic power supply	Biorad
Universal Power Supply	Biorad
mySPIN™ 6 Mini Centrifuge	Thermo
Water bath and Lid	Nüve
pH Meter	Hanna
Microwave oven	Beko
Basic PCR	Life Sciences
Balance - basic	Sartorius
Vortex Mixer	Thermo
Stemi 305 Compact Stereo Microscope	Zeiss
SZX2 Series stereo microscope	Olympus
StabiliTherm™ Ovens and Incubators	Thermo
Dry Bath Incubator	Allsheng
SimpliAmp Thermal Cycler	Applied Biosystems
Borosilicate glass capillary	World Precision Instrument
Flaming/Brown Micropipette Puller	Sutter Instrument
Pasteur pipette glass 145 mm	Isolab
Filter tips (10 µl, 20 µl, 200 µl, 1000 µl)	Vertex
MicroCL 17 Microcentrifuge	Thermo
Mikro 200 Centrifuge	Hettich
Applied Biosystems 7500/7500 Fast	Applied Biosystems

RESULTS

3.1 *nradd* Expression is Regulated by Wnt/ β -Catenin Signaling at Three Developmental Stages: Gastrula, Somitogenesis and Organogenesis

Since many Wnt pathway regulators are transcriptionally regulated by the pathway itself, gene expression profiling of zebrafish embryos was performed after conditional activation and inhibition of Wnt/ β -catenin signaling in embryos at gastrula, somitogenesis, and organogenesis stages to identify novel regulators (MacDonald et al., 2009). Genes positively regulated upon activation and inhibition of the pathway during all three stages were screened and among such broadly regulated genes, neurotrophin receptor-associated death domain (*nradd*) whose function in Wnt/ β -catenin signaling and vertebrate embryonic development has not been described yet, identified in the master thesis of Betül Haykır previously (Haykır, 2017). At late gastrula stages, *nradd* was expressed at the marginal zone and later became concentrated at the posterior sites of the embryo (Figure 4). During organogenesis, its expression was downregulated in the posterior regions and observed at a weaker level throughout the body. To confirm the gene expression profiling results, we investigated *nradd* expression levels at gastrula, somitogenesis and organogenesis by in situ hybridization in heat shock-inducible transgenic zebrafish embryos Tg(hsp70l:Mmu.Axin1-YFP)w35 (hs:Axin1) and Tg(hsp70l:wnt8a-GFP)w34 (hs:Wnt8) by overexpressing pathway inhibitor Axin1 and pathway activator Wnt8, respectively. For all these developmental stages, results showed that inhibition of β -catenin signaling suppressed *nradd* expression while the activation upregulated *nradd* expression level (Figure 4). In situ hybridization results were supported by qPCR which also revealed that *nradd* expression increased with activated Wnt/ β -catenin signaling in hs:Wnt8 embryos, and decreased with inhibition of the signaling in hs:Axin1 embryos at all three stages as in the expression levels of *sp5l* which is known as a Wnt/ β -Catenin target gene (Figure 5A ,B, C). These results supported that *nradd* is a transcriptional target of Wnt/ β -catenin signaling and its expression is broadly regulated by the pathway during development.

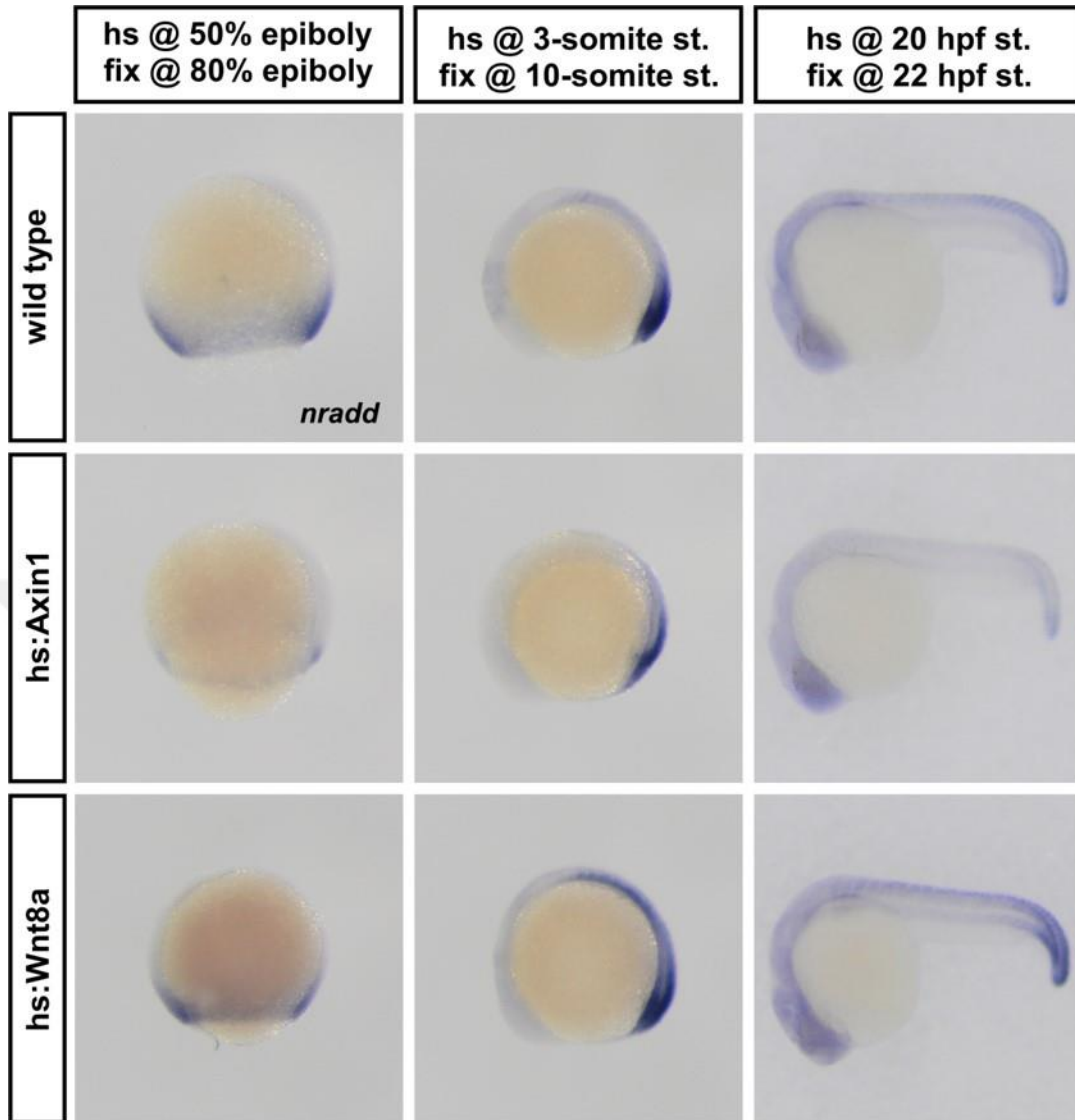


Figure 4: *nradd* is transcriptionally regulated by Wnt/ β -catenin signaling during development. Whole-mount in situ hybridization (WMISH) shows that *nradd* expression is downregulated in transgenic embryos expressing Axin1 (hs:Axin1) and upregulated in transgenic embryos expressing Wnt8a (hs:Wnt8a) at gastrula (80% epiboly), somitogenesis (10-somite) and organogenesis (22 hpf) stages. (n (80% epiboly): wild type 21/21 embryos, hs:Axin1 18/20 embryos, hs:Wnt8a 16/17; n (10-somite st.): wild type 24/24 embryos, hs:Axin1 24/24 embryos, hs:Wnt8a 28/30; n (22 hpf st.): wild type 19/19 embryos, hs:Axin1 23/25 embryos, hs:Wnt8a 19/20) hs: heat shock, fix: fixation, st: stage, hpf: hours post-fertilization. Three independent experiments were conducted.

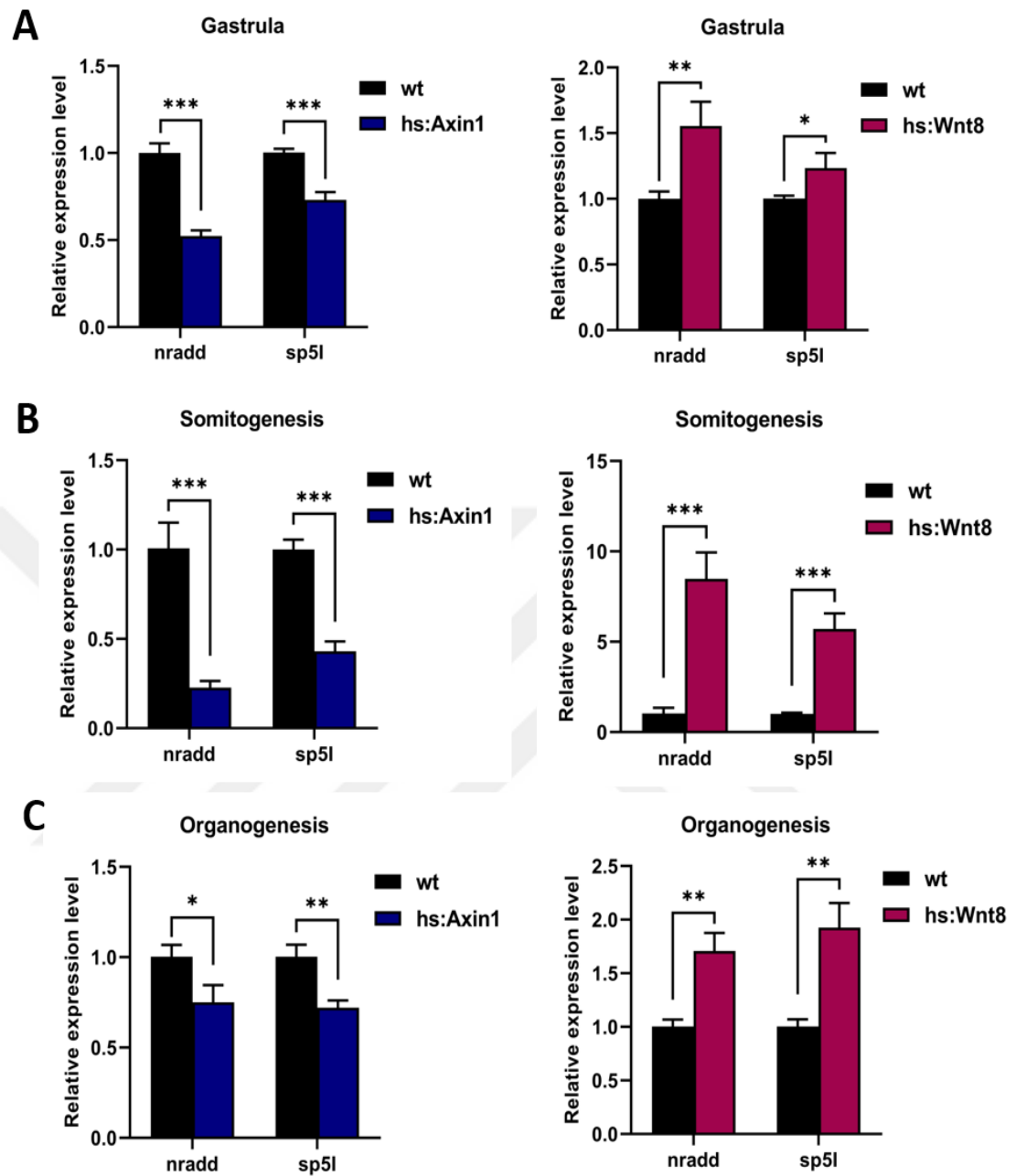


Figure 5: *nradd* is transcriptionally controlled by Wnt/ β -catenin signaling during development. (A–C) *nradd* and *sp5l* expression levels determined by qPCR in hs:Axin1 and hs:Wnt8a transgenic embryos are shown relative to those in wild-type embryos at (B) gastrula, (C) somitogenesis and (D) organogenesis stages. Statistical significance was evaluated using an unpaired t-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Error bars represent $\pm$ standard deviation (SD, $n = 3$). Three independent experiments were conducted.

3.2 Nradd Acts as an Inhibitor of Wnt / β -catenin Signaling Pathway.

Unusually high Wnt signaling activity via *wnt8* overexpression results in distinctive phenotypes ranging from mild posteriorization of the neuroectoderm to loss of forebrain and notochord in zebrafish embryos (classes 2 to 4) (Figure 6A). Therefore, we next asked whether *nradd* overexpression can rescue these phenotypes in 24 hours-post-fertilization (hpf) embryos. For this, we induced β -catenin signaling in hs:Wnt8 transgenic embryos by applying a single heat shock at early gastrula (shield stage), which consistently resulted in mostly class 3 and 4 phenotypes at 24 hpf (Figure 6A). *nradd* overexpression indeed significantly restored the phenotypes to class 2 and 3 (Figure 6B). In addition, we examined the expression of *axin2*, *cdx4*, and *sp5l* genes which are known as the direct Wnt/ β -catenin target genes in these embryos (Weidinger et al. 2005; Pilon et al. 2006). Results showed that *nradd* overexpression reduced the expression of these genes in both wild-type and Wnt8-induced embryos (Figure 7A, B, C). All these results suggested that *nradd* acts as an inhibitor of the Wnt/ β -catenin signaling pathway.

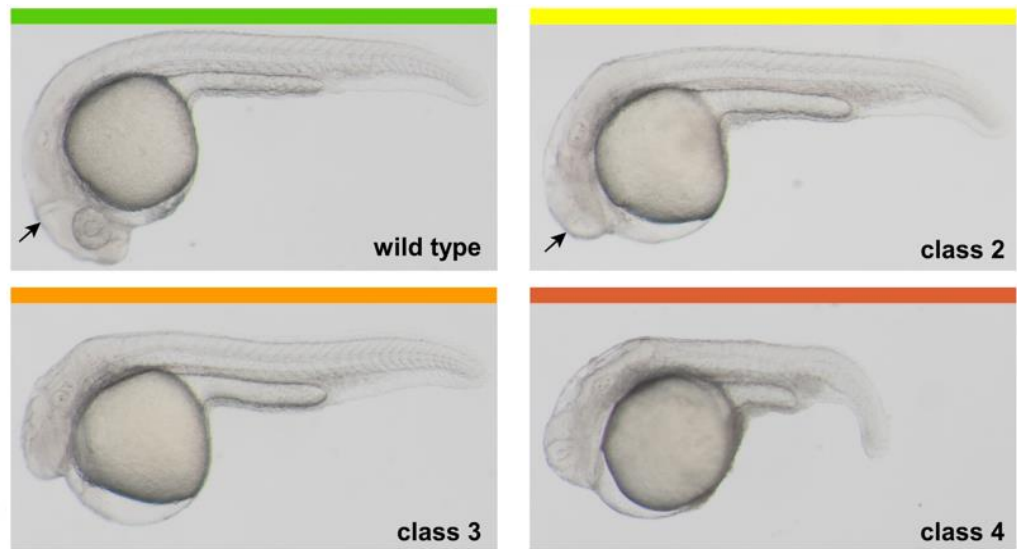
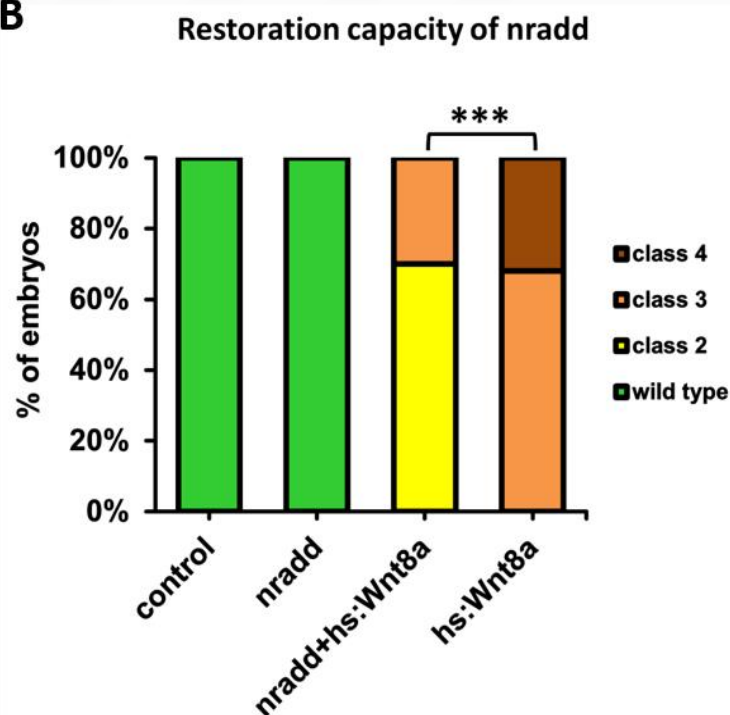
A**B**

Figure 6: *Nradd* acts as an inhibitor of Wnt/ β -catenin signaling. (A) Classification of phenotypes at 24 hpf caused by *wnt8* overexpression in *hs:Wnt8a* transgenic embryos after 1 hour of heat shock during gastrulation. Class 2: no eyes, reduced forebrain, normal midbrain–hindbrain boundary (mhb, arrow), class 3: no forebrain, severely reduced midbrain, no mhb, class 4: abnormal notochord development. (B) Phenotypes in wild-type (wt) or *hs:Wnt8a* transgenic embryos injected with *nradd* mRNA (150 pg) or equimolar amounts of control mRNA heat shocked at shield stage during gastrulation and scored at 24 hpf. *nradd* rescues Wnt8-induced phenotypes. (n (tailbud): control 53 embryos, *nradd* 46 embryos, *nradd*+*hs:Wnt8a* 56 embryos, *hs:Wnt8a* 45 embryos). Three independent experiments were conducted.

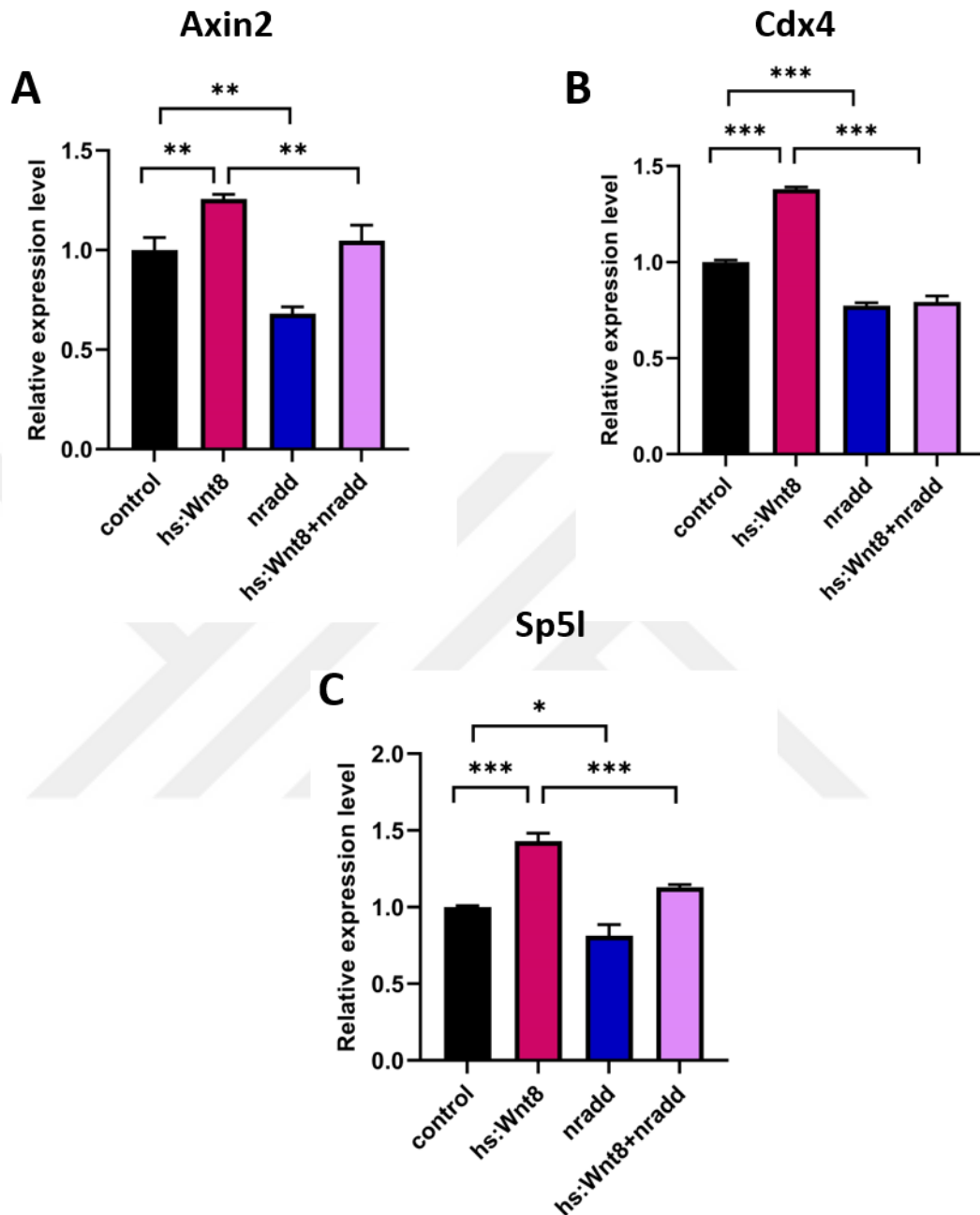


Figure 7: Nradd reduces the expression of Wnt/ β -catenin target genes. (A-C) Expression levels of direct Wnt-target genes axin2, cdx4 and sp5l determined by qPCR in *nradd*-overexpressing wt embryos (*nradd*) relative to those in wt embryos injected with GFP mRNA (control) and in *nradd*-overexpressing hs:Wnt8 embryos (hs:Wnt8+nradd) relative to those in hs:Wnt8 embryos injected with GFP mRNA (hs:Wnt8). All target genes are reduced by nradd in both wt and hs:Wnt8 embryos. Statistical significance was evaluated using an unpaired t-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Error bars represent $\pm$ standard deviation (SD, $n = 3$).

3.3 Nradd Suppresses Wnt8-Mediated Mesoderm and Neuroectoderm Patterning

Wnt8-mediated β -catenin signaling during gastrulation has essential roles in the specification of ventrolateral mesodermal fates and the restriction of the dorsal organizer (Lekven et al. 2001). To test whether *nradd* affects the mesodermal patterning of the embryo, we investigated how the organizer influenced by *nradd* overexpression during early development. *nradd* overexpression resulted in a significant expansion of dorsal mesodermal fates marked by goosecoid (*gsc*) expression, not at late blastula (30% epiboly), but at early gastrula (60% epiboly) stages, suggesting that *nradd* can relieve Wnt8-mediated dorsal organizer restriction (Figure 8A and B). To further analyze the role of *nradd* in neuroectodermal patterning, we examined whether it could rescue the posteriorization of the zebrafish embryo (loss of anterior neuroectodermal specifications) induced by *wnt8* overexpression. To monitor later neuroectodermal fates, we marked the forebrain by *foxg1a* expression, midbrain-hindbrain border by *her* expression, and hindbrain by *krox20* expression in embryos at 10-somite. *nradd* overexpression partially rescued the loss of forebrain observed in *wnt8* overexpressing embryos during mid-somitogenesis (10–13 somites) (Figure 9). Therefore, we conclude that *nradd* acts as a negative regulator of Wnt8-activated β -catenin signaling during mesodermal and neuroectodermal patterning.

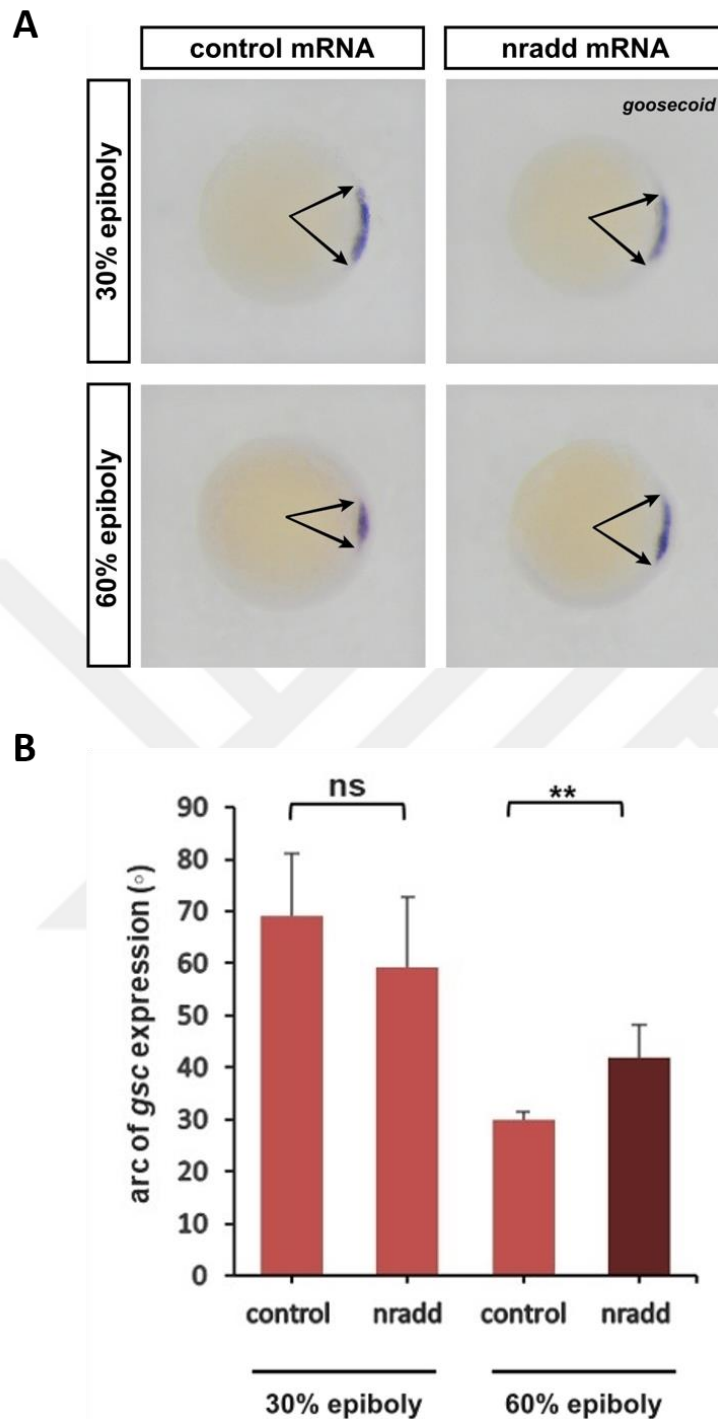


Figure 8: Nradd suppresses Wnt-mediated patterning of the mesoderm. (A) *nradd* mRNA (250 pg) causes expansion of the dorsal organizer domain (arrows) marked by *goosecoid* (*gsc*) WMISH, not at late blastula (30% epiboly, n: control 23/23 embryos, *nradd* 26/29 embryos), but at early gastrula (60% epiboly, n: control 27/27 embryos, *nradd* 32/33 embryos) stage zebrafish embryos. Three independent experiments were conducted. (B) Quantification of *gsc* expression shown in (A) by measurement of arc degree (29 embryos in control 30% epiboly, 33 embryos in *nradd* 30% epiboly, 35 embryos in control 60% epiboly, 37 embryos in *nradd* 60% epiboly). Error bars are SD. ** indicates $p < 0.01$ and ns is non-significant.

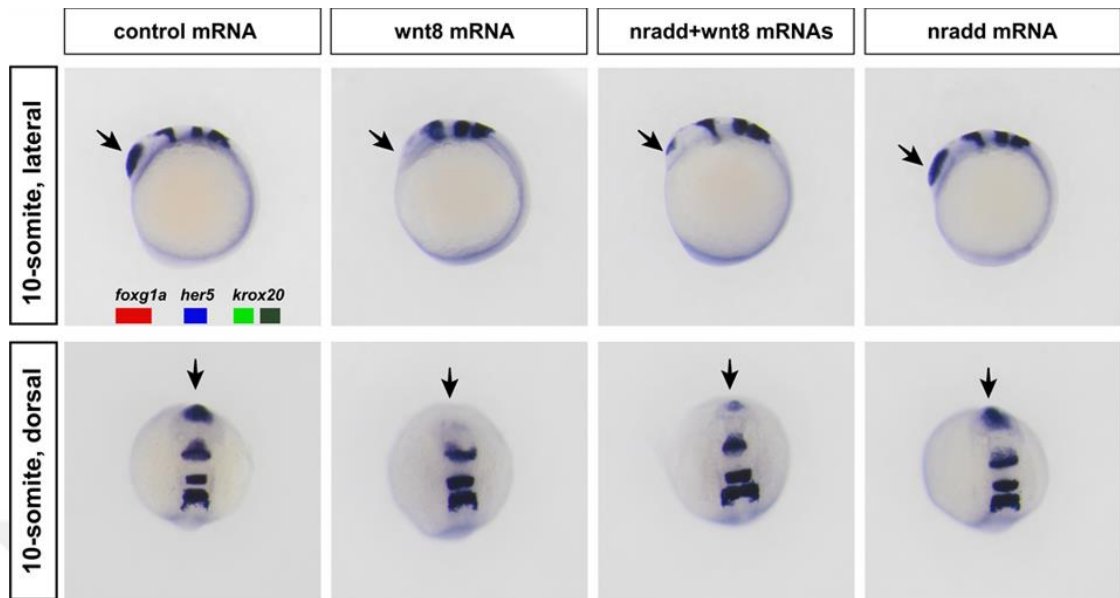


Figure 9: Nradd suppresses Wnt-mediated patterning of the neuroectoderm. *nradd* mRNA (250 pg) restores the telencephalon, marked by *fozg1a* (arrowhead), that is completely abolished by *wnt8* mRNA (20 pg). WMISH is performed to detect expression of three independent RNAs, the forebrain marker *fozg1a* (red bar), the midbrain–hindbrain boundary marker *her5* (blue bar) and the rhombomere 3/5 marker *krox20* (two green bars) in zebrafish embryos at the 10-somite stage (n: control 33/33 embryos, *wnt8* 35/36 embryos, *nradd*+*wnt8* 42/45 embryos). Three independent experiments were conducted.

3.4 Determining the Mutagenesis Efficiency of guide RNAs Designed Based on F0 Knockout Method

To understand better the role of *nradd* in the zebrafish early development, we wanted to investigate the effect of *nradd* loss of function in the *nradd* F0 Knockout model generated by CRISPR/Cas9 gene editing. For this, we followed the targeting approach described previously (Wu et al. 2018). In this approach, predicted coding sequences are divided into five equal fragments and guide RNAs are designed to target each of the first four sections (Figure 10A).

To analyze the mutagenesis efficiency of guide RNAs targeting *nradd* designed by following this strategy, we performed high resolution melting curve analysis (HRMA) of each targeted genomic site in each *nradd* crisprant injected with Cas9/gRNAs mix (F0) (Samarut, Lissouba, and Drapeau 2016). The analysis showed that the melting curves of F0 embryos (blue lines) were shifted or irregular compared to wild-type larvae (pink lines), which is explained by mosaic heteroduplex PCR fragments formed due to the random mutations induced by Cas9/gRNAs mix injection. When we compare the mutagenesis efficiency of each gRNA, gRNAs 4 and 3 have higher mutagenesis efficiency compared to gRNA 1 and gRNA 2 since they showed higher irregularity in melting curves of their targeted sites (Figure 10B).

To further test whether this targeting approach generates F0 embryos showing a phenotype close to germline-transmitted homozygous knockout phenotypes, we targeted *tyrosinase* (*tyr*), whose absence leads to a phenotype including observable pigmentation loss and impaired vision, by following the same strategy. Embryos injected with Cas9/gRNA mix targeting *tyr* showed a severe pigmentation loss in their body and eyes similarly to the homozygous *tyr* knockout phenotype. Hereby, we confirmed that F0 knockout modelling is a suitable approach to characterize a gene of interest in F0 embryos (Figure 10C).

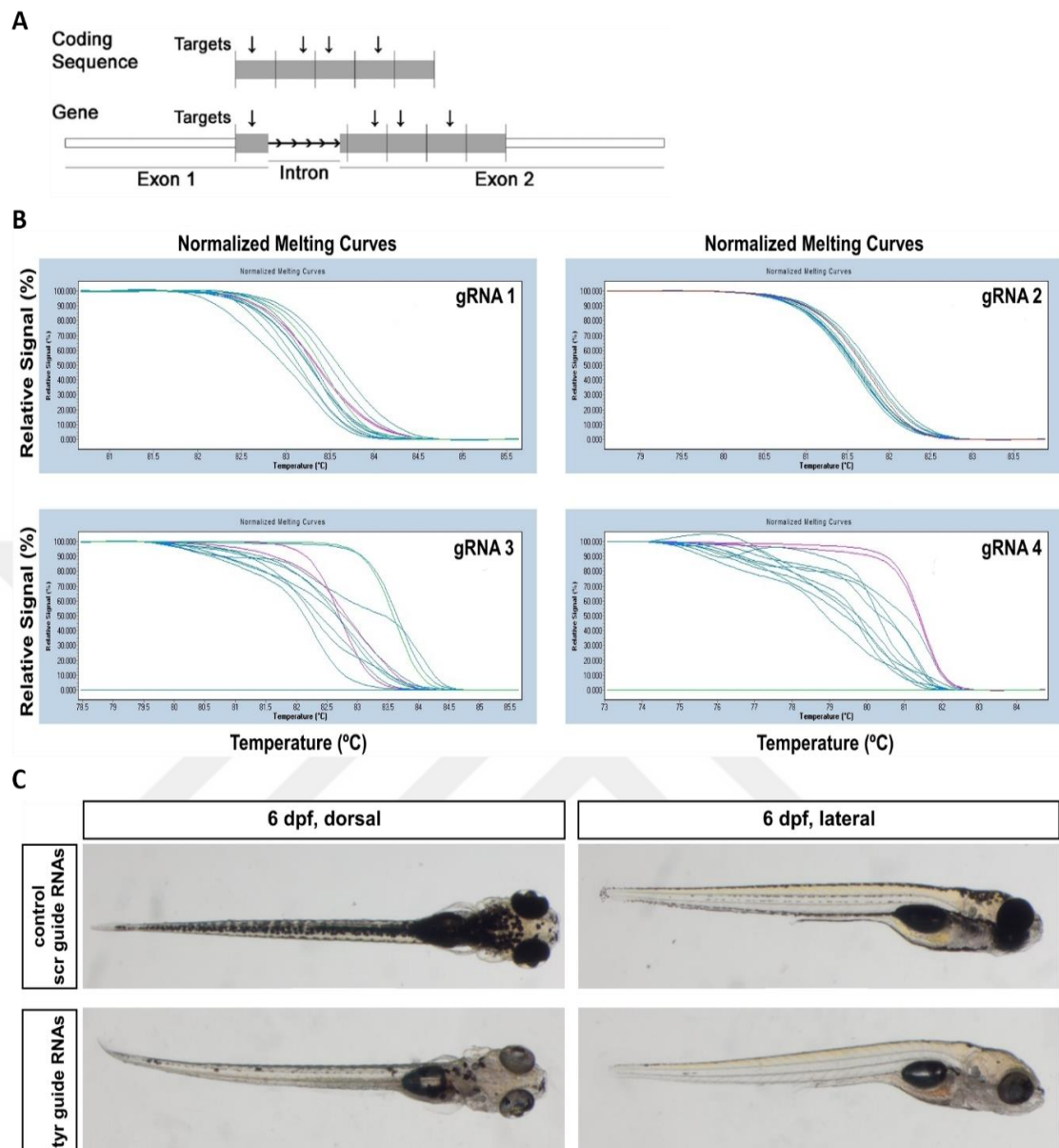


Figure 10: Targeting with 4-gRNA efficiently silenced genes in F0 embryos. (A) Coding sequences of genes are divided into five equal parts and gRNAs (arrows) are targeted to each of the first four parts. (B) HRM curve analysis of uninjected wild type (WT, pink curves) and Cas9/gRNA mix injected (blue curves) embryos at 3dpf. Cas9 protein along with 4-gRNA mix targeting different sections of *nradd* coding sequence are co-microinjected into one-cell stage embryos. The genomic DNA was extracted from each individual F0 embryo (injected with Cas9/4 gRNAs at one-cell stage) at 3 day-post-fertilization (dpf). Following, a 2-steps Evagreen PCR protocol was performed to amplify targeted genomic DNA parts and then HRM analyses were performed. The blue curves are shifted, and irregular compared to the pink WT curves, indicating the presence of indels. (C) Pigmentation phenotype of embryos injected Cas9/ 4-gRNA mix targeting different sections of *tyr* coding sequence compared to WT. F0 *tyr* knockout embryos showed reduced level of pigmentation.

3.5 Characterization of *nradd* crispants (F0 *nradd* Knockout model)

After the evaluation of mutagenesis efficiency for guide RNAs designed based on the F0 Knockout strategy, to test whether targeting *nradd* with this strategy affects the expression level of *nradd*, we investigated the mRNA expression level of *nradd* in F0 embryos at 24, 72, and 120 hpf. The injection of CRISPR components targeting *nradd* significantly reduced *nradd* mRNA expression level in all stages that we investigated (Figure 11A). However, F0 zebrafish with a decreased level of *nradd* expression did not show a visible phenotypic difference compared to wild-type embryos from embryonic development to adulthood. To investigate whether the decreased level of *nradd* expression affects Wnt/ β -catenin signaling activity, we examined the expression of *axin2*, *cdx4*, and *sp5l* as Wnt/ β -catenin target genes in F0 embryos at 24 hpf. The reduced expression level of *nradd* led to a significant increase in the expression of Wnt-target genes, pointing out again the negative regulatory role of *nradd* in the pathway (Figure 11B). Moreover, we also investigated the level of apoptosis in *nradd* crispants. To evaluate the defined apoptotic function of Nradd by several papers, previously, we examined the level of cleaved-caspase-3 immunoreactivity which is one of the most reliable markers of apoptotic cells and we observed a significant increase in apoptotic cells in the embryonic brain of *nradd* crispants at 24 hpf (Figure 12A). In addition, we revealed that decreased level of *nradd* expression causes a significant upregulation in the expression of pro-apoptotic marker genes, Baxa and Bcl2like1, in F0 embryos at 24 hpf (Figure 12B). Since our previous results have revealed an apoptosis-promoting role of Nradd in cooperation with Wnt/ β -catenin signaling, overexpression or misexpression of *nradd* likely disrupts the balance of apoptosis (Ozalp et al. 2021). However, further investigations are still needed especially in F2 homozygous *nradd* knockout zebrafish to eliminate possible complications of mosaicism in F0 *nradd* crispants. Hence, our results can form a whole picture with the outcomes of previous studies.

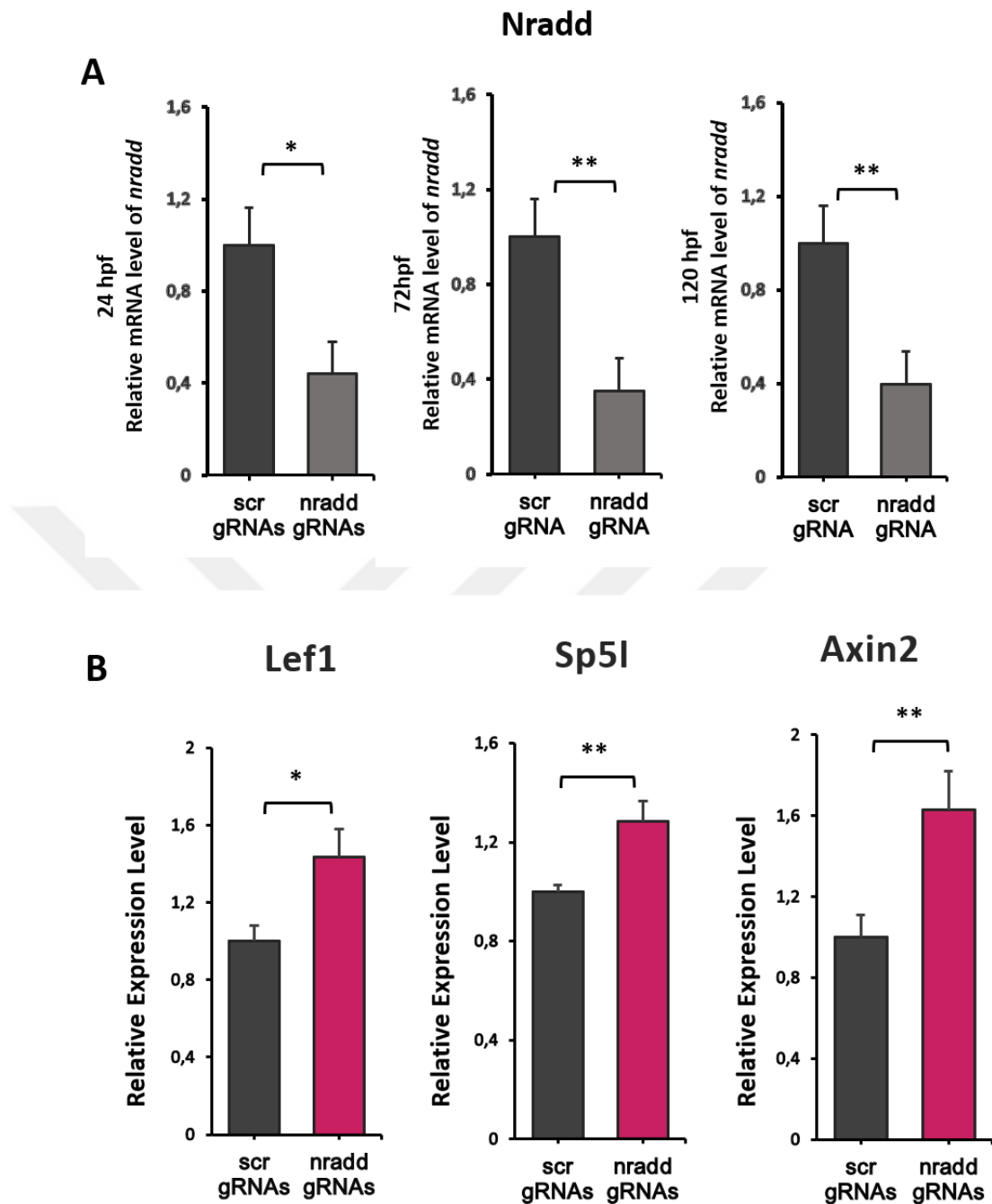
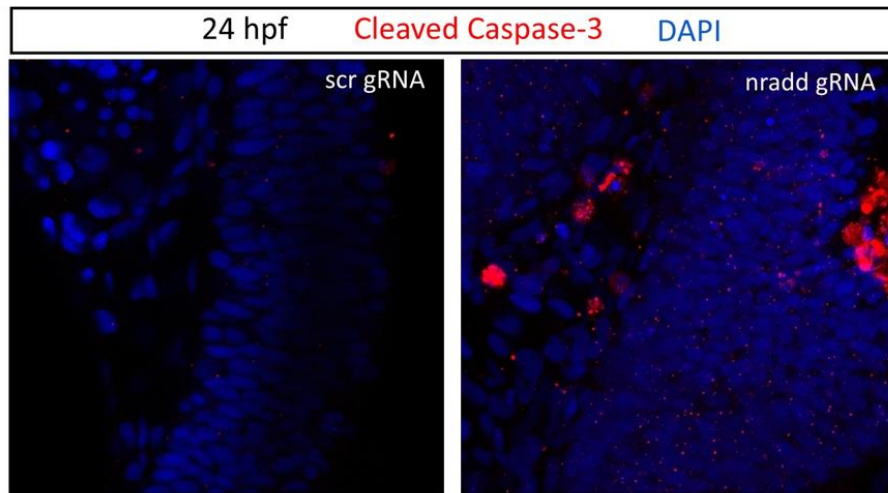


Figure 11: *nradd* crispants have an increased level of Wnt/ β -catenin signaling activity. (A) The expression level of *nradd* determined by qPCR in F0 *nradd* crispants (*nradd* gRNAs) relative to control embryos injected with guide RNAs targeting no site on the zebrafish genome (*scr* gRNA). (B) Expression levels of direct Wnt-target genes *axin2*, *cdx4* and *sp5l* determined by qPCR in F0 *nradd* crispants (*nradd* gRNAs) relative to control embryos injected with guide RNAs targeting no site on the zebrafish genome (*scr* gRNA). All target genes are increased in F0 *nradd* crispants

A



B

Baxa

Bcl2like11

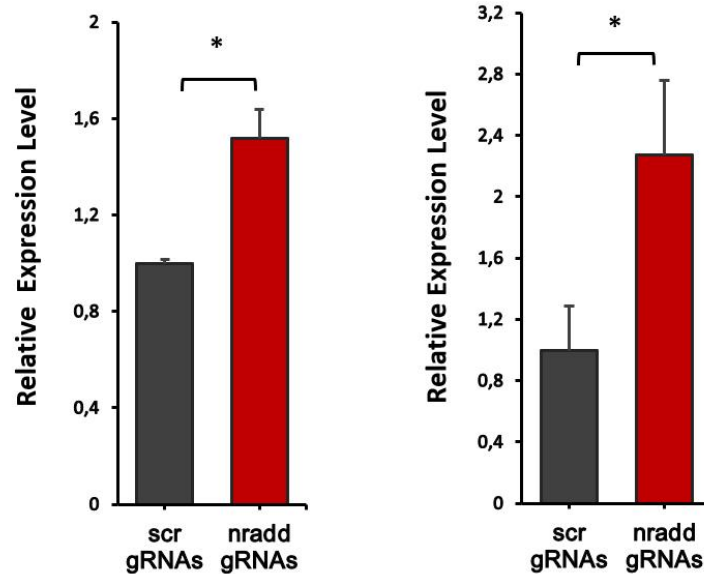


Figure 12: *nradd* crispants have an increased level of apoptosis. (A) Anti-cleaved caspase 3 stainings of F0 *nradd* crispants (nradd gRNAs) relative to control embryos injected with guide RNAs targeting no site on the zebrafish genome (scr gRNA). Sections are counterstained for DAPI. (B) Expression levels of pro-apoptotic marker genes, Baxa and Bcl2like11 determined by qPCR in F0 *nradd* crispants (nradd gRNAs) relative to control embryos injected with guide RNAs targeting no site on the zebrafish genome (scr gRNA). Three independent experiments were conducted. Statistical significance was evaluated using an unpaired t-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Error bars represent $\pm$ standard deviation (SD, $n = 3$).

DISCUSSION

Since Wnt/ β -catenin signaling plays essential roles in embryonic development and the maintenance of tissue homeostasis, the pathway is subjected to tight regulation by various modulators to keep the balance. In this study, we revealed the functional role of *Nradd*, a poorly characterized homolog of p75NTR, in the pathway regulation during embryonic development and apoptosis by examining the overexpression and F0 Knockout models of *nradd* in zebrafish. Our study suggests that *nradd* is a Wnt/ β -catenin target gene and acts as a suppressor in the pathway during Wnt-driven patterning of the mesoderm and the neuroectoderm. At the same time, our data showed that *nradd* overexpression can restore the loss of anterior brain parts induced by *wnt8* overexpression in transgenic zebrafish embryos. To further characterize the role of *nradd* in development, we generated *nradd* F0 knockout models named crispants. *nradd* crispants showed a significant increase in the expression levels of Wnt target genes, which is consistent with the outcome of overexpression experiments revealing the inhibitory role of Nradd in the pathway regulation. In addition, F0 embryos expressing a decreased level of *nradd* exhibited a significant rise in the number of apoptotic cells during the development of embryonic brain regions.

How does Nradd suppress Wnt/ β -catenin signaling? Nradd is known as a membrane-bound death receptor in TNFR superfamily. The family members possess a death domain that interacts with Fas-associated death domain protein (FADD) for the activation of initiator caspases and executioner caspases which induce apoptosis (Ichim, Tauszig-Delamasure, and Mehlen 2012; Haase et al. 2008; Jansen et al. 2007). However, these receptors do not only function in the initiation of programmed cell death but affect many biological processes including cell survival, differentiation, proliferation, and inflammation since interacting with wide variety of proteins included in many diverse signaling pathways. P75NTR which is the homolog of Nradd is an exceptional example in the TNFR family due to its differential roles in both apoptosis and cell survival. P75NTR support either the pro-survival actions of the Trk receptors or the initiation of apoptosis with sortilin and mediate crucial cell fate decisions especially in development of nervous system through several signaling pathways (Meeker and

Williams 2015). Our results reveal that Nradd, a p75NTR homolog, negatively regulates Wnt/ β -catenin signaling but the question of "through which mechanism" still needs further investigations. Our recent paper, which is partially included in the results of this thesis, showed that Nradd localized at the plasma membrane and physically interacted with both the FZD8a receptor and LRP6 co-receptor, the Wnt–receptor complex components (Ozalp et al. 2021). These results can be used as a starting point to investigate and explain the mechanism of pathway inhibition. Moreover, in a recent study, it has been shown that P75NTR overexpression upregulates the level of nuclear β -catenin in rat ectomesenchymal stem cells (EMSC) and enhances EMSC osteogenic differentiation. In the same study, they also demonstrated that silencing P75NTR causes a decreased level of nuclear β -catenin and weakens EMSC osteogenic differentiation, suggesting that the Wnt/ β -catenin pathway was affected by P75NTR functions (G. Li et al. 2017). In another study, it has been reported that Nradd forms a stable heterodimer with P75NTR through its transmembrane domain (TMD) and death domain (DD) within the intracellular domain (ICD) by breaking p75NTR dimers. p75NTR needs to dimerize through both its TMD and DD to recruit proteins that function in several pathways, so this heterodimer complex formation (Nradd-P75NTR) inhibits many downstream signaling pathways of P75NTR by blocking dimerization (Vilar et al. 2014). Consistent with both studies, we demonstrated the inhibitory role of Nradd in Wnt/ β -catenin signaling using both gain of function and loss-of function models. In addition, we reported that the death domain within the intracellular region of Nradd is necessary for the suppression of Wnt/ β -catenin signaling and the induction of apoptosis (Ozalp et al. 2021). When we evaluate these results together, it can be speculated that Nradd might suppress Wnt/ β -catenin signaling by blocking the function of P75NTR through its ICD. To test this possibility, the effect of Nradd on Wnt/ β -catenin signaling can be examined in the presence and absence of p75NTR in future investigations.

How does Nradd affect apoptosis? Mouse NRADD has been reported to stimulate cell death signaling pathways in primary neuronal cells and neuroblastoma cell lines through interaction with P75NTR and sortilin (Wang et al. 2003; Frankowski et al. 2002; Gowrishankar, Zeidler, and Vincenz 2004). Consistently with this, data shown in Ozalp *et al* demonstrated that the *nradd* overexpression induced apoptosis in early zebrafish

brain development, human embryonic and neuroblastoma cell lines. However, another study using transgenic mice overexpressing NRADD reported that NRADD decreased the apoptosis induced by FasL, by interacting with FADD and inhibiting the recruitment of pro-caspase-8 in contradistinction to studies suggesting NRADD as an apoptosis inducer. Interestingly, these mice showed a rapid recovery and rather reduced neuronal cell death after spinal cord injury (Sung et al. 2013). Regarding these studies, our overexpression data suggest a pro-apoptotic role for Nradd whereas F0 *nradd* knockout model data showed that reduced *nradd* expression induces apoptosis in crispants (Ozalp et al. 2021). However, these controversial outcomes might be explained by several hypotheses. One possibility is Nradd's inhibitory role in downstream signaling pathways of P75NTR. Since P75NTR has a dual role in both cell survival and apoptosis, and the emergence of these roles is regulated in a context-dependent manner in cells, the inhibition of P75NTR by Nradd can promote both cell survival and apoptosis depending on cellular conditions. Thus, further investigations are needed to reveal whether Nradd induce apoptosis or cell survival under which conditions according to this hypothesis.

In this study, to investigate *nradd* loss of function in zebrafish, we used a novel technique, F0-Knockout modeling (Wu et al. 2018). Although this method provides so many advantages with being less laborious, easy, and rapid, crispant embryos are highly mosaic. This mosaicism mostly does not cause a problem to obtain gene loss of function phenotype in zebrafish embryos. However, in some cases, it could affect some of the biological and developmental processes like apoptosis in the organism without only depending on the gene loss. Regarding this, proper zebrafish embryonic anterior-posterior (AP) patterning requires a gradual decrease in Wnt/ β -catenin activity from posterior (high Wnt activity) to anterior (low Wnt activity) sites. However, the appearance of cells with unfit Wnt/ β -catenin activity (high or low) produces noise in the Wnt/ β -catenin-gradient and affects membrane β -catenin and cadherin protein levels, disrupting the balance of cadherin levels between unfit and neighboring cells (Akieda et al. 2019). This induces TGF- β -Smad signaling and production of reactive oxygen species (ROS) and consequently ROS-mediated apoptosis in Wnt/ β -catenin-abnormal cells in the AP axis. Thus, unfit cells having excess low or high Wnt signaling activity among neighboring cells are eliminated by apoptosis (Akieda et al. 2019). Based on

this information, we speculated that the increased apoptosis level observed in the early brain of *nradd* crispants might be caused by the mosaic spread of *nradd* KO cells in the organism. This mosaicism might disrupt the Wnt activity gradient in anterior brain regions and might promote the elimination of *nradd* KO cells with high Wnt activity among normal anterior cells with low Wnt activity by ROS-mediated-apoptosis. To eliminate this possibility and understand the real role of Nradd in cell death, we need to further investigate apoptosis levels in homozygous *nradd* KO zebrafish embryos generated by CRISPR/Cas9 editing.

β -catenin signaling have important roles in neural development that starts with the early induction of the neural plate and continues throughout subsequent patterning of the neuroectoderm during central nervous system (CNS) development (Brafman and Willert 2017). Our data suggests Nradd as a new effective modifier in the canonical Wnt-mediated posteriorization of the neuroectoderm. Since the β -Catenin signaling dynamics regulate cell fate in differentiating neural stem cells in early development and adult brain, the misregulation of the pathway have been implicated in many neurodegenerative and neuro-oncological diseases, which is raising the importance of the specific pathway targeting for efficient treatment strategies (Rosenbloom et al. 2020). Thus, Nradd's modulatory roles in Wnt signaling along with cell death and survival may serve as a promising target for therapeutic interventions in CNS trauma and neurological disorders.

CONCLUSION & PERSPECTIVES

In this study, we revealed *nradd*, homolog of p75NTR, acts as Wnt target gene and a suppressor of the Wnt/ β -catenin signaling pathway in zebrafish embryos. Nradd can considerably suppress phenotypes mediated by Wnt signaling in mesodermal and neuroectodermal patterning during zebrafish gastrulation. Our F0 *nradd* loss of function model generated by CRISPR/Cas9 technique showed that the absence of *nradd* promote cell death in early brain during zebrafish development and leads to elevated Wnt signaling activity. With these features, Nradd can be considered as a promising target for the discovery of treatments against diseases where Wnt/ β -catenin signaling is affected. As an example:

Wnt/ β -catenin signaling is one of the major signaling cascades that drive proliferation and differentiation of oligodendrocytes during embryonic development. Several studies showed that the majority of oligodendrocyte precursor cells (OPCs) are affected by dysregulation of β -catenin signaling and hence cannot differentiate into myelin-producing mature oligodendrocytes. OPCs found in active plaques of multiple sclerosis (MS) patients likewise have unusually high Wnt/ β -catenin signaling activity, and do not differentiate into mature oligodendrocytes in response to injury, resulting in failure of re-myelination. Strikingly, rebalancing β -catenin signaling can induce differentiation of these OPCs into mature oligodendrocytes, resulting in successful remyelination. Our current study revealed that Nradd protein, a homolog of p75 neurotrophin receptor (p75NTR), acts as a negative feedback regulator of Wnt/ β -catenin signaling. Besides this novel role, several studies suggested that Nradd has a function in both neurotrophin-mediated neuronal survival and pro-neurotrophin-mediated cell death by physically interacting with p75NTR, TrkA, and Sortilin proteins. Thus, based on Nradd's negative regulatory role in Wnt signaling and its role in neurotrophin-mediated neuronal survival through TrkA signaling, we aim to test whether Nradd has the potential to induce OPC differentiation and myelination. This aim has been supported by FEBS as a summer fellowship project to be carried out in the Center for Clinical Brain Sciences at The University of Edinburgh in 2020. Unfortunately, it could not be realized due to the COVID19 restrictions.

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APPENDIX

Ethical Approval Form



**İZMİR BİYOTIP VE GENOM MERKEZİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
(İBG-HADYEK)
KARARI**

TOPLANTI TARİHİ	09/01/2019	TOPLANTI GÜNÜ	Çarşamba
TOPLANTI SAYISI	1	TOPLANTI SAATİ	15.30

Sayın Doç. Dr. Güneş ÖZHAN BAYKAN,

01/2019 Protokol No'lu; yürütücüsü olduğunuz “ Nörotrofin reseptörü ile ilişkili ölüm bölgesi proteininin Wnt/B-katenin sinyal iletiminin düzenlenmesindeki rolünün zebrafalığı embriyonik modeli ve CRISPR/Cas9 knockout modeli ile araştırılması ” başlıklı projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

(Proje Yürütücüsü) Doç. Dr. H. Güneş ÖZHAN BAYKAN Başkan	Vet. Hek. Kerem ESMEN Başkan Yardımcısı
Prof. Dr. H. Ayfer BAĞRIYANIK Üye	Dr. Gülçin ÇAKAN AKDOĞAN Üye
(Katılmamıştır) Dr. Öğr. Üyesi Ayşe Banu DEMİR Üye	Prof. Dr. Enişari GÜNELİ Üye
Doç. Dr. Kasım DİRİL Üye	Vet. Hek. Umur KELEŞ Üye
H. Muammer KARAGÖZ Üye	

EK 2

İBG-Enstitü TEZ DEĞERLENDİRME FORMU (Jüri Üyeleri İçin)

ÖĞRENCİ BİLGİLERİ

Adı: Özge

Anabilim/Bilim Dalı: Genom Bilimleri ve Moleküler Biyoteknoloji

Soyadı: Çark

Programı: Moleküler Biyoloji ve Genetik

Öğrenci No: 2017850048

Yüksek Lisans / Doktora

Tez Kodu: DEU.HSI.MSc/2017850048

Danışmanı: Doç. Dr. Güneş Özhan
İkinci Danışmanı (Varsa): -



TEZ BAŞLIĞI: Investigation of the role of Neurotrophin receptor associated death domain protein in regulation of Wnt/β-catenin signaling using the embryonic and CRISPR/Cas9 knockout models of zebrafish

SUNUM	Tez başlığı çalışma konusunu açık ve yeterli olarak tanımlamakta mıdır? ☒ Evet ☐ Düzeltmesi Gerekir
	Tez kolaylıkla okunup anlaşılıyor mu ? ☒ Evet ☐ Kısmen düzeltilmesi gerekir ☐ Yeniden yazılması gerekir
	Tablo, şekil ve grafikler tez yazım kurallarına uygun olarak hazırlanmış mı ? ☒ Evet ☐ Düzeltmesi gerekir
	Kaynaklar Dizini ☒ Doğru ☐ Hatalı Açıklayınız:
BÜTÜNLÜK	Tez bölümleri birbirlerine mantıksal ve analitik bir bütünlük ve akış içinde bağlanıyor mu? ☒ Evet ☐ Hayır Açıklayınız:
ÖZGÜNLÜK ve YARATICILIK	Aday, sizce bu çalışma sonunda bilimsel araştırma yapma, bilgiye erişme, değerlendirme ve yorumlama yeteneği kazanmış mıdır? ☒ Evet ☐ Hayır Doktora tezleri, ayrıca aşağıda belirtilen niteliklerden en az birini sağlamalıdır. Bu tez çalışması : ☒ Bilime yenilik getirmiştir ☐ Yeni bir bilimsel yöntem geliştirmiştir ☐ Bilinen bir yöntemi yeni bir alana uygulamıştır
GİRİŞ	Araştırmaya konu olan problem tanımlanmıştır: ☒ Evet ☐ Hayır Problemin çözümüne yönelik hipotez/hipotezler açık olarak belirtilmiş ya da araştırma soruları tanımlanmış mıdır? : Evet ☒ Hayır ☐

G.O.

GENEL BİLGİLER (Literatür Bilgisi)	Literatür bilgileri özümserenek derlenmiş midir? ☒ Evet ☐ Hayır Görüşlerinizi Tez Değerlendirme Kriterleri 'ne uygun olarak belirtiniz <i>Uygundur.</i>
GEREÇ ve YÖNTEM	Görüşlerinizi Tez Değerlendirme Kriterleri'ne ugun olarak belirtiniz <i>Uygundur.</i>
BULGULAR	Görüşlerinizi Tez Değerlendirme Kriterleri'ne ugun olarak belirtiniz <i>Uygundur.</i>
TARTIŞMA	Görüşlerinizi Tez Değerlendirme Kriterleri'ne ugun olarak belirtiniz <i>Uygundur.</i>
SONUÇ VE ÖNERİLER	Sonuç/sonuçlar net olarak belirtilip baştaki hipotez ve/veya araştırma soruları ile ilişkilendirilmiş midir? <i>İlişkilendirilmiştir.</i>
KAYNAKLAR	Kaynaklar uygun yazılmış mı? Yeterli mi? Güncel kaynak var mı? <i>Uygun yazılmıştır.</i>
EKLER	Eksik var mı? (Doktora tezleri için makale kabul yazısı ve makale metni), tüm tezler için etik kurul onayları, Arbis formatında özgeçmiş ve yayın listesi <i>Eksik bulunmamaktadır.</i>
DİĞER	Tez hakkında önemli gördüğünüz diğer hususları bu kısımda belirtebilirsiniz: <i>Literatüre yur. veriler ve mutant hatlar kazandırmıştır.</i>
JÜRİ DEĞERLENDİRME SONUCU	Tarafımdan incelenen bu tez, Dokuz Eylül Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliği uyarınca : ☒ Kabul edilebilir niteliktedir. ☐ Ek süre verilerek düzeltilmesi gerekir. ☐ Ret edilmesi gerekir.

G.Ö.

JÜRİ ÜYESİNİN

Adı Soyadı: Doç. Dr. H. Güneş ÖZHAN

Anabilim / Bilim Dalı: Biyotıp ve Sağlık Teknolojileri

Üniversitesi / Enstitüsü: Dokuz Eylül Üniversitesi

Tarih: 08.06.2021

İmza



EK 2

İBG-Enstitü TEZ DEĞERLENDİRME FORMU (Jüri Üyeleri İçin)

ÖĞRENCİ BİLGİLERİ

Adı: Özge

Anabilim/Bilim Dalı: Genom Bilimleri ve Moleküler Biyoteknoloji

Soyadı: Çark

Programı: Moleküler Biyoloji ve Genetik

Öğrenci No: 2017850048

Yüksek Lisans / Doktora

Tez Kodu: DEU.HSI.MSc/2017850048

☒ ☐

Danışmanı: Doç. Dr. Güneş Özhan

İkinci Danışmanı (Varsa): -

TEZ BAŞLIĞI: Investigation of the role of Neurotrophin receptor associated death domain protein in regulation of Wnt/ β -catenin signaling using the embryonic and CRISPR/Cas9 knockout models of zebrafish

SUNUM	Tez başlığı çalışma konusunu açık ve yeterli olarak tanımlamakta mıdır? ☒ Evet ☐ Düzeltilmesi Gerekir
	Tez kolaylıkla okunup anlaşılıyor mu ? ☒ Evet ☐ Kısmen düzeltilmesi gerekir ☐ Yeniden yazılması gerekir
	Tablo, şekil ve grafikler tez yazım kurallarına uygun olarak hazırlanmış mı ? ☒ Evet ☐ Düzeltilmesi gerekir
	Kaynaklar Dizini ☒ Doğru ☐ Hatalı Açıklayınız:
BÜTÜNLÜK	Tez bölümleri birbirlerine mantıksal ve analitik bir bütünlük ve akış içinde bağlanıyor mu? ☒ Evet ☐ Hayır Açıklayınız :
ÖZGÜNLÜK ve YARATICILIK	Aday, sizce bu çalışma sonunda bilimsel araştırma yapma, bilgiye erişme, değerlendirme ve yorumlama yeteneği kazanmış mıdır? ☒ Evet ☐ Hayır Doktora tezleri, ayrıca aşağıda belirtilen niteliklerden en az birini sağlamalıdır. Bu tez çalışması : ☒ Bilime yenilik getirmiştir ☒ Yeni bir bilimsel yöntem geliştirmiştir ☐ Bilinen bir yöntemi yeni bir alana uygulamıştır
GİRİŞ	Araştırmaya konu olan problem tanımlanmıştır: ☒ Evet ☐ Hayır Problemin çözümüne yönelik hipotez/hipotezler açık olarak belirtilmiş ya da araştırma soruları tanımlanmış mıdır? : Evet ☒ Hayır ☐
GENEL BİLGİLER (Literatür Bilgisi)	Literatür bilgileri özümseyerek derlenmiş midir? ☒ Evet ☐ Hayır Görüşlerinizi Tez Değerlendirme Kriterleri'ne uygun olarak belirtiniz

GEREÇ ve YÖNTEM	Görüşlerinizi Tez Değerlendirme Kriterleri'ne uygun olarak belirtiniz <i>Uygundur</i>
BULGULAR	Görüşlerinizi Tez Değerlendirme Kriterleri'ne uygun olarak belirtiniz <i>Uygundur</i>
TARTIŞMA	Görüşlerinizi Tez Değerlendirme Kriterleri'ne uygun olarak belirtiniz <i>Uygundur</i>
SONUÇ VE ÖNERİLER	Sonuç/sonuçlar net olarak belirtilip baştaki hipotez ve/veya araştırma soruları ile ilişkilendirilmiş midir? <i>ilişkilendirilmiştir</i>
KAYNAKLAR	Kaynaklar uygun yazılmış mı? Yeterli mi? Güncel kaynak var mı? <i>Uygundur</i>
EKLER	Eksik var mı? (Doktora tezleri için makale kabul yazısı ve makale metni), tüm tezler için etik kurul onayları, Arbis formatında özgeçmiş ve yayın listesi <i>Uygundur</i>
DİĞER	Tez hakkında önemli gördüğünüz diğer hususları bu kısımda belirtebilirsiniz: <i>Başarılı bir tezdır</i>
JÜRİ DEĞERLENDİRME SONUCU	Tarafımdan incelenen bu tez, Dokuz Eylül Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliği uyarınca : ☒ Kabul edilebilir niteliktedir. ☐ Ek süre verilerek düzeltilmesi gerekir. ☐ Ret edilmesi gerekir.
JÜRİ ÜYESİNİN Adı Soyadı: Dr. Yavuz Oktay Anabilim / Bilim Dalı: Tıbbi Biyoloji Üniversitesi / Enstitüsü: Dokuz Eylül Üniversitesi Tarih: 08.06.2021 İmza 	

EK 2**İBG-Enstitü TEZ DEĞERLENDİRME FORMU
(Jüri Üyeleri İçin)****ÖĞRENCİ BİLGİLERİ**

Adı: Özge

Anabilim/Bilim Dalı: Genom Bilimleri ve Moleküler Biyoteknoloji

Soyadı: Çark

Programı: Moleküler Biyoloji ve Genetik

Öğrenci No: 2017850048

Yüksek Lisans / Doktora

Tez Kodu: DEU.HSI.MSc/2017850048



Danışmanı: Doç. Dr. Güneş Özhan

İkinci Danışmanı (Varsa): -

TEZ BAŞLIĞI: Investigation of the role of Neurotrophin receptor associated death domain protein in regulation of Wnt/ β -catenin signaling using the embryonic and CRISPR/Cas9 knockout models of zebrafish

SUNUM	Tez başlığı çalışma konusunu açık ve yeterli olarak tanımlamakta mıdır? ☒ Evet ☐ Düzeltmesi Gerekir
	Tez kolaylıkla okunup anlaşılıyor mu ? ☒ Evet ☐ Kısmen düzeltilmesi gerekir ☐ Yeniden yazılması gerekir
	Tablo, şekil ve grafikler tez yazım kurallarına uygun olarak hazırlanmış mı ? ☒ Evet ☐ Düzeltmesi gerekir
	Kaynaklar Dizini ☒ Doğru ☐ Hatalı Açıklayınız:
BÜTÜNLÜK	Tez bölümleri birbirlerine mantıksal ve analitik bir bütünlük ve akış içinde bağlanıyor mu? ☒ Evet ☐ Hayır Açıklayınız:
ÖZGÜNLÜK ve YARATICILIK	Aday, sizce bu çalışma sonunda bilimsel araştırma yapma, bilgiye erişme, değerlendirme ve yorumlama yeteneği kazanmış mıdır? ☒ Evet ☐ Hayır
	Doktora tezleri, ayrıca aşağıda belirtilen niteliklerden en az birini sağlamalıdır. Bu tez çalışması : ☒ Bilime yenilik getirmiştir ☒ Yeni bir bilimsel yöntem geliştirmiştir ☐ Bilinen bir yöntemi yeni bir alana uygulamıştır
GİRİŞ	Araştırmaya konu olan problem tanımlanmıştır: ☒ Evet ☐ Hayır Problemin çözümüne yönelik hipotez/hipotezler açık olarak belirtilmiş ya da araştırma soruları tanımlanmış mıdır? : Evet ☒ Hayır ☐

GENEL BİLGİLER (Literatür Bilgisi)	Literatür bilgileri özümzenerek derlenmiş midir? ☒ Evet ☐ Hayır Görüşlerinizi Tez Değerlendirme Kriterleri'ne uygun olarak belirtiniz
GEREÇ ve YÖNTEM	Görüşlerinizi Tez Değerlendirme Kriterleri'ne uygun olarak belirtiniz <i>Uygundur.</i>
BULGULAR	Görüşlerinizi Tez Değerlendirme Kriterleri'ne uygun olarak belirtiniz <i>Uygundur.</i>
TARTIŞMA	Görüşlerinizi Tez Değerlendirme Kriterleri'ne uygun olarak belirtiniz <i>Uygundur.</i>
SONUÇ VE ÖNERİLER	Sonuç/sonuçlar net olarak belirtilip baştaki hipotez ve/veya araştırma soruları ile ilişkilendirilmiş midir? <i>İlişkilendirilmiştir.</i>
KAYNAKLAR	Kaynaklar uygun yazılmış mı? Yeterli mi? Güncel kaynak var mı? <i>Uygundur.</i>
EKLER	Eksik var mı? (Doktora tezleri için makale kabul yazısı ve makale metni), tüm tezler için etik kurul onayları, Arbis formatında özgeçmiş ve yayın listesi <i>Uygundur.</i>
DİĞER	Tez hakkında önemli gördüğünüz diğer hususları bu kısımda belirtebilirsiniz: <i>Başarılı bir tezdır.</i>
JÜRİ DEĞERLENDİRME SONUCU	Tarafımdan incelenen bu tez, Dokuz Eylül Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliği uyarınca : ☒ Kabul edilebilir niteliktedir. ☐ Ek süre verilerek düzeltilmesi gerekir. ☐ Ret edilmesi gerekir.

JÜRİ ÜYESİNİN

Adı Soyadı: Doç Dr. Özden Yalçın-Özuysal

Anabilim / Bilim Dalı: Moleküler Biyoloji ve Genetik

Üniversitesi / Enstitüsü: İzmir Yüksek Teknoloji Enstitüsü

Tarih: 08.06.2021

İmza

ÖZGEÇMİŞ

ADI SOYADI: Özge Çark

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Yazışma Adresi :	Balçova-İzmir
Telefon :	+90
Faks :	
e-posta :	@gmail.com

EĞİTİM BİLGİLERİ

Ülke	Üniversite	Fakülte/Enstitü	Öğrenim Alanı	Derece	Mezuniyet Yılı
Türkiye	Dokuz Eylül Üniversitesi	İzmir Uluslararası Biyotıp ve Genom Enstitüsü	Moleküler Biyoloji ve Genetik	Yüksek Lisans	2021
Türkiye	İYTE	Fen Fakültesi	Moleküler Biyoloji ve Genetik	Lisans	2017

AKADEMİK/MESLEKTE DENEYİM

Kurum/Kuruluş	Ülke	Şehir	Bölüm/Birim	Görev Türü	Görev Dönemi
Dokuz Eylül Üniversitesi	Türkiye	İzmir	İBG-Moleküler Biyoloji ve Genetik	Yüksek Lisans Öğrencisi	Şubat 2018- Haziran 2021
Dokuz Eylül Üniversitesi	Türkiye	İzmir	İBG-Moleküler Biyoloji ve Genetik	Stajer Öğrenci	Haziran 2016 - Ağustos 2016
				.	

UZMANLIK ALANLARI

Uzmanlık Alanları
Moleküler Biyoloji, Gelişim Biyolojisi ve Zebra balığı

DİĞER AKADEMİK FAALİYETLER

Son Bir Yılda Uluslararası İndekslere Kayıtlı Makale/Derleme İçin Yapılan Danışmanlık Sayısı			
Son Bir Yılda Projeler İçin Yapılan Danışmanlık Sayısı			
Yayınlara Alınan Toplam Atıf Sayısı			
Danışmanlık Yapılan Öğrenci Sayısı		Tamamlanan	Devam Eden
	Yüksek Lisans		
	Doktora		
	Uzmanlık		
Diğer Faaliyetler (Eser/görev/faaliyet/sorumluluk/olay/üyelik vb.)			

ÖDÜLLER

	Ödülün Adı	Alındığı Kuruluş	Yılı
☐	FEBSYaz Stajı Bursu	FEBS	2020

YAYINLARI**SCI, SSCI, AHCI indekslerine giren dergilerde yayınlanan makaleler**

Ozalp, O.; Cark, O.; Azbazdar, Y.; Haykir, B.; Cucun, G.; Kucukaylak, I.; Alkan-Yesilyurt, G.; Sezgin, E.; Ozhan, G. Nradd Acts as a Negative Feedback Regulator of Wnt/ β -Catenin Signaling and Promotes Apoptosis. Biomolecules 2021, 11, 100.
<https://doi.org/10.3390/biom11010100>

Diğer dergilerde yayınlanan makaleler

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Hakemli konferans/sempozyumların bildiri kitaplarında yer alan yayınlar

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