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# INVESTIGATION OF THE ROLE OF NEUROTROPHIN RECEPTOR ASSOCIATED DEATH DOMAIN PROTEIN IN REGULATION OF WNT/ß-CATENIN SIGNALING USING THE ZEBRAFISH MODEL

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MOLECULAR BIOLOGY AND GENETICS

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İZMİR ULUSLARARASI BİYOTIP VE
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Ph.D. THESIS

**Ozgun Ozalp**

Supervisor: Associate Professor Dr. Gunes OZHAN

(This research is supported by TUBITAK 1001 Project No: 217Z123)

**THESIS CODE: DEU.HSI. PhD/2017850006**

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# INVESTIGATION OF THE ROLE OF NEUROTROPHIN RECEPTOR ASSOCIATED DEATH DOMAIN PROTEIN IN REGULATION OF WNT/ß-CATENIN SIGNALING USING THE ZEBRAFISH MODEL

## ABSTRACT

Wnt/β-catenin signalling pathway is crucial for developmental processes and tissue homeostasis. Abnormal regulation of this signalling transduction might lead to several disorders. Hence, the understanding mechanisms underlying Wnt-related diseases might provide benefits for novel therapeutic approaches. For this purpose, elucidation of the roles of the modulators in this pathway has a significant importance for discovering novel drug targets.

In the context of this thesis, the role of the neurotrophin receptor associated death domain protein (Nradd), which is a homolog of p75 neurotrophin receptor (p75NTR), in regulation of Wnt/ß-catenin signaling was investigated using the zebrafish model. Since the Nradd is a transmembrane protein with an intracellular death domain, it is able to induce apoptosis in healthy and cancerous cells. Therefore, the relationship between regulatory role as part of the Wnt/ß-catenin signaling and induction of apoptosis in cell culture and zebrafish model has been aimed to be unravelled. As a result of this modulation mechanism, the interaction between Nradd and other Wnt components was determined as a indicator of the dual role of this novel death domain protein.

Based on our biochemical and molecular biological results, Nradd was identified as a novel feedback regulator of the Wnt/β-catenin signalling pathway. Consequently, we propose that targeting Wnt signalling via modulation of Nradd in novel treatment strategies could be beneficial for eradicating neurological disorders.

**Keywords:** Wnt/β-catenin signalling, neurotrophin receptor associated death domain (nradd) gene, apoptosis, zebrafish, regulator

# NÖROTROFİN RESEPTÖRÜ İLE İLİŞKİLİ ÖLÜM BÖLGESİ PROTEİNİNİN WNT/ß-KATENİN SİNYAL İLETİMİNİN DÜZENLENMESİNDEKİ ROLÜNÜN ZEBRA BALIĞI MODELİNDE ARAŞTIRILMASI

## ÖZET

Wnt/β-katenin sinyal yolağı, gelişimsel süreçler ve doku homeostazı için çok önemlidir. Bu sinyal iletiminin anormal düzenlenmesi çeşitli bozukluklara yol açabilir. Bu nedenle, Wnt ile ilişkili hastalıkların altında yatan mekanizmaların anlaşılması, yeni terapötik yaklaşımlar için fayda sağlayabilir. Bu amaçla, bu yolaktaki modülatörlerin rollerinin aydınlatılması, yeni ilaç hedeflerinin keşfedilmesi açısından büyük önem taşımaktadır.

Bu tez kapsamında, p75 nörotrofin reseptörünün ($p75^{NTR}$) bir homologu olan nörotrofin reseptörü ilişkili ölüm alanı proteininin (Nradd) Wnt/ß-katenin sinyalleşmesinin düzenlenmesindeki rolü zebra balığı modeli kullanılarak araştırılmıştır. Nradd, hücre içi ölüm alanına sahip bir transmembran proteini olduğundan, sağlıklı ve kanserli hücrelerde apoptozu indükleyebilir. Bu nedenle, Wnt/ß-katenin sinyal iletiminin bir bölümü olarak olarak düzenleyici rolü ve apoptoz indüksiyonu arasındaki ilişkinin hücre kültürü ve zebra balığı modelinde ortaya çıkarılması amaçlanmıştır. Bu modülasyon mekanizmasının bir sonucu olarak, Nradd ve diğer Wnt bileşenleri arasındaki etkileşim, bu yeni ölüm bölgesi proteininin ikili rolünün bir göstergesi olarak belirlenmiştir.

Biyokimyasal ve moleküler biyolojik sonuçlarımıza dayanarak, Nradd, Wnt/β-katenin sinyal yolunun yeni bir geri besleme düzenleyicisi olarak tanımlandı. Sonuç olarak, yeni tedavi stratejilerinde Nradd'ın modülasyonu yoluyla Wnt sinyalini hedeflemenin nörolojik bozuklukların ortadan kaldırılması için faydalı olabileceğini öneriyoruz.

**Anahtar sözcükler:** Wnt/β-katenin sinyal iletimi, nörotrofin reseptörü ile ilişkili ölüm bölgesi proteini, apoptoz, zebra balığı, regülatör

# 1. INTRODUCTION AND AIM

Wnt/β-catenin signal transduction presents critical functions during several stages of embryonic development and in maintenance of cellular homeostasis and regeneration of many adult tissues. (Logan and Nusse, 2004; Ozhan and Weidinger, 2014; Ozhan and Weidinger, 2015, Yu et al., 2021) Previous studies have shown that dysregulation of the Wnt/β-catenin signalling was involved in several diseases such as congenital malformations and oncological disorders. (Wang et al., 2012; Clevers and Nusse, 2012; Le et al., 2019) Despite the identification of significant components of this pathway, there are still many biological questions for understanding the modulation mechanisms of signal transduction. Thereby, a targeted treatment of Wnt pathways-related diseases has not been established yet (Yu et al., 2021; Taciak et al., 2018)

Since crucial components of signaling pathways possess various essential cellular functions, a significant approach for invention of novel therapeutics is to characterize regulatory components (modulators) that can modify the signaling transduction (Kahn, 2014). These modulators are favourably specialised for the signaling pathway and not necessary for the vital processes in the cell, thereby they tend to be very proper targets for therapeutic development (Jung and Park, 2020). Therefore, the molecular determination of new modulators has a great importance for developing new drugs. In this thesis, the novel role of a neurotrophin receptor associated death domain protein in the modulation of Wnt/β-catenin signalling pathway has been elucidated.

Neurotrophins are proteins which have fundamental roles in neuronal growth, survival, differentiation and function. (Ichim et al., 2012; Liu et al., 2012; Sahay et al., 2020). The members of neurotrophins are comprised of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (Bothwell, 2019). These growth factors act to elicit cellular reactions by interacting with the members of the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (TrkA, TrkB, and TrkC) to trigger cell survival and neurotrophin receptor p75 to induce cell death (Liu et al., 2012). Previous studies have implied that neutrotrophins may interact with Wnt signalling in different perspectives (Arevalo et al., 2005; Hiester et al., 2013; Yan et al., 2021; Zhou et al., 2020). However, there was no robust data to

explain the regulatory mechanism in respect of an interaction between a neurotrophin receptor and a Wnt signalling pathway component.

Based on our previous RNA-seq transcriptome profile analysis technique, *nradd* was selected as a Wnt/β-catenin signal transduction pathway target gene. In terms of understanding the interaction between Nradd and other Wnt signaling components, we focussed on the possibility a feedback mechanism in the membrane.

For this purpose, our first goal was to investigate the novel function of a neurotrophin receptor-associated death domain (*nradd*) gene as a feedback regulator of the Wnt/β-catenin signalling pathway by using several molecular methods including whole-mount in situ hybridization in zebrafish embryos, molecular cloning, in vitro capped sense RNA synthesis, microinjection into zebrafish embryos and fluorescent microscopy.

Secondly, the molecular characterization of the relationship between Nradd and the Wnt/β-catenin signal transduction pathway was analysed by cell culture techniques, confocal fluorescence microscopy, luciferase activity measurement, and western blotting. Following the Western blot for lysates, co-immunoprecipitation (Co-IP) method was carried out to determine the interaction between Nradd and other Wnt signaling components, which are Lrp and Fzd, at the cell membrane.

Next, since Nradd has an ability to induce apoptosis in cell culture, we investigated the possible relationship between apoptosis and Wnt/β-catenin signaling pathway upon Nradd overexpression. Hence, we carried out the apoptosis assay by flow cytometry to unravel the apoptotic ability of Nradd in various cell lines including neuroblastoma cell lines. Concordantly, we performed immunoflorescence staining to detect apoptotic cells in zebrafish embryos at 24 dpf (days post fertilization) after microinjection of *nradd* mRNA at one cell stage.

In conclusion, we aimed to unravel the modulatory role of Nradd in Wnt/β-catenin signalling pathway and the contributory act in apoptosis by using cell culture methods and zebrafish model organism. Based on our comprehensive data, we suggest that Nradd may be a promising therapeutic target in treatment of disorders caused by abnormal Wnt signaling.

# 2. LITERATURE

## 2.1. Wnt Signalling Pathways

The historical first steps for elucidation of Wnt signalling has been started through genetic studies on mouse and *Drosophila*. (Nusse and Varmus, 2012; Nusse and Varmus, 1982; Nüsslein-Volhard and Wieschaus, 1980). A variety of scientists have been made valuable efforts to sort out unknown parts of the Wnt signaling. Despite our principal knowledge about of this important pathway, understanding the molecular roles of novel modulators in signal transduction is still needed.

Wnt signaling pathway is an evolutionarily conserved, developmental pathway which regulates major biological processes such as cellular proliferation and differentiation, tissue regeneration and tumorigenesis (Acebron et al., 2014; Atlasi et al., 2013; Demirci and Cucun et al., 2020; Zhan et al., 2017).

Based on the dependence of β-catenin, Wnt signalling pathways are principially classified into two sub-groups as canonical Wnt signalling pathway (Wnt/β-catenin signalling pathway) and non-canonical Wnt signalling pathway (Katoh, 2017). While canonical Wnt signaling mostly modulates cell proliferation, non-canonical Wnt signaling manages cell polarization and cell migration (Rim et al., 2022; Franco et al., 2016)

### 2.1.1 Canonical Wnt signalling pathway

The Wnt signaling pathway is initiated by highly-conserved lipid-modified secreted proteins as an intercellular signaling cascade. Basically, this pathway includes a Wnt ligand from a secreting cell, its compatible receptors on the surface of a receiving cell, and signal transducers in the receiving cell. Following the recognition of the ligand and intracellular transfer of the signal, pathway activation causes different cellular responses such as cell proliferation or cell polarisation. These cellular responses are carried out within the scope of a molecular schedule driving crucial events in the development of organism (Rim et al., 2022; Mehta et al., 2021).

Since the canonical Wnt signaling pathway is a crucial regulator during development in all existing metazoans, it has a primary role in organization of the anterior-posterior axis (Ryan and Baxevanis, 2007; Schier and Talbot, 2005). Studies based on the mouse and zebrafish models show that loss of Wnt signaling leads to a severely truncated body, which can generate only the head and anterior part of the trunk (Martin and Kimelman, 2009). Canonical Wnt signalling pathway, which is also named as the Wnt/β-catenin signalling pathway, necessitates the existence of β-catenin as a major downstream effector (Wiese et al., 2018).

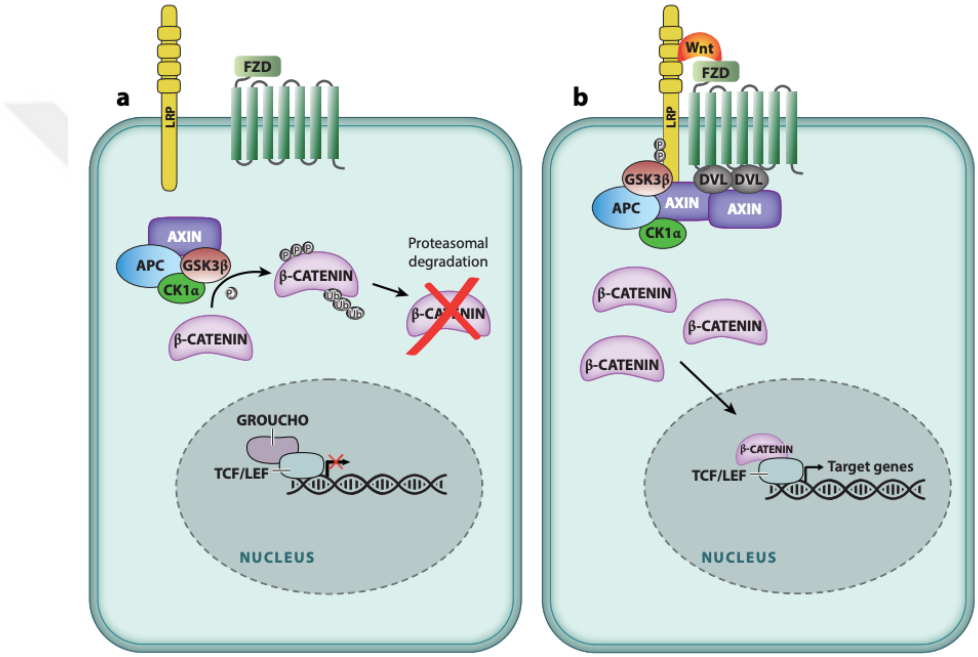



**Figure 1:** Canonical Wnt signalling pathway (Rim et al., 2022)

When there is no Wnt signal, Wnt signaling is not activated to prohibit abnormal cellular responses. In that case, this molecular status is executed via persistent phosphorylation and degradation of the major effector β-catenin (Liu et al., 2002; Stamos and Weis, 2013). Next, the destruction complex is formed upon the phosphorylation the β-catenin by serine/threonine kinase glycogen synthase kinase 3 beta (GSK3β) under favour of casein kinase 1 alpha (CK1α), axis inhibition protein (AXIN), and adenomatous polyposis coli (APC). β-catenin undergo ubiquitylation and then proteasomal degradation as a result of phosphorylation at N-terminal serine and threonine residues. (van Noort et al., 2002; Ha et al., 2004) (Figure 1a).

In contrast, when there is a Wnt signal, Wnt proteins generally exert for binding to Frizzled/Lrp receptor complexes in adjacent cells to evoke signal transduction (Bhanot et al., 1996; Wehrli et al., 2000). Next, Gsk3 and Ck1 phosphorylate Lrp6 leading to binding of Axin (Tamai et al., 2004; Zeng et al., 2005). In the context of this molecular interaction, Dishevelled (Dvl) connects the Frizzled. In this way, signalling starting from the membrane ends up to form signalosomes (Bilic et al., 2007; Gammons et al., 2016; Gerlach et al., 2018). These events lead to the stabilization and accumulation of β-catenin in the cell by translocation or disruption of the destruction complex. Following the translocation of β-catenin into the nucleus, transcriptional activation of target genes is drived by interaction of β-catenin with Tcf/Lef transcription factors (Behrens et al., 1996; Brunner et al., 1997; Molenaar et al., 1996) (Figure 1b).

### 2.1.2 Non-canonical Wnt signalling pathway

Wnt proteins are also able to activate signal pathway independently from β-catenin, thus they are categorized as non-canonical Wnt signaling pathways (Ackers and Malgor, 2018). One of the well defined type of non-canonical Wnt signaling is the planar cell polarity (PCP) pathway (Carvalho et al., 2019). Another significant one of non-canonical pathways is the Wnt/$Ca^{2+}$pathway (Sandsmark et al., 2017). Previous studies indicate that the non-canonical Wnt pathway can block the canonical Wnt signaling (Maye et al., 2004; Kremenevskaja et al., 2005).

While the PCP pathway is frequently triggered by Wnt5a and Wnt11 via FZD and DVL, Wnt/$Ca^{2+}$ pathway includes the activation of phospholipase C by Wnt5a/FZD2 via the heterotrimeric G proteins. PCP pathway modulates cell polarity in morphogenesis steps such as gastrulation and neural tube closure. On the other hand, Wnt/$Ca^{2+}$ signaling pathway supports ventral cell fate in embryogenesis (Axelrod, 2009; Gómez-Orte et al., 2013; Kühl et al., 2000).

### 2.1.3 Feedback regulation of Wnt signaling in development

The canonical Wnt pathway enables the modulation of signaling in diverse developmental aspects via highly immensely different feedback mechanisms. For instance, Axin2 protein has a inhibitory function to attenuate the excessive Wnt signaling in vertebrates (Jho et al., 2002; Lustig et al., 2002). Previous studies have revealed the molecular mechanism of novel proteins, which are able to regulate the Wnt/β-catenin signaling pathway. It was shown that *waif1a* gene coding the Wnt-activated inhibitory factor 1 transmembrane protein is a new negative regulator of this pathway. Besides its role in the canonical Wnt pathway, Waif1a protein also is involved in the cell polarity via non-canonical Wnt pathway as a positive regulator. (Kagermeier-Schenk et al., 2011).

Another study has unravelled that Lypd6 protein acts for the control of Lrp6 phosphorylation as a novel positive regulator of the Wnt/β-catenin signaling pathway (Ozhan et al., 2013). The fact that these genes encoding significant modulators function as transcriptional target genes of the Wnt/β-catenin signal transduction pathway implies the importance of the feedback mechanism in the regulation of the signaling pathway and their value in identifying new therapeutics.

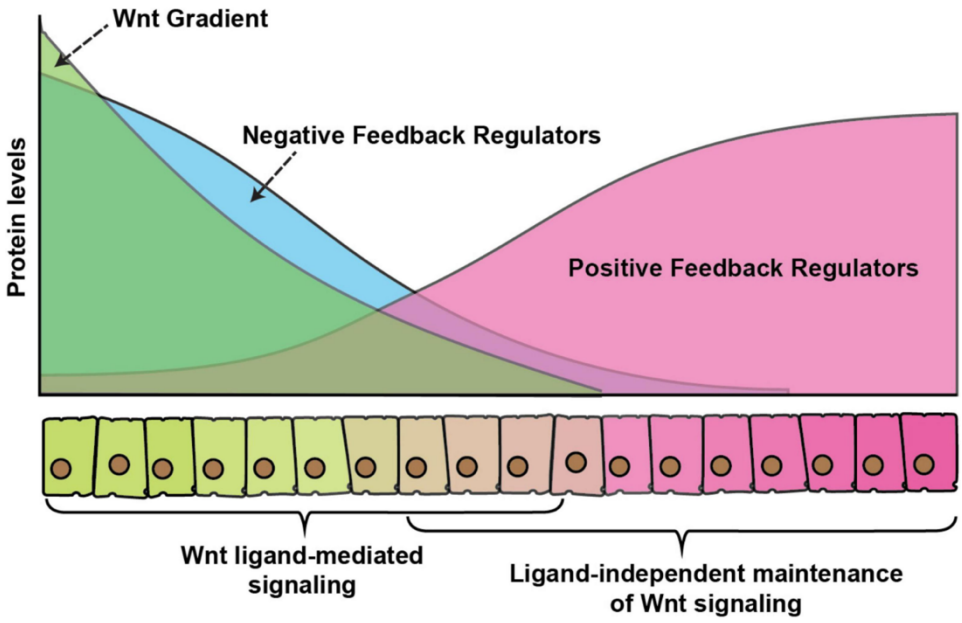



**Figure 2:** Feedback regulation of Wnt signaling (Mehta et al., 2021)

Since the Wnt/β-catenin signaling controls cell proliferation, cell fate determination, and cell differentiation during developmental stages, there are various Wnt target genes diverse in context dependent manner (Vlad et al., 2008; Logan and Nusse, 2004). The establishment of balanced signalling might be complicated in developing tissues due to a set of change in ligand level. It has been known that several signaling pathways benefit from positive and negative feedback regulators to sustain developmental processes. Thus, positive feedback is needed to amplify low signals, while negative feedback alleviate signaling. Furthermore, signaling pathways are responsible for regulating the expression of feedback modulators in the mechanism by supressing the positive modulators or activating negative modulators (Mehta et al., 2021) (Figure 2).

### 2.1.4 Targeting of Wnt/β-catenin signaling for cancer therapy

Wnt signaling is prompted normally as result of binding of WNT ligands to LrpP5/6 co-receptors and Fzd receptors (He et al., 2004). Under pathological conditions, Wnt signaling is dysregulated via mutations of its components resulting in diseases such as cancer (Braune et al., 2018; White et al., 2012; Sandberg et al., 2013). Mostly, upregulation of Wnt signaling is detected in most types of cancers (Clevers, 2006; Clevers and Nusse, 2012). Furthermore, acquired drug resistance might accompony with the Wnt signaling (Amawi et al., 2021). Therefore, current therapeutic strategies propose that the inhibition of Wnt signaling might bring together hopeful responses for cancer treatment (Jung and Park, 2020).

Defects in genetic and epigenetic regulation of Wnt/β-catenin signaling leads to cancer. Thus, discovery of novel drugs targeting this pathway has gained importance via inhibition of its components. Recently, to inhibit WNT ligands or receptors to pave the way for cancer treatment, WNT ligand inhibitors, PORCN (a membrane-bound o-acyltransferase family protein that regulates Wnt signaling via palmitoylation) inhibitors, and FZD antagonists have been investigated by clinical trials of several Wnt signaling-related human cancers (Jung and Park, 2020) (Figure 3).

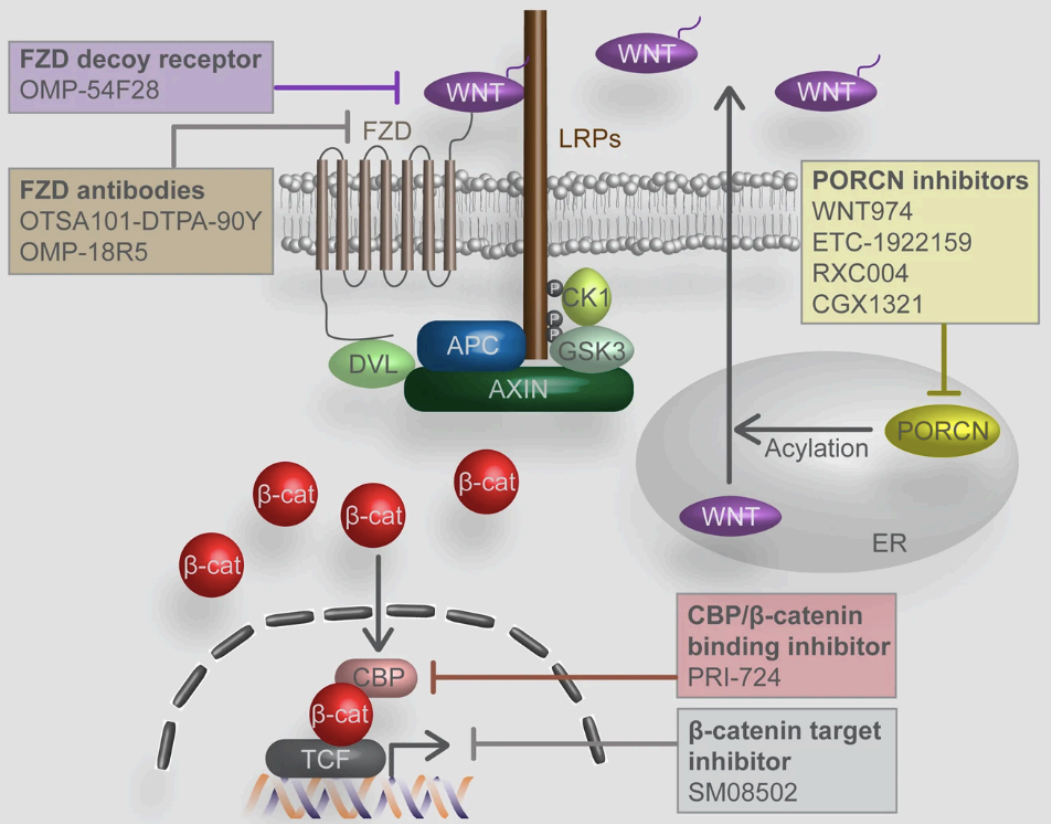



**Figure 3:** Targeting of Wnt/β-catenin signaling for cancer therapy (Jung and Park, 2020)

### 2.2 Neurotrophins

Neurotrophins constitute growth factors derived a group of dimeric secreted proteins, which are involved in regulation of pivotal biological hallmarks of vertebrate neurons, such as differentiation and survival characteristics. (Sherwood et al., 1997; Bibel and Barde, 2000; Huang and Reichardt, 2001).

Mammalian neurotrophins consist of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4). These proteins have many functional properties that classical neurotransmitters possess, such as their release at synapses and required in activity-dependent synaptic plasticity (Mitre et al., 2017; Poo, 2001; Todd et al., 2007). On the other hand, pro-neurotrophins are proteolytically degraded inside or outside the cell to remove the "Pro" region, which is the main site of interaction with the co-receptor Sortilin (Feng et al., 2010).

The wide range of biological activities controlled by neurotrophins is derived from the ability of these proteins to bind to receptors that are structurally different to each other. These secreted neurotrophins bind to two types of receptor, which are tropomyosin receptor kinase (Trk) family receptors of tyrosine kinases and p75 neurotrophin receptor ($p75^{NTR}$) (Barford et al., 2017; Dechant and Barde, 2002; Naito et al., 2017; Reichardt, 2006; Bothwell 2016). Neurotrophins promote neuronal survival by activating Trk receptors or cause cell death by activating $p75^{NTR}$ (Bothwell, 2019).

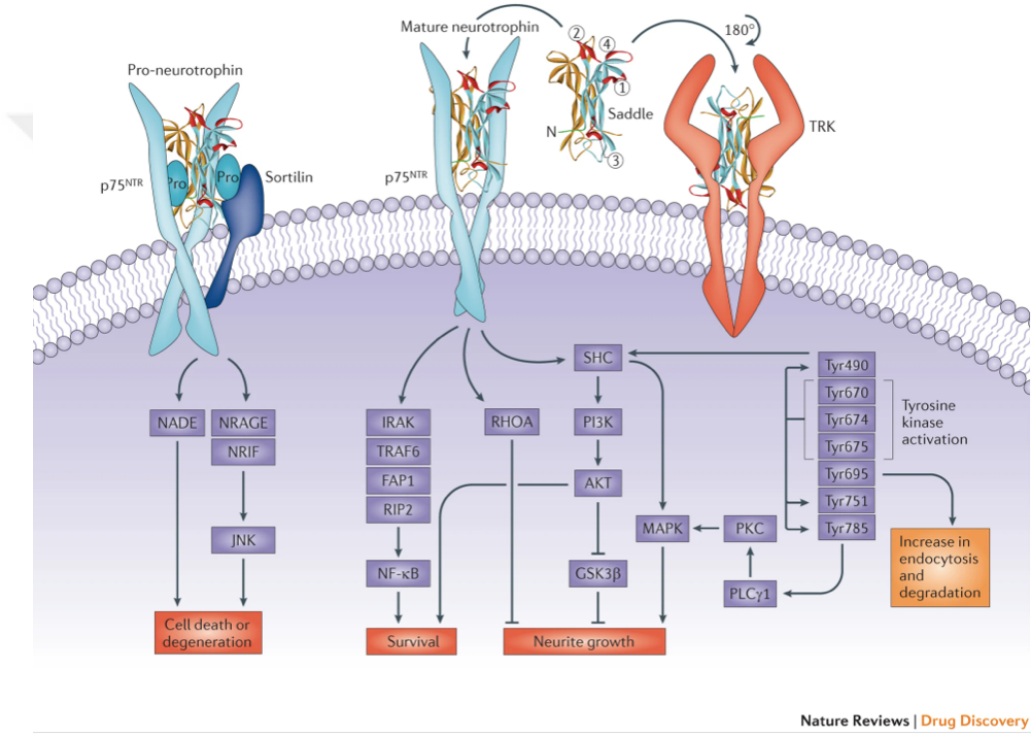



**Figure 4:** Neurotrophins, their receptors and signalling pathways (Longo and Massa, 2013).

Trk ligand binding by mature neurotrophins causes the phosphorylation of a series of tyrosine residues, which trigger kinase activity, resulting in further receptor autophosphorylation. In addition, phosphorylation at distinct residues generates adaptor binding sites which link the receptor to mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K) and phospholipase Cγ1 (PLCγ1) pathways. Then, these pathways may act through signalling endosomes that are

transported to the nucleus, to eventually enhance neurite outgrowth, differentiation and cell survival.

While mature neurotrophins binding to $p75^{NTR}$ may increase neurotrophin binding to Trk receptors, strengthen Trk signalling via AKT and MAPKs, and then supports survival through the nuclear factor-κB (NF-κB) pathway, or inhibits the actions of Trk through the activation of JUN N-terminal kinase (JNK) and RHOA pathway, pro-neurotrophin binding in complex with Sortilin specifically initiates cell-death-related pathways (Longo and Massa, 2013; Pedraza et al., 2005; Teng et al., 2010) (Figure 4).

### 2.2.1 Nradd

$p75^{NTR}$ is a member of the tumor necrosis factor receptor (TNFR) superfamily (TNFRSF) (Locksley et al., 2001; Wiens and Glenney 2011) and binds to every type of neurotrophins with similar nanomolar affinities (Bothwell et al., 1995, 2016, Roux and Barker, 2002). Besides that p75NTR is a transmembrane protein which includes a cytoplasmic death domain (DD) near the carboxy terminus, it acts as one of the pivotal components in apoptotic signaling (Locksley et al., 2001). Neurotrophin receptor associated death domain (*nradd*), which is a mammalian homologue of $p75^{NTR}$, has been also known as neurotrophin receptor homolog 2 (*nhr2*) or p75-like apoptosis inducing death domain (*plaidd*). Interestingly, Nradd has a distinctively a short amino end (Frankowski et al., 2002, Wang et al., 2003). Furthermore, the transmembrane portion and cytoplasmic region of the protein reflects a significant similarity to these regions in $p75^{NTR}$ (Hashimoto et al., 2004; Murray et al., 2004).

The *nradd* gene is generally highly expressed during the embryonic period, while its expression decreases in adulthood (Frankowski et al., 2002; Wang et al., 2003). Previous studies have shown that *nradd* is co-expressed with $p75^{NTR}$ in distinct cell populations such as dorsal root ganglion or cultured sympathetic neurons (Kanning et al., 2003; Murray et al., 2004). The high expression of *sortilin* in these regions and the detection of cell death induced by pro-neurotrophins suggest that *nradd* may play a role in pro-neurotrophin-mediated cell death (Nakamura et al., 2007). Interestingly, the

*nradd/nrh2* gene constitutes a pseudogene in human (https://www.ncbi.nlm.nih.gov/gene/100129354).

Although the Nradd protein is defined as a receptor, there might be no direct interaction with secreted ligands due to its unique extracellular domain (Frankowski et al., 2002; Kanning et al., 2003; Murray et al., 2004; Wang et al., 2003). However, previous studies indicate that Nradd protein plays a role in neurotrophin signaling by interacting physically with $p75^{NTR}$, Sortilin and TrkA proteins (Hashimoto et al., 2004; Kim ve Hempstead, 2009; Murray et al., 2004). Moreover, *nradd* overexpression supports that this protein induces apoptosis through a caspase-8 by the extrinsic apoptotic pathway in primary neuron cells (Kim ve Hempstead, 2009; Murray et al., 2004).

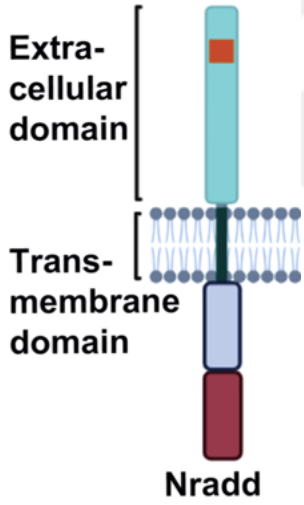



**Figure 5:** The structure of Nradd protein (Created with BioRender.com)

### 2.3 Apoptosis

Previously, cell death mechanisms were basically classified into two different processes based on the biological outcomes, which are apoptosis (programmed cell death) or necrosis (uncontrolled cell death). In contrast, recent articles suggest that many other cell death types such as oncosis, pyroptosis and autophagy might have important roles in different contexts. Apoptosis includes a variety of unique changes in the cellular morphology, as well as a range of enzyme-dependent biochemical

processes (D'Arcy, 2019; Bottone et al., 2013). The apoptotic program, which is functionally conserved in eukaryotes, constitute a crucial role for the proper survival of an organism (Aravind et al., 1999; Portt et al., 2011). Particularly, apoptosis is required for the removal many structures and adaptation for distinct functions during the development (Meier et al., 2000; Doseff, 2004). Hence, the failure on the regulation of apoptosis has been involved in different diseases including cancer. However, elevated apoptosis may cause neurodegenerative and neuromuscular diseases derived from deprivation of neurons.

The initiation of apoptosis relies on the activation of an array of cysteine-aspartic proteases known as caspases. There are two groups of caspases, which are the initiator caspases and the executioner caspases (Elmore, 2007). After the cell detects damage, the initiator caspases (caspases 8 and 9) are activated from inactive procaspases type and continue to activate the executioner caspases (caspases 3, 6 and 7). As a result of the activation of the executioner caspases, a cascade of events initiates several morphological and biochemical apoptotic hallmarks, including DNA fragmentation, phosphatidylserine externalization and generation of apoptotic bodies (Nagata, 2005; Coleman et al., 2001).

Extrinsic pathway of apoptosis, which is also named as the death receptor (DR) pathway, is activated by production of death ligands derived from natural killer cells or macrophages. Next, these ligands bind to DRs in the membrane of target cell to trigger apoptotic pathway through the activation of pro-caspase to caspase 8. (Igney and Krammer, 2002; Kim et al., 2004).

DRs are comprised of members of the tumour necrosis factor (TNF) superfamily, as well as Fas cell surface death receptor (FAS) (Bossen et al., 2006; Wajant, 2002). Activation of a caspase 8 requires the binding of a death ligand to a DR. Next, procaspase 8 is recruited to a death-inducing signal complex (DISC), which is dynamic complex protein at the intracellular tail of the DR (Muzio et al., 1996; Dickens et al., 2012). This complex also may contain either an adaptor protein called as FAS-associated death domain (FADD) or TNF receptor (TNFR)-associated death domain (TRADD), which manages the assembly of procaspase 8 to the DISC (Kim et al.,

2004). Finally, the dimerisation of many procaspase 8 monomers in the DISC entails the induction of apoptosis by caspase 8 (D'Arcy, 2019).

### 2.3.1 The relationship between apoptosis and Wnt signaling pathway

Wnt pathway have importance during normal development of the neural tissues and organs (Hari et al., 2002; Abu-Khalil et al., 2004; Bastidas et al., 2004). Wnt signaling is also responsible for regulation of the embryonic cell patterning, cellular proliferation and differentiation, survival and apoptosis (Masckauchán et al., 2005; Pećina-Šlaus et al., 2010). The role of Wnt signaling in apoptosis depends on the cell fate by resulting either induce or supress the processes of apoptosis (Trejo-Solis et al., 2021).

Prior studies have displayed that activation and inhibition of the Wnt/β-catenin pathway induce antitumoural effects in the similar cancer type. Thereby, misbalance in the fine adjustment of this pathway may present novel therapeutic modalities (Anastas and Moon, 2013; Duffy et al., 2016). It has been shown that activation or inhibition of the Wnt/β-catenin signaling might lessen the survival through the elevation of apoptosis after the signaling is triggered. In addition, induction of the signalling pathway has been contributed to cell cycle arrest in melanoma and neuroblastoma (Duffy et al., 2016). Besides, the Wnt/β-catenin signaling has been involved in expansion or reduction of tumours via metastasis or drug resistance mechanisms depending on the tumour type (Anastas and Moon, 2013). Previously, a synergistic correlation between the activation of the Wnt/β-catenin pathway and the induction of apoptosis has been indicated (Zimmerman et al., 2013). Furthermore, the connection between the Wnt/β-catenin pathway and apoptotic pathways has been reported via the activation of caspase caspase-3, -8 and -9 (Rubio et al., 2017). Since the functional outcome of Wnt/β-catenin signaling pathway is multifaceted, it is able to support or block carcinogenesis in different biological themes (Trejo-Solis et al., 2021).

## 2.4 Zebrafish as a model organism in developmental biology

The zebrafish (*Danio rerio*) has been used as a vertebrate model organism to study developmental biology and genetics (Mayden et al., 2007; Meyers, 2018). Because,

the zebrafish genome has been entirely sequenced, and it has developmental behaviors that can be easily observed and tested (Howe et al., 2013; Norton and Bally-Cuif, 2010).

The advantages of zebrafish model also includes rapid and ex-utero embryonic development, robust and transparent embryo structure (Marshall and Osborn, 2016; Dahm and Geisler, 2006). Since the zebrafish has a short generation time, it is able to reach the maturity that can reproduce rapidly after 2-4 months (Lawrence et al., 2012).

Moreover, there are many well characterized transgenic zebrafish lines to study specific biological topics (Stuart et al., 1990). Also, the size of the organism remains stable during during the early development and thereby enables to track cells via basic staining protocols. Due to its similar circadian rhythm, zebrafish model holds similarity in terms of toxicity assays and daily sleep cycle to the human models (Jones, 2007; Vatine et al., 2011). To collect developmental and genetic information based on several scientific studies, there has been a specialised database called as The Zebrafish Information Network (ZFIN) (http://zfin.org/).

Studies by using zebrafish model on developmental biology, genetics, oncology and regeneration have provided significant contributions to find out unknown biological mechanisms (Özhan and Weidinger, 2014; Demirci and Cucun et al., 2017). In addition, comparative genomic analyses uncovered that nearly 70% of human genes have at least one zebrafish ortholog. (Howe et al., 2013). This genetic similarity of embryonic development processes during the evolution supports the robustness of the zebrafish as a convenient model organism to enlighten the regulatory machinery in the signalling pathways. Therefore, we benefitted from this useful model to conduct our experiments and support our hypothesis.




**Figure 6:** Developmental stages in zebrafish (biorender.com)

# 3. MATERIALS AND METHODS

## 3.1 Type of the research

The research type in the thesis was designed and performed as an experimental study.

## 3.2 Location and the time frame of the research

The research was performed between 1 May 2019 and 1 May 2022 at İzmir Biomedicine and Genome Center.

## 3.3 Research population, sampling, and experimental groups

This research did not cover human studies.

## 3.4 Research materials

The HEK293T, SH-SY5Y cells were cultured in cell culture and obtained from Ozhan lab's stocks. Wnt3a CM (Wnt3a-enriched condition media) was obtained from Ozhan lab's stocks. Zebrafish model organism was used for *in vivo* assays and obtained from the zebrafish core facility (WT, Tg(7xTcf- Xla.Siam:nlsm-Cherryia) and Tg(hsp70l:wnt8a-GFP)$^{w34}$ were obtained from Ozhan lab's stocks.

## 3.5 Research variables

The variables of this research include WT Nradd and mutant Nradd constructs which are Nradd DDD and Nradd GSD. Also, different Wnt/β-catenin signaling components such as Lrp and Fzd are involved in the research variables.

## 3.6 Materials and methods

### 3.6.1 Reagents, devices, tools

Desktop centrifuge (Thermo Fisher)

Laminar cabinet (Thermo Scientific MSC Advantage)

$CO_2$ incubator (Thermo Scientific Steri Cycle)

Water bath (Nüve NB9)

Shaking Mixer (Labnet)

-80°C Freezer (Thermo Scientific)

Liquid Nitrogen Cell Storage Tank (Thermo Cryoplus 2)

pH metre (Hanna Instruments HI8521)

Microplate Reader (Thermo Varioskan® Flash)

Confocal microscope (Zeiss 780 Upright FCS/Confocal Microscope)

Applied Biosystems 7500 Fast Real-Time PCR Machine (Foster City, CA, USA)

ChemiChem chemiluminescent detection system (BioRad)

Cell culture 10 cm petri dish (Corning) 24-well cell culture plate (Corning) 6-well cell culture plate (Corning)

50 mL centrifuge tube (Corning)

15 mL centrifuge tube (NEST biotechnology)

Cryotube (Corning)

Dulbecco's Modified Eagle Medium High/Low glucose (DMEM) (Gibco) Dulbecco's Modified Eagle Medium: Nutrient Mixture (DMEM F12) (Gibco)

Fetal bovine serum (FBS) (Gibco)

Trypsin-EDTA (0.25%) (Gibco)

Penicillin Streptomycin (Pen/Strep) (Thermo Fisher Scientific 100X) Agarose (Merck)

SureBeadsTM Protein G Magnetic Beads Co-Immunoprecipitation Kit (BioRad, Hercules, CA, USA)

SP6 mMessage mMachine Kit (Thermo Fisher Scientific)

NucleoSpin Plasmid Miniprep Kiti (Macherey-Nagel)

SP6 mMessage mMachine Kit (Thermo Fisher Scientific)

NucleoSpin Plasmid Miniprep Kiti (Macherey-Nagel)

Gel DNA Recovery Kit (Zymoclean)

Dual-luciferase reporter assay system (Promega)

### 3.6.2 Methods

#### 3.6.2.1 Zebrafish lines

Transgenic zebrafish (*Danio rerio*) lines Tg(hsp70l:wnt8a-GFP)$^{w34}$ was outcrossed to wild type (wt) AB zebrafish (Kagermeier-Schenk et al., 2011; Weidinger et al., 2005). Based on a previously published protocol, the embryos were exposed to a heat shock and then characterized (Kagermeier-Schenk et al., 2011). The Tg(7XTcf-Xla.Siam:nlsmCherry)$^{ia}$ transgenic line was utilised as a reporter of Wnt/β-catenin signaling, outcrossed to wt zebrafish and embryos were separated as previously reported in a revelant protocol (Moro et al., 2012).

#### 3.6.2.2 Cloning

RNA was isolated from whole embryos at 24 h post-fertilization (hpf) using a Direct-Zol RNA kit (Zymo Research, Irvine, CA, USA) and cDNA was synthesized with an iScript reverse transcription kit (Biorad, Hercules, CA, USA) using a 1:1 mixture of oligo (dT) and random primers. iScript reverse transcriptase (RT) was replaced with water for (-RT) controls. The following products were amplified with the corresponding primer pairs using 1 µL of cDNA. Zebrafish wt Nradd-EGFP: forward primer 5′-AAAAAGGATCCCCACCATGAAAGGAGCAACTGAAGC-3′ and reverse primer 5′-AAAAAGAATTCGACCACTGATACTCCTTGCG-3′; Nradd death domain deleted (DDD)-EGFP: forward primer 5′-AAAAAGGATCCCCACCATGAAAGGAGCAACTGAAGC-3′ and reverse primer 5′-AAAGAATTCATCCTGTTTGCTGTCCCGTTTA-3′. Both PCR products were digested with BamHI and EcoRI and ligated into a pCS2P+ vector that has EGFP. The Nradd DDD construct was generated through the deletion of a 95 amino acid region between the 318th and 413th amino acids at the C-terminal region. Nradd glycosylation site deleted (GSD)-EGFP was generated by site-directed mutagenesis at wt Nradd-GFP using overlap extension PCR. The Nradd GSD construct was generated through the deletion of a 101 amino acid region between the 22nd and 123rd amino acids at the N-terminal region. The first round of PCR was conducted with the two primer pairs forward1 5′-AAAAAGGATCCCCACCATGAAAGGAGCAACTGAAGC-3′, reverse1 5′-ACACTCCCCATCCTGGCCCACAGCCAAAGGCCATCTT-3′ and forward2 5′-

AAGATGGCCTTTGGCTGTGGGCCAGGATGGGGAGTGT-3′, reverse2 5′-AAAAAGAATTCGACCACTGATACTCCTTGCG-3′. The second round of PCR was performed by using the purified PCR product of the first round as the template and the primers forward1 and reverse2. The purified PCR product of the second round of PCR was again digested with BamHI and EcoRI and ligated into a pCS2P+ vector that has EGFP. For Fz8a-EGFP, zebrafish Fz8a was amplified with the forward primer 5′-AGAATTCAACCACCATGGAGTGCTACCT-3′ and the reverse primer 5′-GGATCCTCAGACTTGGGACAAAGGC-3′. The PCR product was digested with EcoRI and BamHI and ligated into a pCS2P+ vector that has EGFP. Successful cloning was verified by sequencing, restriction digestion and agarose gel electrophoresis.

#### 3.6.2.3 Capped sense mRNA synthesis and microinjection

Capped sense RNAs of GFP, wt Nradd, Nradd-GFP, Nradd DDD-EGFP and Nradd GSD-EGFP were synthesized using an mMessage mMachine Kit (Thermo Fisher Scientific, Waltham, MA, USA). mRNAs were injected into 1-cell zebrafish embryos as 250 pg for *nradd*.

#### 3.6.2.4 Cell culture

Human embryonic kidney 293T (HEK293T) and neuroblastoma SH-SY5Y cell lines were obtained from Ozhan lab's stocks. HEK293T cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) and SH-SY5Y cells in DMEM F-12 at 37°C in a 5% (v/v) CO2 humidified environment. All media for cell culture were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin.

#### 3.6.2.5 Transfection and luciferase assay

HEK293T were seeded on 24-well plates and transfected in triplicate with 20 ng of Wnt8, with and without 300 ng of Nradd. SH-SY5Y cells were seeded on 24-well plates and transfected in triplicate with 250 ng of Nradd and stimulated with Wnt3a conditioned media (CM) for 6h. All cells were co-transfected with 20 ng of firefly luciferase reporter pGL3 BAR [49] and 5 ng of renilla luciferase reporter pGL4.73

hRLuc/SV40 (Promega, Madison, WI, USA) using Fugene HD Transfection Reagent (1 µg/1 µL, Promega, Madison, WI, USA). Twenty-four hours after transfection, reporter activity was measured with the dual luciferase reporter assay kit (Promega, Madison, WI, USA) in a Varioskan Flash multimode reader (Thermo Fisher Scientific, Waltham, MA, USA). Statistical analysis was performed using a Student's t-test. Error bars represent SD, where * and ** indicate $p < 0.05$ and $p < 0.01$, respectively.

#### 3.6.2.6 Subcellular localization

Zebrafish embryos were injected with 250 pg Nradd-EGFP and 250 pg RFP-GPI at the 1-cell stage, dechorionated at 3 hpf (hours post fertilization), mounted in 3% methylcellulose, and imaged using a Zeiss LSM 880 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

#### 3.6.2.7 Co-Immunoprecipitation and western blotting

HEK293T cells were seeded in 6-well plates and transfected with 500 ng Nradd-GFP and 500 ng Lrp6-HA. At 48 h, Wnt induction was performed for 6h and cells were washed with ice-cold PBS and lysed with NOP buffer (10 mM HEPES KOH pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP40 (Igepal CA-630, Sigma-Aldrich, St. Louis, MO, USA). Lysate was centrifuged at 300 g for 5 min at 4 °C. Supernatant was precipitated with a SureBeadsTM Protein G Magnetic Beads Co-Immunoprecipitation Kit (BioRad, Hercules, CA, USA) according to the kit protocol. For western blotting, samples were dissolved in 5X loading dye and separated by SDS gel electrophoresis on a 10% acrylamide-bis acrylamide gel. Proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare Life Science, Chicago, IL, USA). Membranes were blocked in 5% milk powder for 45 min at room temperature (RT) and incubated with the following antibodies at the corresponding dilutions. Primary antibodies: rabbit anti-GFP ((D5.1) XP, 1:1000; Cell Signaling Technology, Danvers, MA, USA) and mouse anti-HA (1: 2000; OriGene Technologies, Rockville, MD, USA). Secondary antibodies: anti-rabbit IgG, horseradish peroxidase (HRP)-linked and anti-mouse IgG, HRP-linked (both 1:2500; Cell Signaling Technology, Danvers, MA, USA).

### 3.6.2.8 Immunofluorescence staining

Zebrafish embryos produced by crossing wt AB and Tg(hsp70l:wnt8a-GFP)$^{w34}$ were injected with the capped sense RNAs of control GFP (100 pg), wt Nradd-GFP (250 pg), Nradd-DDD (250 pg) or Nradd-GSD (250 pg) at the 1-cell stage. Injected embryos were heat shocked at 60% epiboly, dechorionated at 24 hpf by adding 100 µL of 10 mg/mL pronase in 10 mL E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) and fixed in 4% PFA in PBST (1× PBS, 0.1% Tween-20) overnight at 4 ◦C. Embryos were incubated in ice-cold methanol at −20 ◦C for 24 h. Methanol was removed and embryos were washed twice with 1 mL 1× PDT (1× PBST, 0.3% Triton-X, 1% DMSO) for 30 min by rocking gently at RT. Next, embryos were incubated with 500 µL blocking buffer (1× PBST, 10% heat-inactivated fetal bovine serum, 2% bovine serum albumin) at RT for 1 h 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 1× PDT, incubated with rhodamine (TRITC)-AffiniPure donkey anti-rabbit IgG (1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h at RT, re-washed with 1× PDT, mounted in 50% glycerol in PBS and imaged using a Zeiss LSM 880 confocal microscope.

### 3.6.2.9 Annexin V-FITC Apoptosis Assay

HEK293T or SH-SY5Y cells were seeded in 6-well plates. For the control and Nradd, cells were transfected with firefly luciferase reporter pGL3 BAR (100 ng) and the plasmid containing Nradd without GFP tags (250 ng), respectively, according to the manufacturer's protocol. For the positive controls, cells were treated with 0.3 mM $H_2O_2$ and 1 µM staurosporine for 18 h. Relevant cells were treated with Wnt3a CM overnight to induce Wnt signaling. Twenty-four hours after transfection, supernatants and trypsinized cells were collected. Cells were washed and stained with annexin V-FITC and 7AAD using an FITC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, San Diego, CA, USA). Flow cytometry was performed using a BD Fortessa and data were analyzed with FlowJo 8.8.6 software (Tree Star Inc., Ashland, OR, USA). Experiments were performed in three biological replicates and data are representative of at least three independent experiments.

### 3.7. Research plan and timeline

**Table 1:** Experimental timeline

| Date range | Experiments |
|---|---|
| 01/05/2019 – 01/02/2020 | The developmental transcriptional regulation of Nradd by Wnt/β-catenin signaling |
| 01/02/2020 – 01/11/2021 | The localization of Nradd in the plasma membrane and interaction with the Wnt-receptor complex |
| 01/11/2021 – 01/08/2021 | The dual role of Nradd in Wnt/β-catenin signaling to promote apoptosis during development |
| 01/08/2021 – 01/05/2022 | The apoptotic effect of zebrafish Nradd on human embryonic and neuroblastoma cell lines |

### 3.8. Data analysis

Image J, GraphPad Prism7 and Student's T Test were used.

### 3.9. Limitations of the research

There is no limitation for the thesis.

### 3.10. Ethical committee approval

Animal experiments were approved by the Animal Experiments Local Ethics Committee of Izmir Biomedicine and Genome Center (IBG-AELEC)

# 4. RESULTS

## 4.1 Plasmid maps

The maps of plasmids cloned in this thesis are shown in Figures 7 to 14.

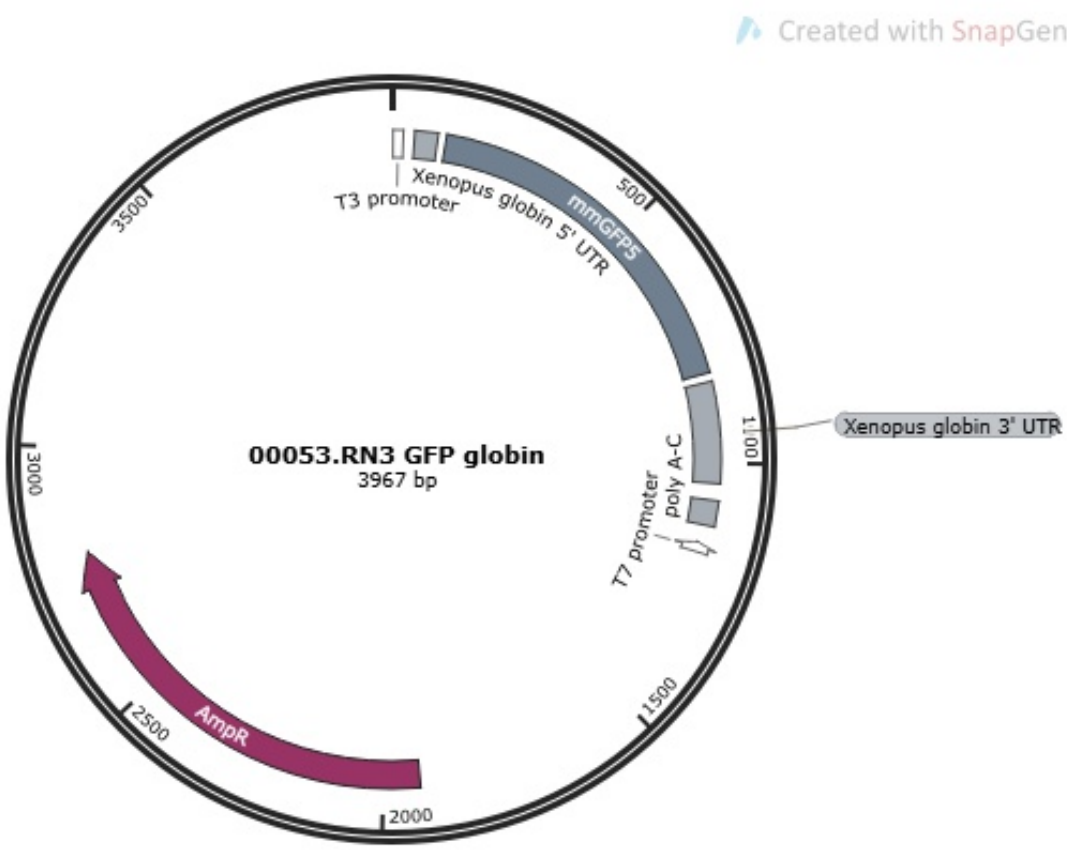



**Figure 7:** Plasmid map of GFP (SNAPGENE).

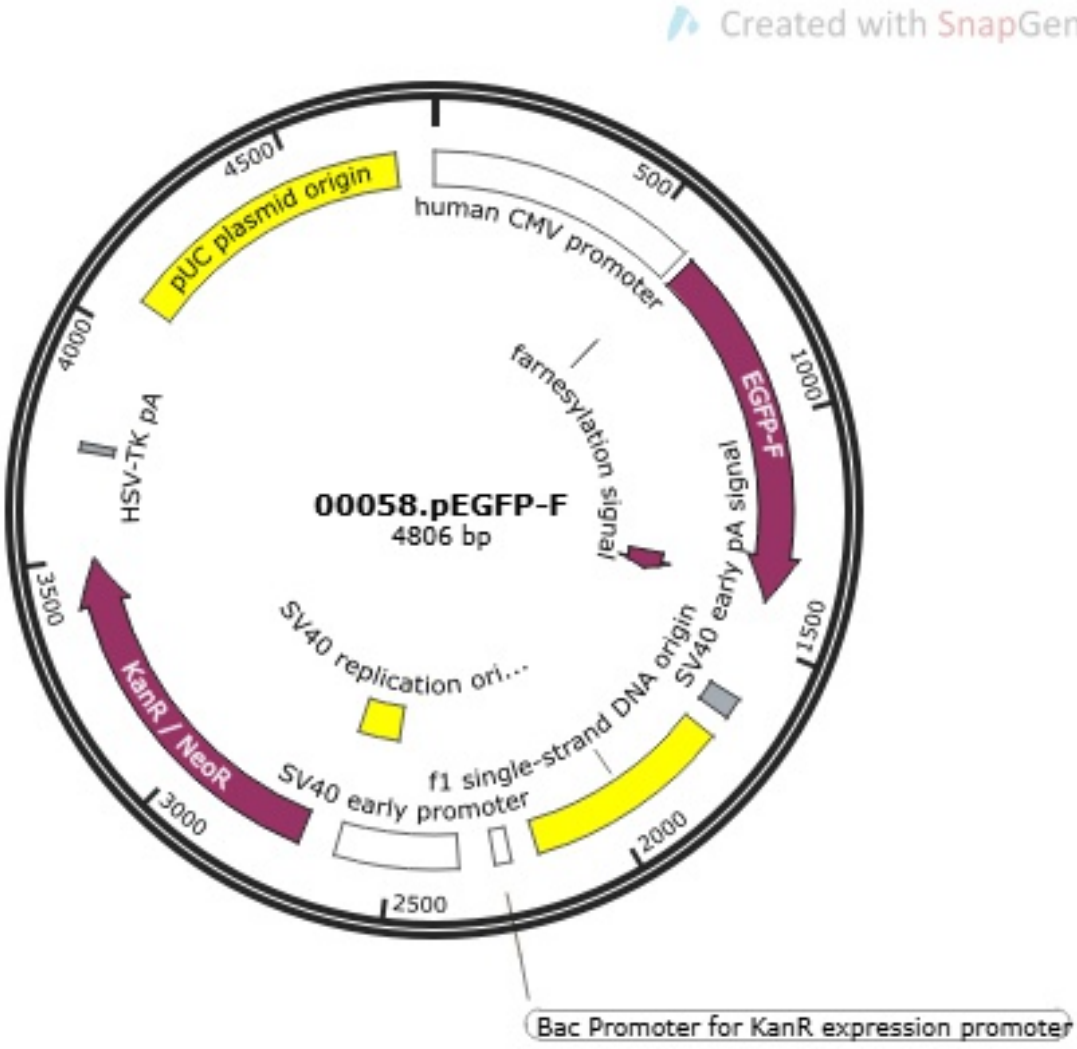



**Figure 8:** Plasmid map of EGFP (SNAPGENE)

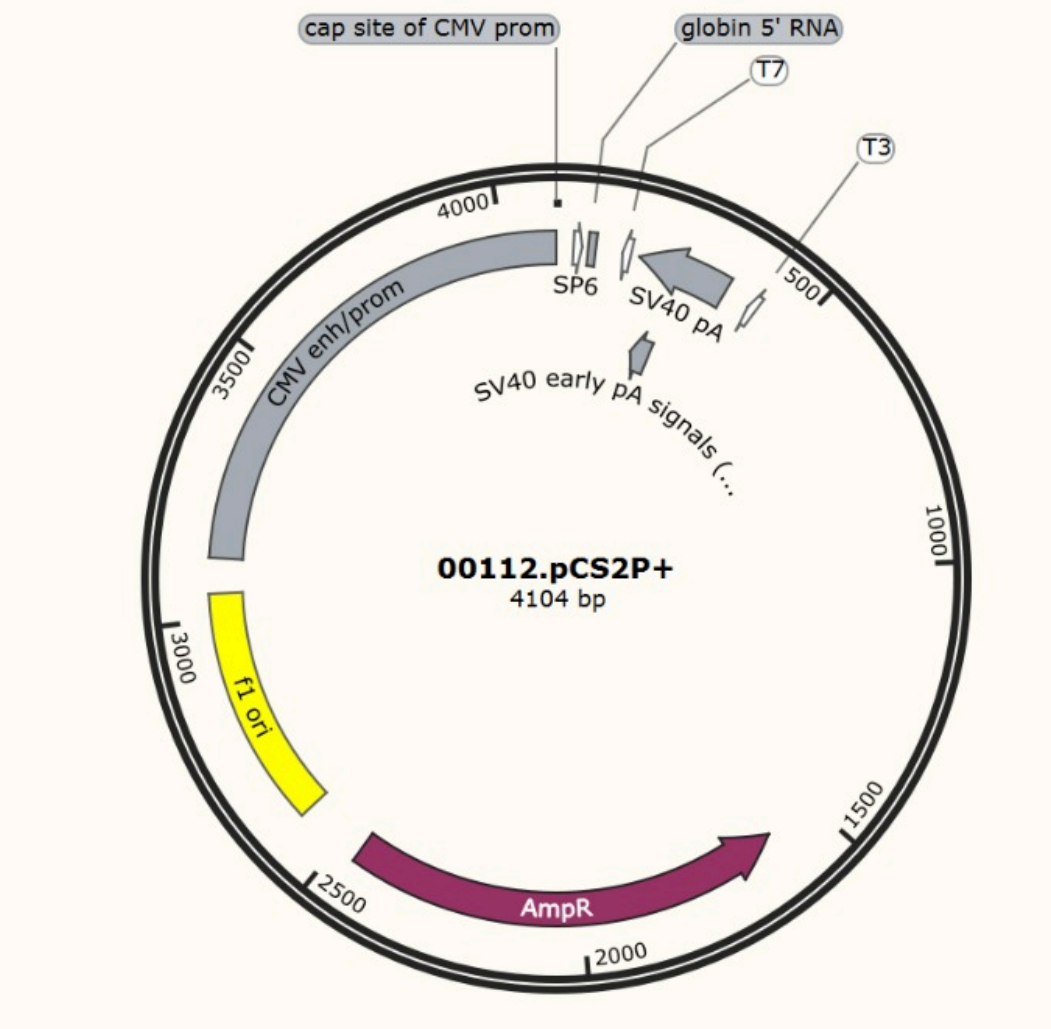



**Figure 9:** Plasmid map of blank plasmid (control plasmid) (SNAPGENE)

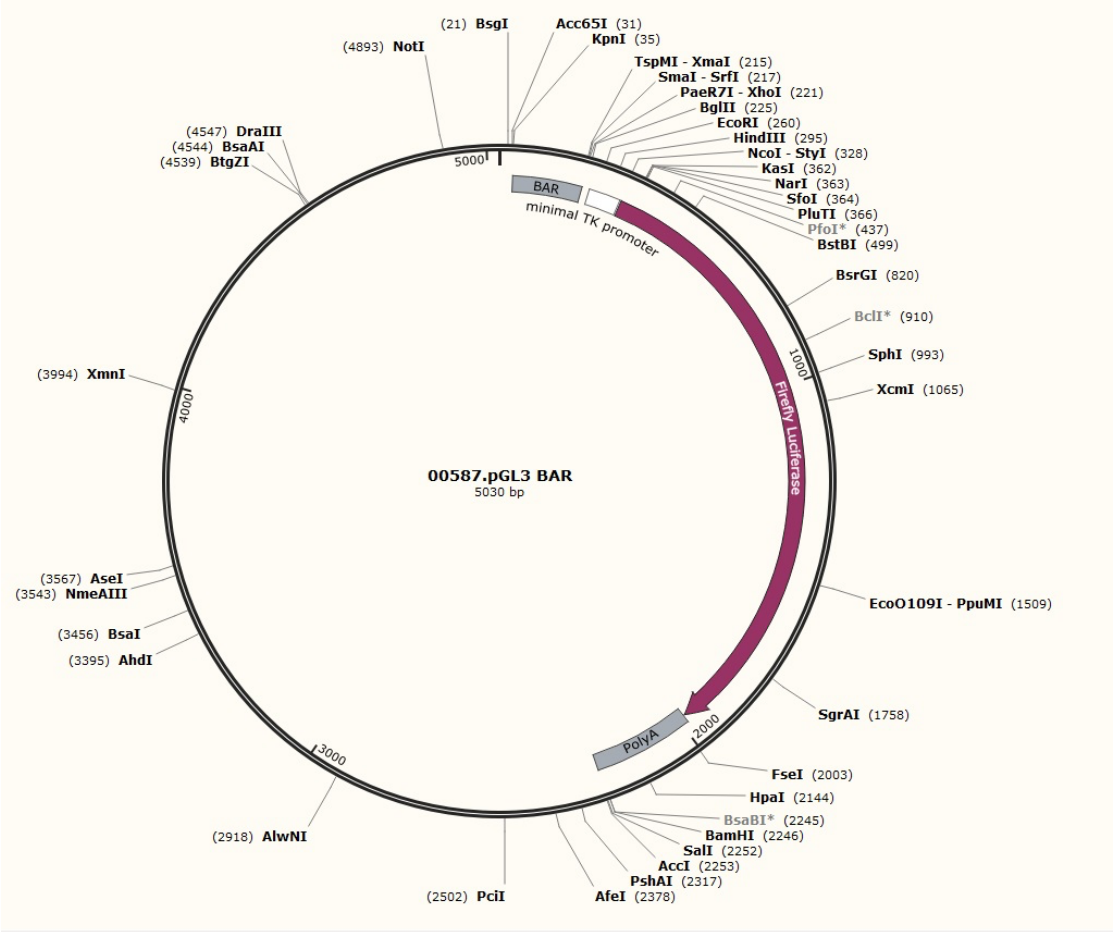



**Figure 10:** Plasmid map of firefly luciferase (SNAPGENE)

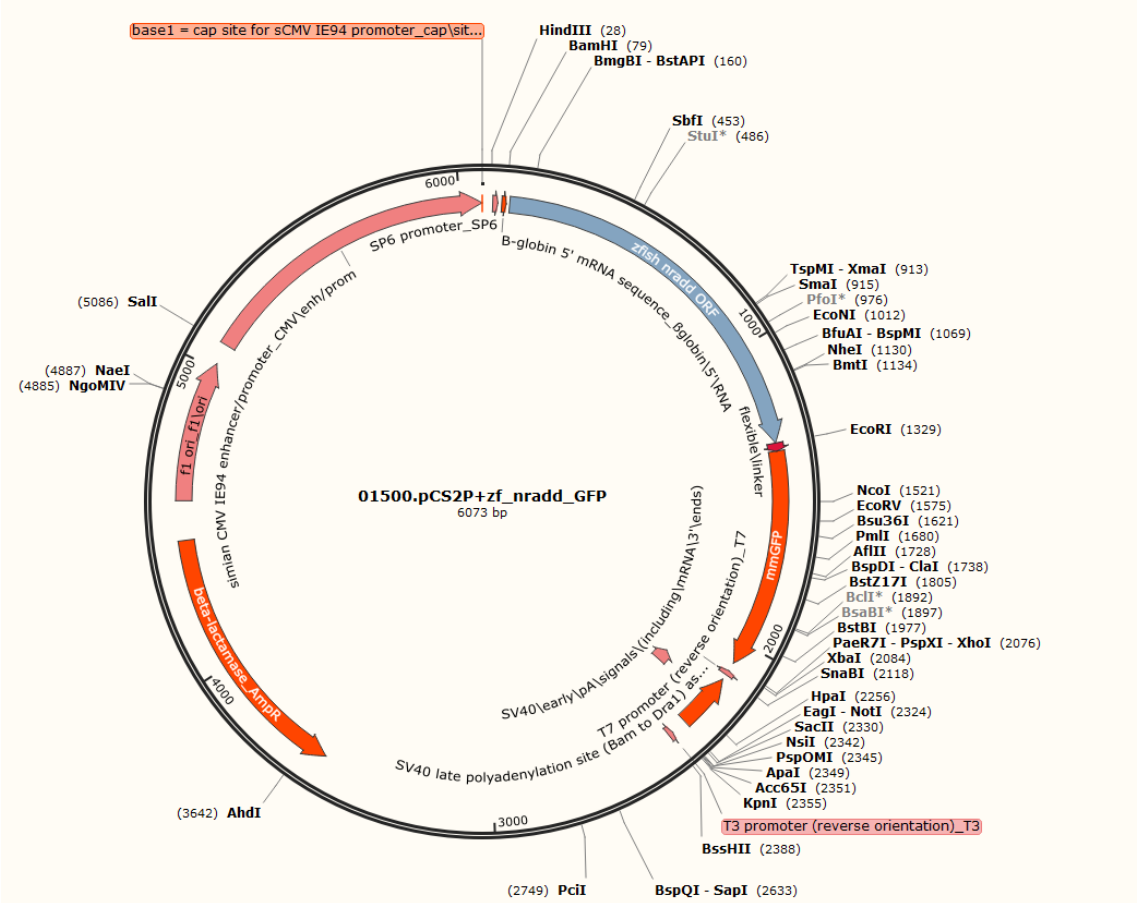



**Figure 11:** Plasmid map of WT Nradd.GFP (SNAPGENE)

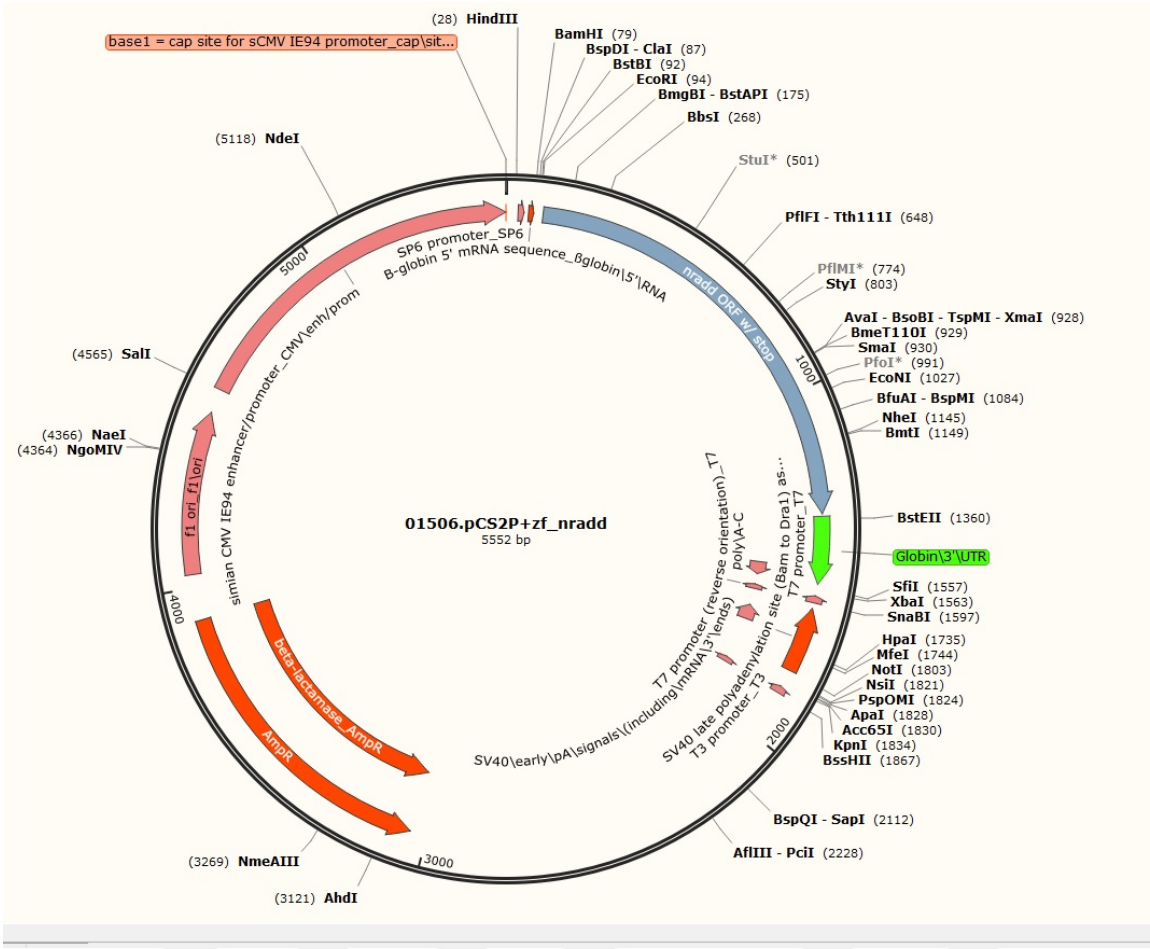



**Figure 12:** Plasmid map of WT Nradd (SNAPGENE)

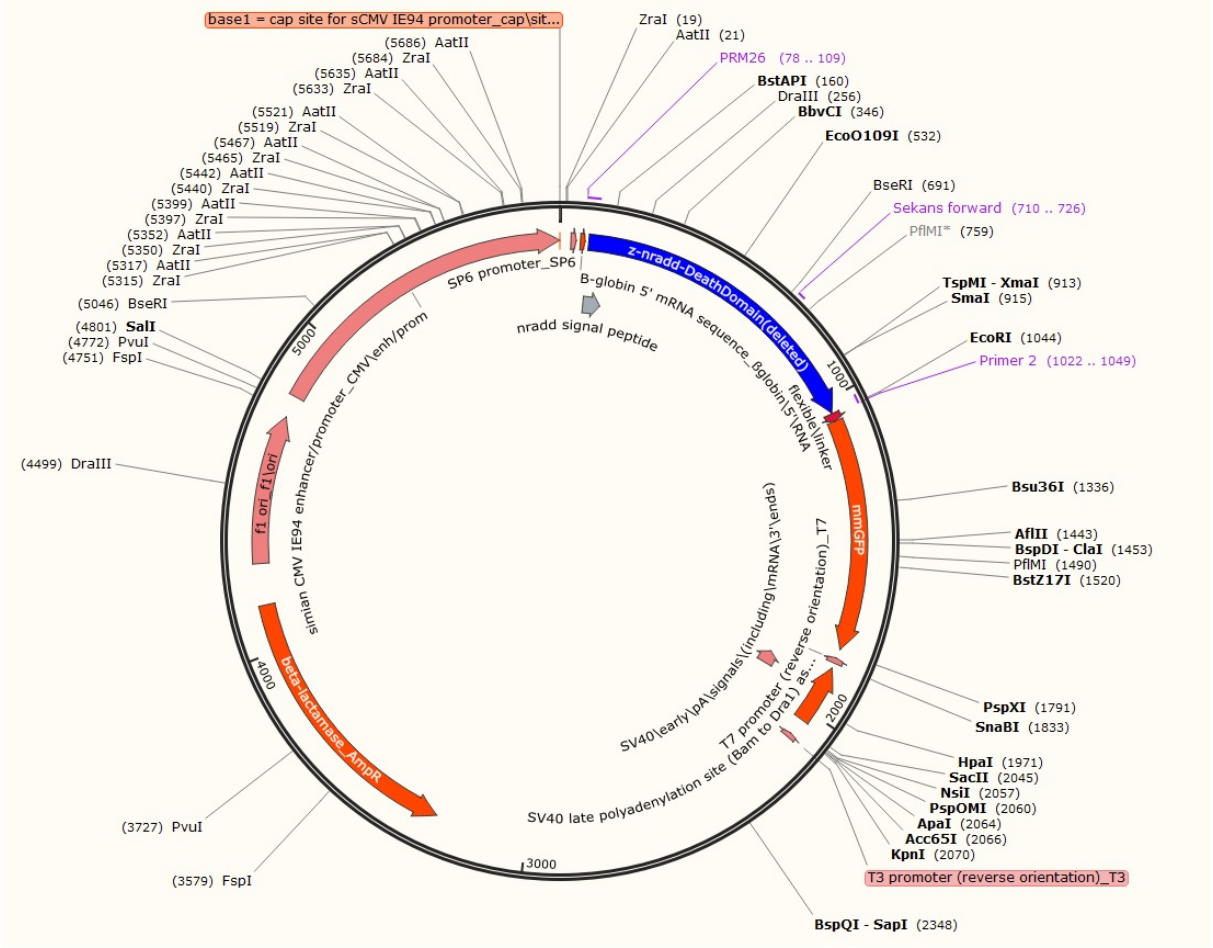



**Figure 13:** Plasmid map of Nradd DDD (SNAPGENE)

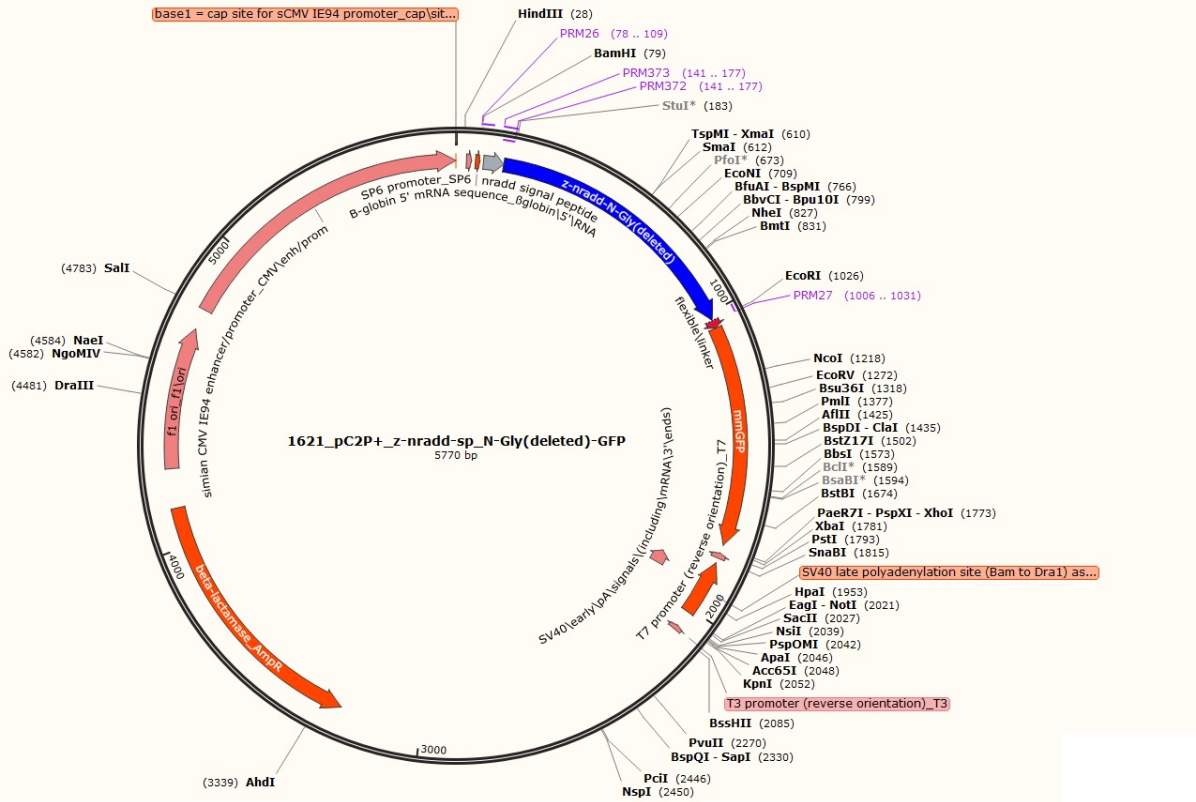



**Figure 14:** Plasmid map of Nradd GSD (SNAPGENE)

## 4.2 The developmental transcriptional regulation of Nradd by Wnt/β-catenin signaling

Our first aim was to investigate the role of nradd in Wnt/β-catenin signaling. Based on previous RNA sequencing data, nradd has been selected as a candidate Wnt target gene. Because, the expression of nradd has changed during gastrula, somitogenesis and organogenesis stages, when Wnt/β-catenin signaling was manipulated positively and negatively. Thus, we have previously performed 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) to overexpress Wnt pathway inhibitor Axin1 and pathway activator Wnt8, respectively. As a result, we found that nradd expression was increased and decreased in Wnt dependent manner (Ozalp et al., 2021) (Figure 15).

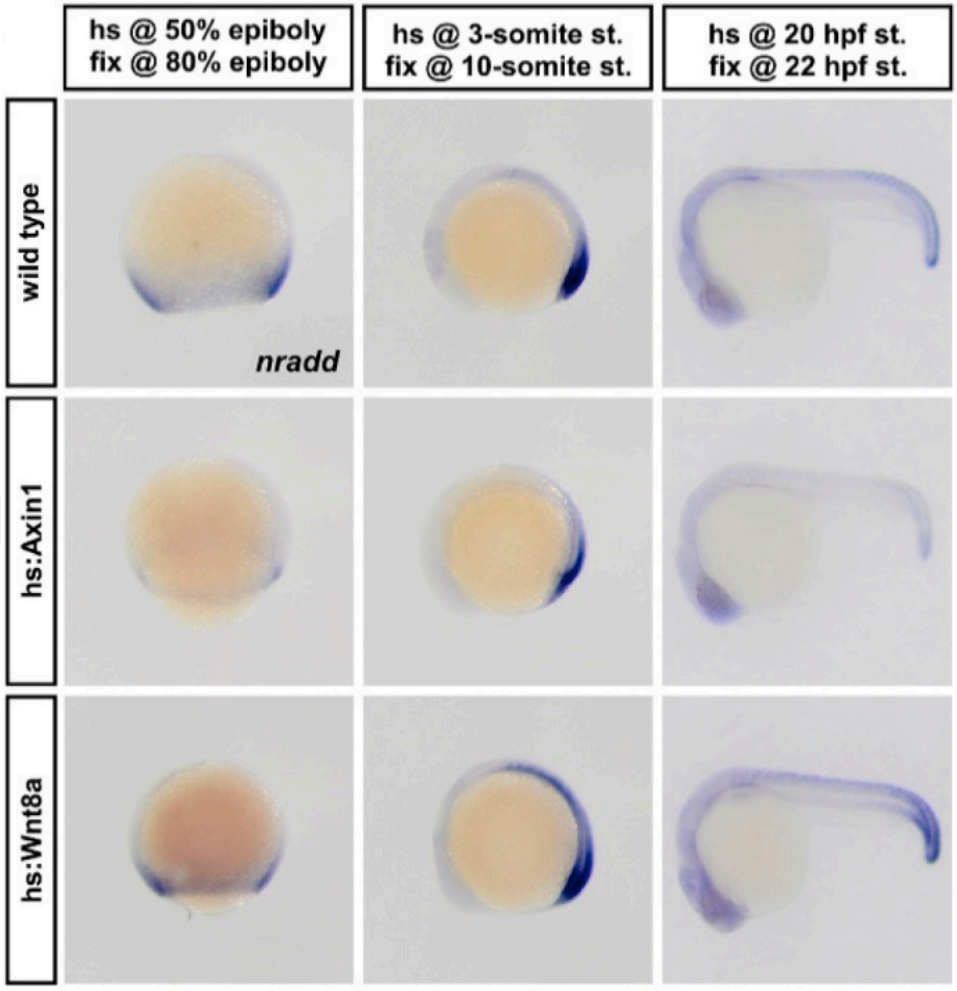



**Figure 15:** Whole-mount in situ hybridization (WMISH) to detect the regulation of *nradd* expression by Wnt/β-catenin signaling during development (Ozalp et al., 2021)

Next, we aimed to validate this regulation mechanism of Nradd by Wnt/β-catenin signaling. Hence, we performed qPCR (quantitative PCR) method in hs:Axin1 and hs:Wnt8a transgenic embryos to show that the expressional change of *nradd* is regulated by this pathway quantitatively. As a result, we have confirmed that *nradd* is a target gene of Wnt/β-catenin signaling pathway based on the transcriptional regulation by comparison with the *sp5l* gene, which is a Wnt target gene (Figure 16) (Ozalp et al., 2021).

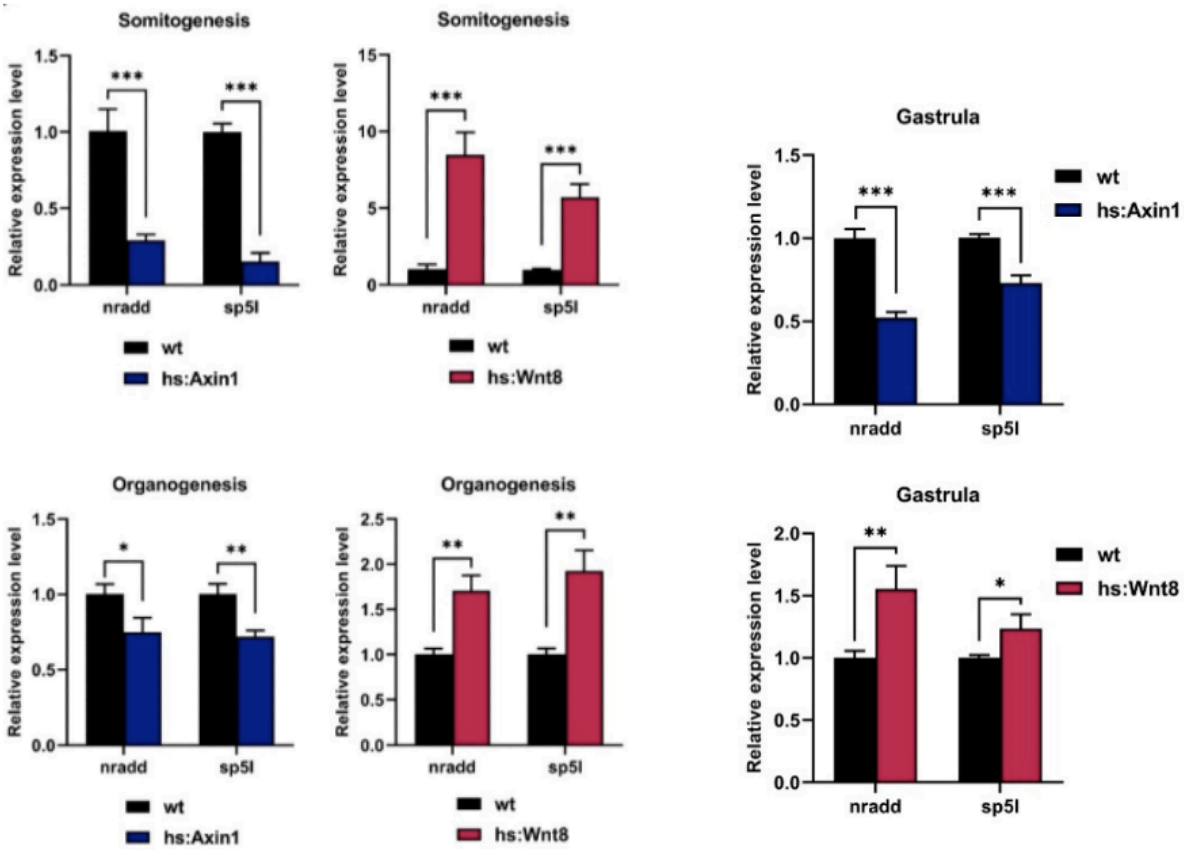



**Figure 16:** The determination of *nradd* expression in Wnt/β-catenin signaling by comparison with the *sp5l* gene (Ozalp et al., 2021)

Next, we aimed to investigate the inhibitory role of *nradd* acts in Wnt/β-catenin signaling. We found that Wnt/β-catenin reporter activity is reduced in 7xTcf:mCherry embryos injected with *nradd* mRNA (Figure 17) (Ozalp et al., 2021). We also found that Nradd is able to inhibit Wnt signaling significantly in both cell lines (Figure 18) (Ozalp et al., 2021).

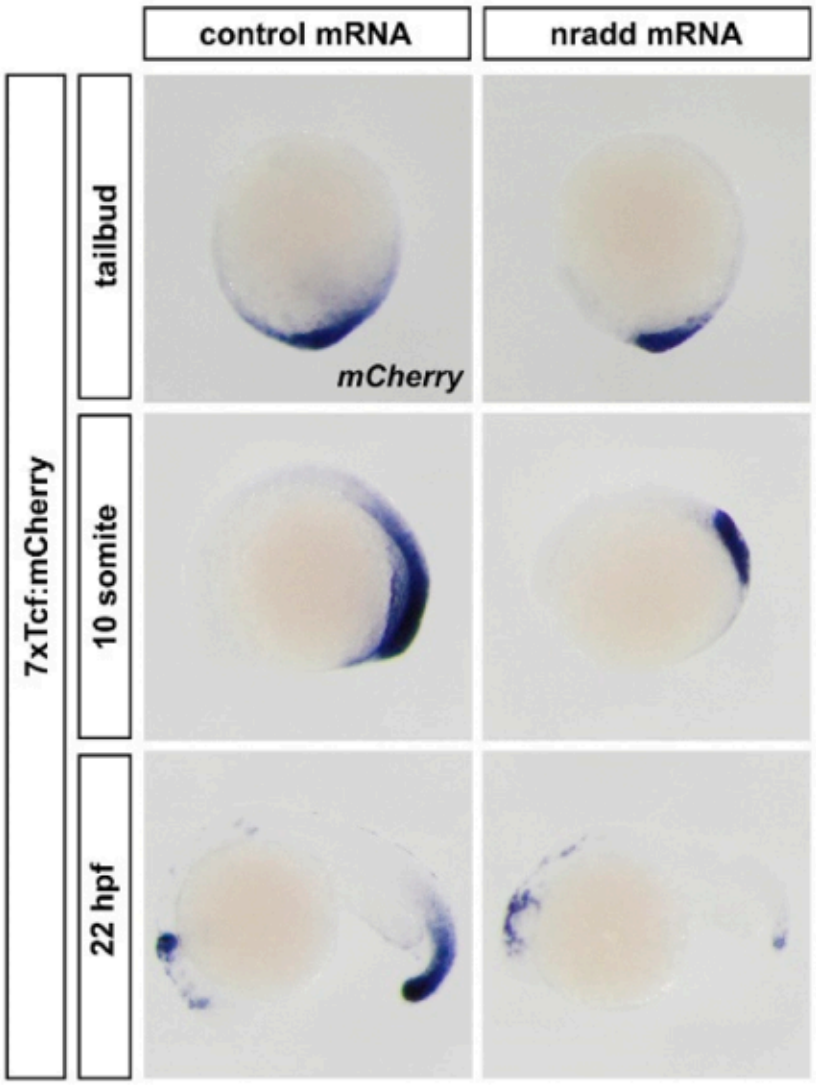



**Figure 17:** The determination of Wnt/β-catenin reporter activity in 7xTcf:mCherry embryos injected with *nradd* mRNA

Next, we measured the Wnt/β-catenin signaling activity in HEK293T and SH-SY5Y cells upon *nradd* overexpression, besides co-induction with Wnt8 or Wnt3a conditioned media (CM) (Figure 18). We found that Nradd is able to inhibit Wnt signaling significantly in both cell lines by assessing pBAR luciferase reporter activity. Statistical significance was evaluated using an unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Error bars represent SD. Three independent experiments were performed.

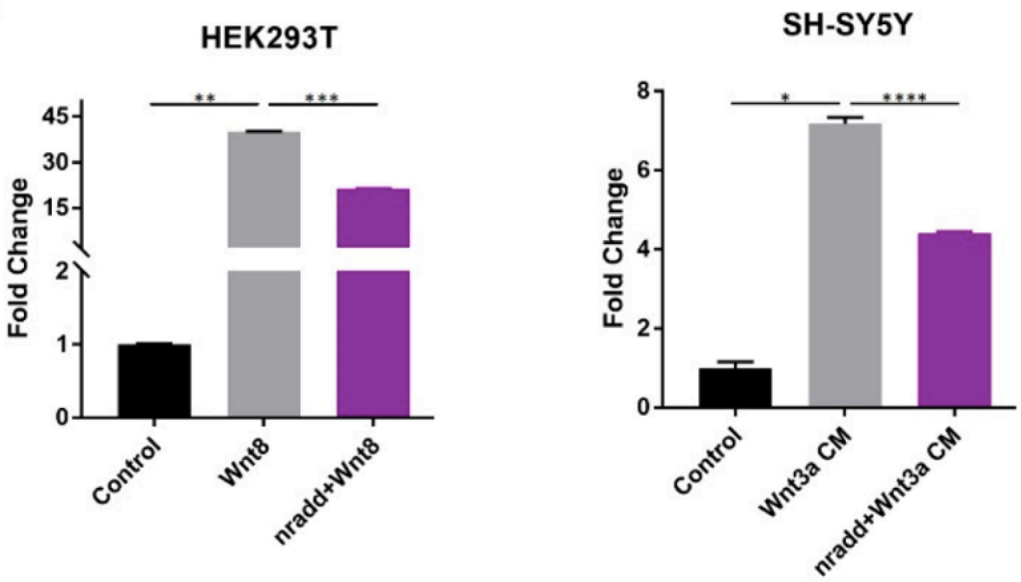



**Figure 18:** The assessment of pBAR luciferase reporter activity to investigate the inhibitory role of *nradd* in Wnt signaling in cell culture.

### 4.3 The localization of Nradd in the plasma membrane and interaction with the Wnt-receptor complex

Since Nradd encodes a transmembrane protein, we aimed to examine its localization in the cell and to test whether it interacts with the Wnt-receptor complex. We found that Nradd-EGFP is localized in the plasma membrane and co-localized with membrane-bound RFP-GPI (glycosylphosphatidylinositol) in early blastula stage zebrafish embryos (Figure 19).

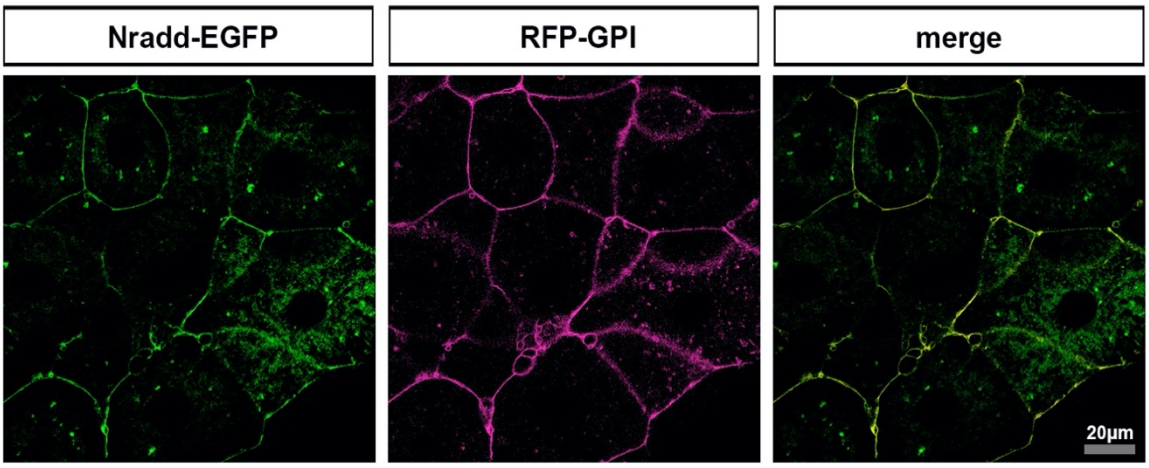



**Figure 19:** Localization of Nradd-EGFP and RFP-GP at the plasma membrane of cells of zebrafish embryos at 4 hpf

Next, we investigated whether there is a physical interaction between Nradd and components of the Wnt-receptor complex at the plasma membrane. First, Nradd was co-precipitated with the canonical Wnt co-receptor Lrp6, demonstrating a potential interaction between these two proteins (Figure 20).

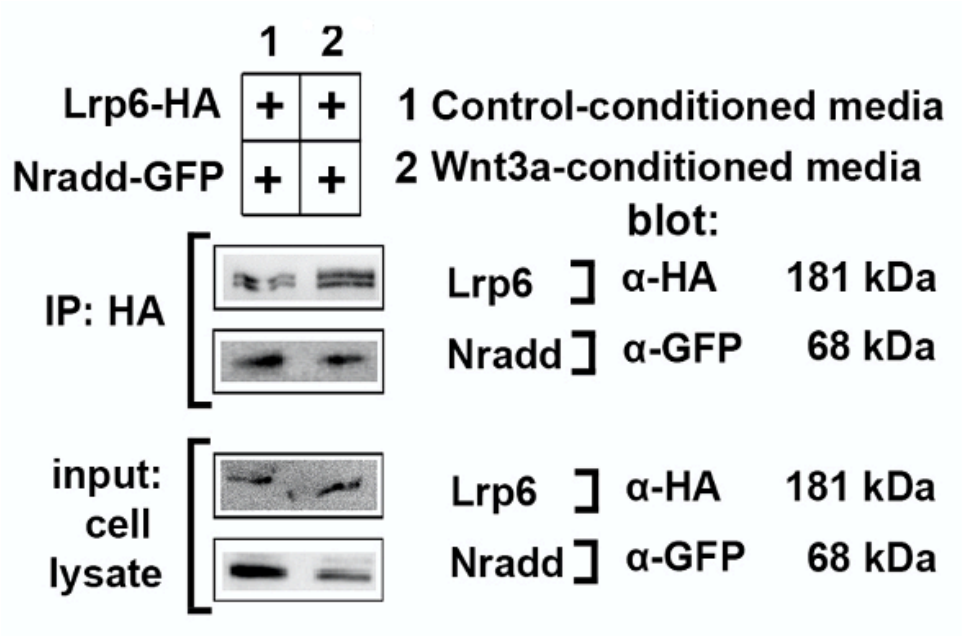



**Figure 20:** Western blot representation of Nradd-GFP co-precipitated with Lrp6-HA in HEK293T cells

### 4.4. The dual role of Nradd in Wnt/β-catenin signaling to promote apoptosis during development

Although Nradd has been shown to promote apoptosis in neuroblastoma cell lines, its potential role in embryonic development has not been elucidated (Wang et al, 2003). Nradd has four amino-terminal cysteine-rich domains (CRDs) conserved among TNFRSF members located between amino acids 23 and 184, and a carboxy-terminal death region located between amino acids 326 and 404.

In addition to the wild-type Nradd, two other constructs were thus generated; Nradd-DDD and Nradd-GSD, where the C-terminal region containing the death region and the N-terminal region including the N-glycosylation region and two CRD regions were deleted, respectively (Figure 21).

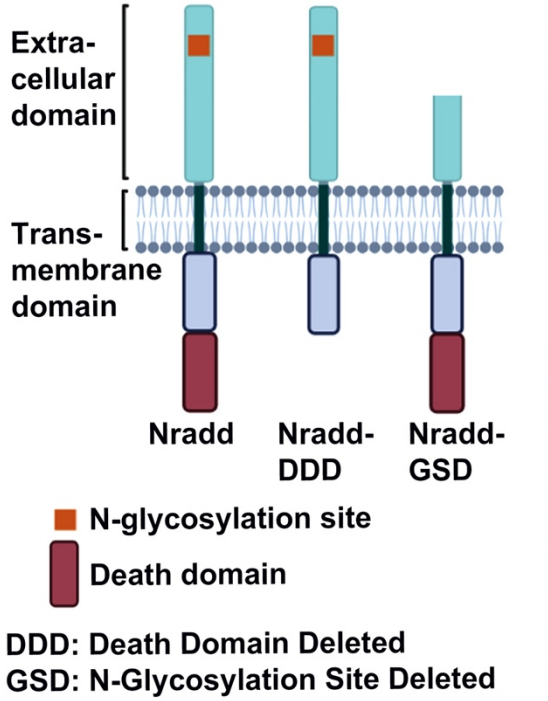



**Figure 21:** Schematic representation of foreign-type Nradd constructs without death domain (Nradd death domain deleted (DDD)) and Nradd constructs without N-glycosylated N-terminal domain (Nradd glycosylation site deleted (GSD)) (Created with BioRender.com)

Firstly, we injected wild type nradd-GFP mRNA into the wild type zebrafish embryos at 1-cell stage and imaged them at 24hpf to detect whether our nradd-GFP construct works properly after in vitro transcription (Figure 22).

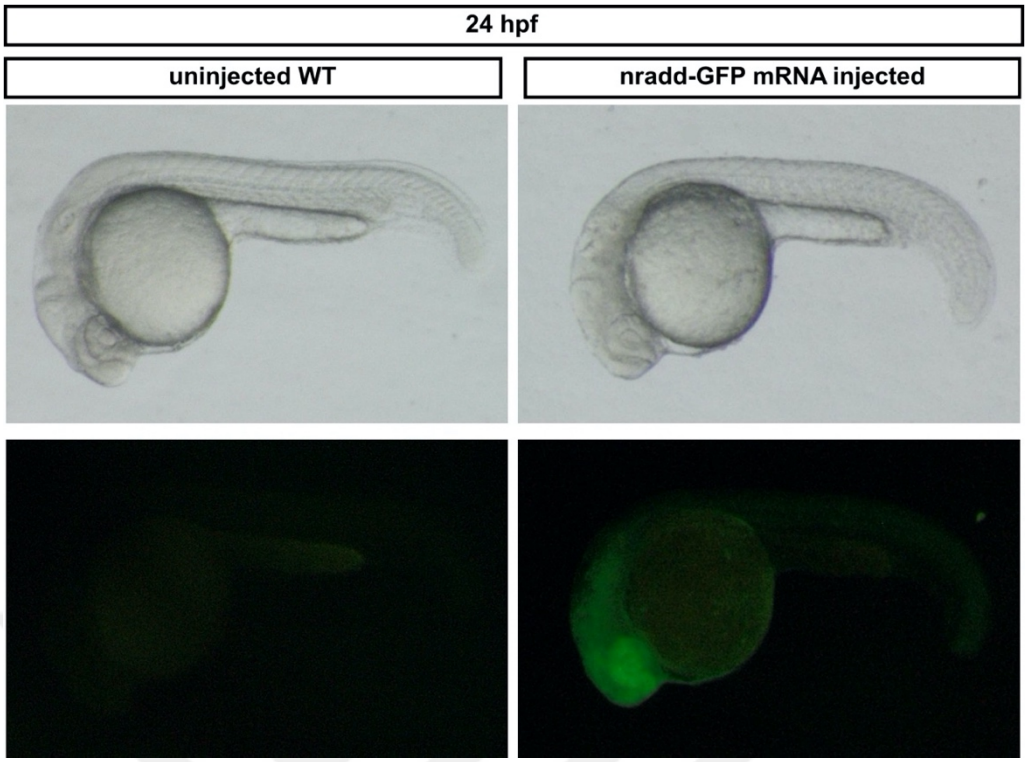



**Figure 22:** The demonstration of *nradd-GFP* expression in WT zebrafish embryos at 24 hpf

Next, we injected mRNAs of wild type nradd, nradd DDD and nradd GSD into the wild type zebrafish embryos at 1-cell stage and imaged them at 4hpf to detect whether our nradd-GFP construct works properly after in vitro transcription (Figure 23).

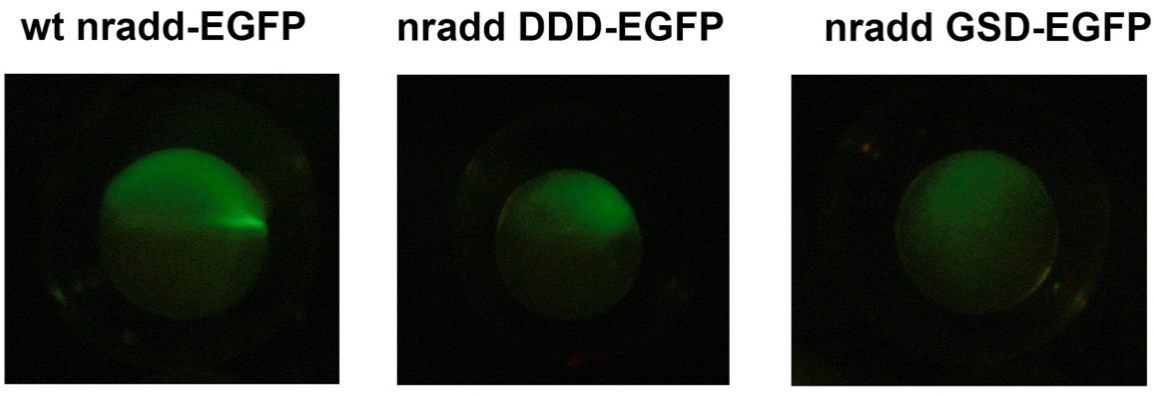



**Figure 23:** The demonstration of *nradd-EGFP*, *nradd DDD-EGFP* and *nradd GSD-EGFP* expression WT zebrafish embryos at 4hpf

Firstly, to address whether Nradd can induce apoptosis during development, we overexpressed the wild-type nradd mRNA in zebrafish embryos and performed

immunostaining for active caspase 3, a marker of apoptosis. In this step, we focused on the brain regions of the zebrafish embryos at 24hpf, as Nradd is able to suppress the Wnt8-mediated pattern of the neuroectoderm during development. As a result, we unravelled that Nradd is sufficient by itself to increase apoptosis significantly and is also involved in Wnt8-mediated β-catenin signaling activated in hs:Wnt8 transgenic zebrafish embryos exposed to a heat shock at 60% epiboly (Figure 24).

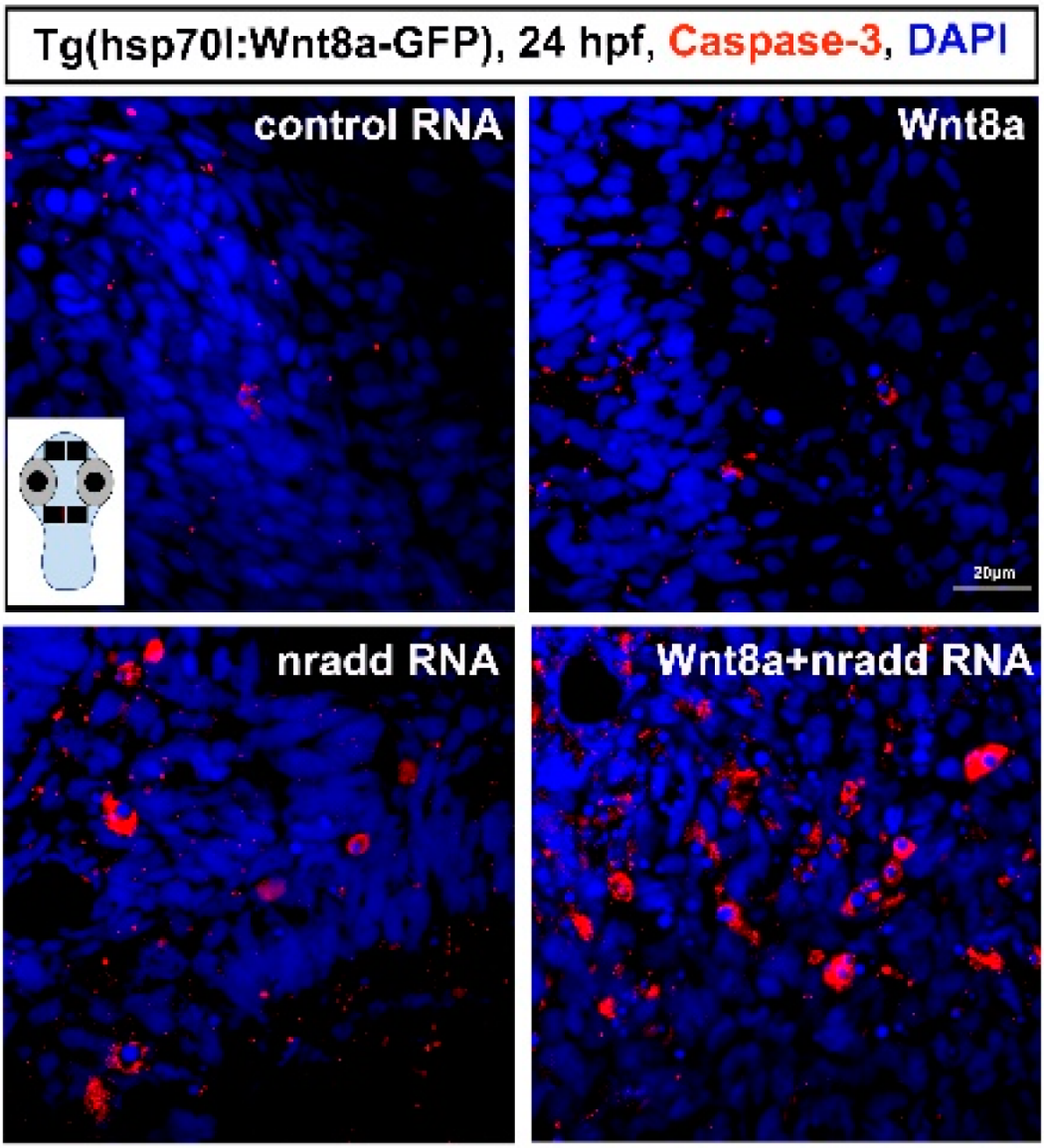



**Figure 24:** Confocal microscopy of nradd-induced apoptosis by performing anti-cleaved caspase 3 staining at 24 hpf with activated Wnt8-mediated β-catenin signaling in hs:Wnt8 transgenic zebrafish embryos. The attached thumbnail shows a representative embryo with four different regions indicated by black rectangles used for counting cleaved caspase 3-positive cells.

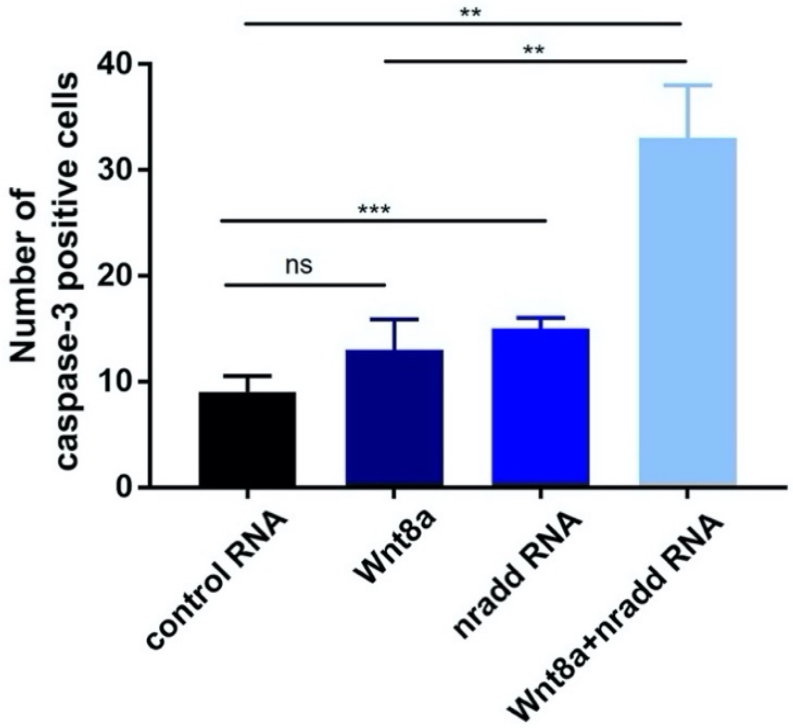



**Figure 25:** Quantitative evaluation of cleaved caspase 3-positive apoptotic cells. Numbers represent the mean number of apoptotic cells counted from four different sites of 20 embryos for each group. ** $p < 0.01$ and *** $p < 0.001$.

In contrast to wild-type Nradd, we found that Nradd-DDD and Nradd-GSD can not inhibit canonical Wnt signaling and induce apoptosis in wild-type zebrafish embryos (Figure 26).

Thus, Nradd appears to promote apoptosis along with Wnt/β-catenin signaling. Furthermore, both the C-terminal death domain and the N-glycosylated N-terminal domain are required for its apoptotic function.

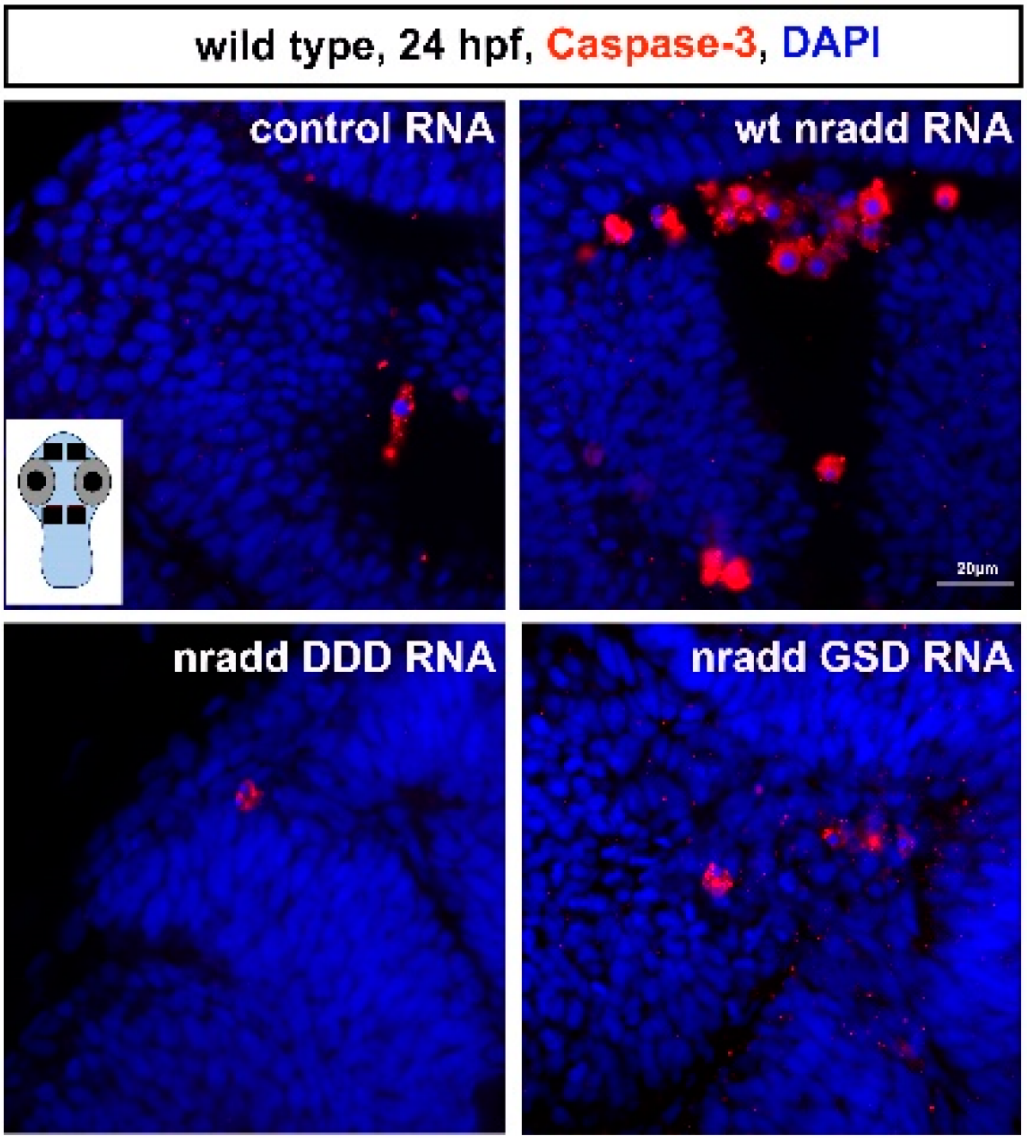



**Figure 26**: Demonstration of apoptosis by confocal microscopy by anti-cleaved caspase 3 staining at 24 hpf with activated wnt8-mediated β-catenin signaling of wild-type nradd, nradd DDD, and nradd GSD in wild-type zebrafish embryos

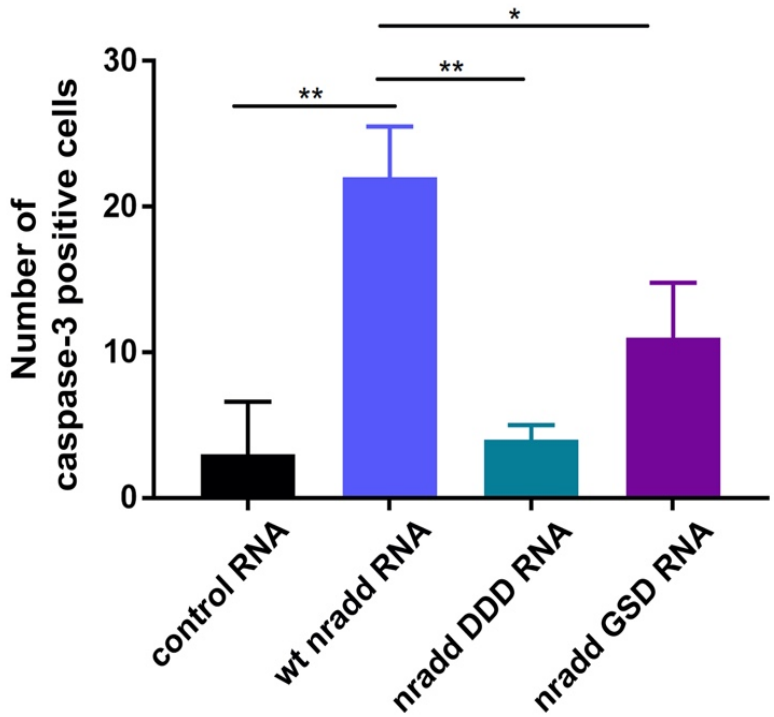



**Figure 27:** Quantitative evaluation of cleaved caspase 3-positive apoptotic cells upon overexpression of wild-type nradd, nradd DDD, and nradd GSD in wild-type zebrafish embryos. Numbers represent the mean number of apoptotic cells counted from four different sites of 20 embryos for each group. ** $p < 0.01$ and *** $p < 0.001$.

### 4.5 The apoptotic effect of zebrafish Nradd on human embryonic and neuroblastoma cell lines

In this part of the thesis, we transfected human embryonic kidney 293T (HEK293T) cells and neuroblastoma (SH-SY5Y) cells with zebrafish Nradd to test whether Nradd in zebrafish is functional in humans, although it is normally a pseudogene in humans (Figure 28).

In this way, Nradd significantly induced apoptosis alone, but could not co-induce with Wnt3a in those cell lines (Figure 29,30).

In addition, we showed that Nradd in zebrafish is able to increase apoptosis significantly in human neuroblastoma SH-SY5Y cells independently of the Wnt3a induction (Figure 31,32).

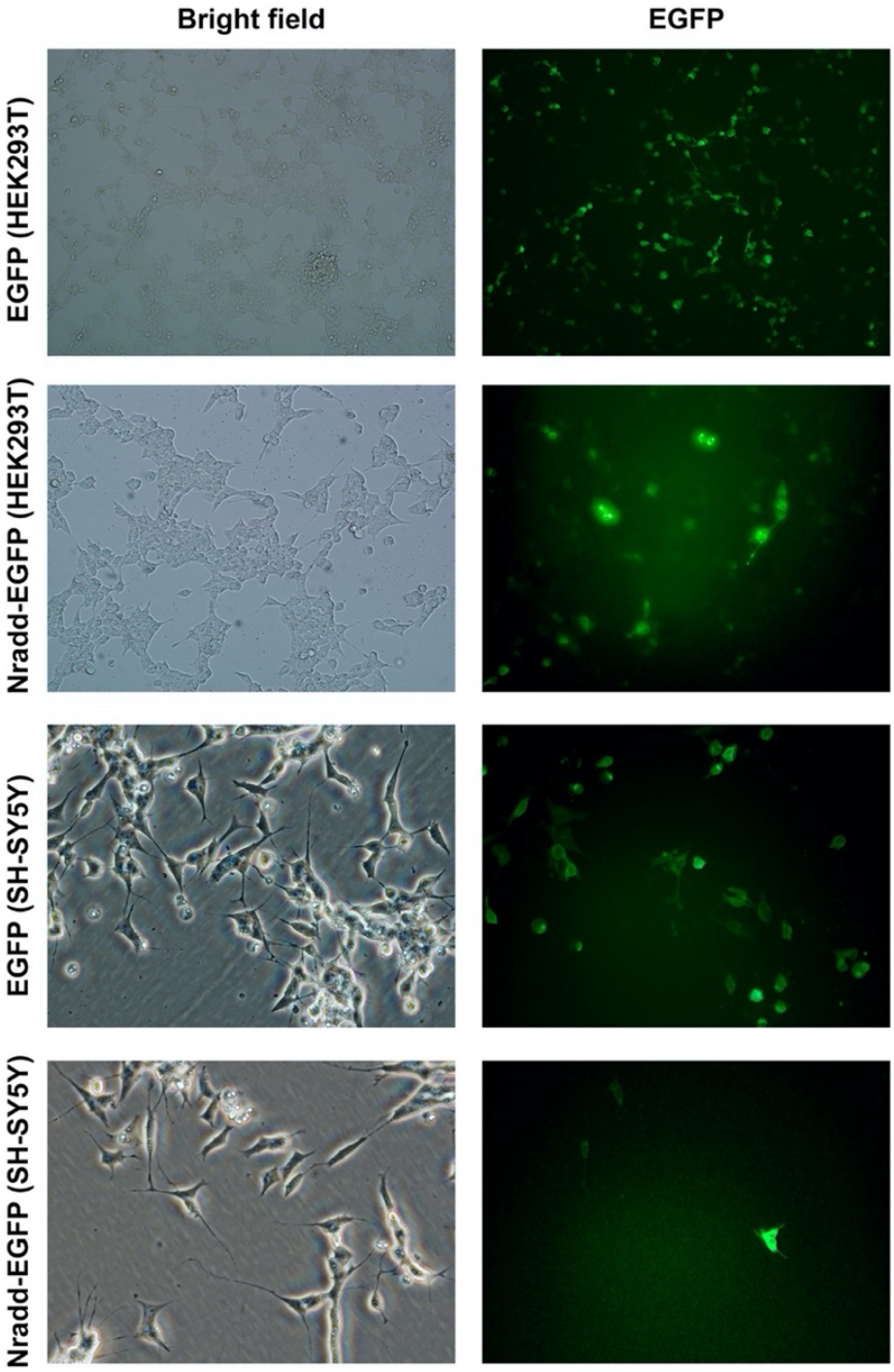



**Figure 28:** The demonstration of *nradd-EGFP* and *EGFP* expression in HEK293T and SH-SY5Y cells by fluorescence microscopy

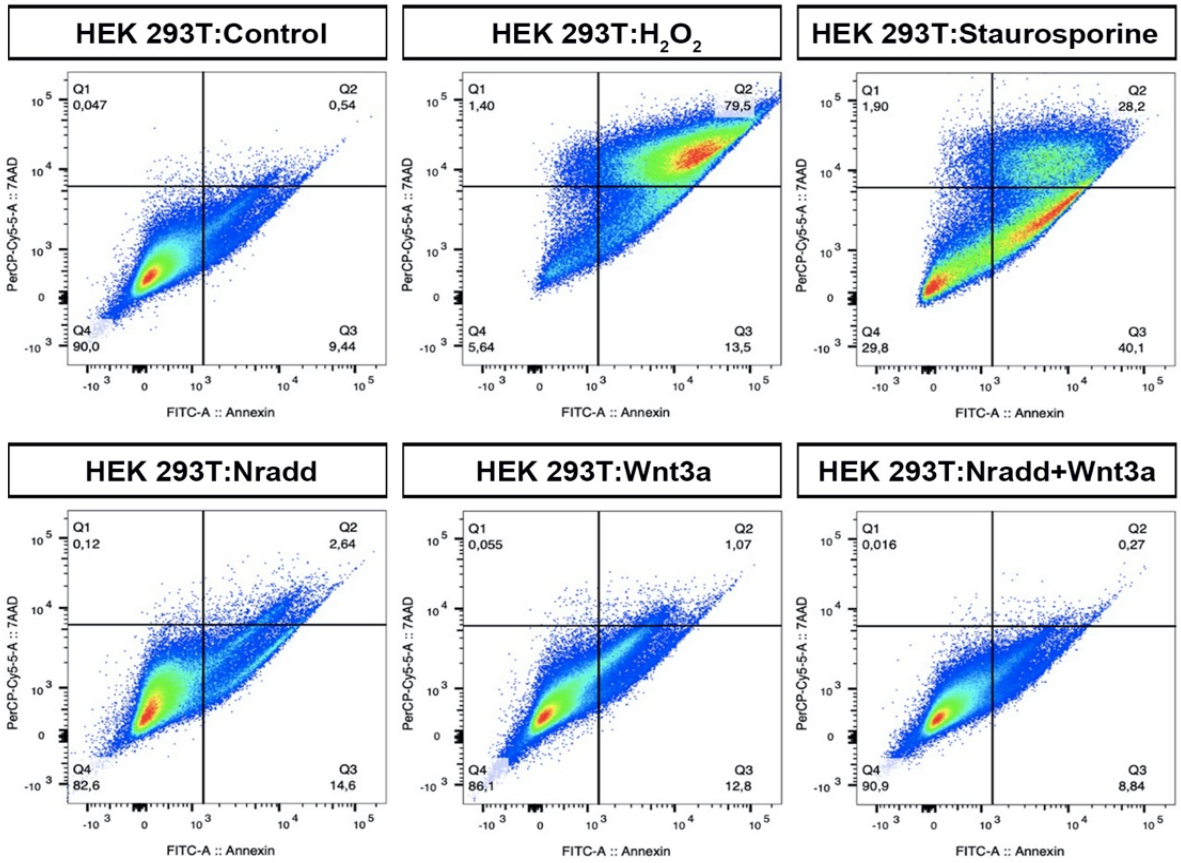



**Figure 29:** Analysis of apoptosis in HEK293T cells using flow cytometry after staining with Annexin V-FITC and 7AAD. Representative scatter plots between 7AAD (y-axis) and annexin V-FITC (x-axis).

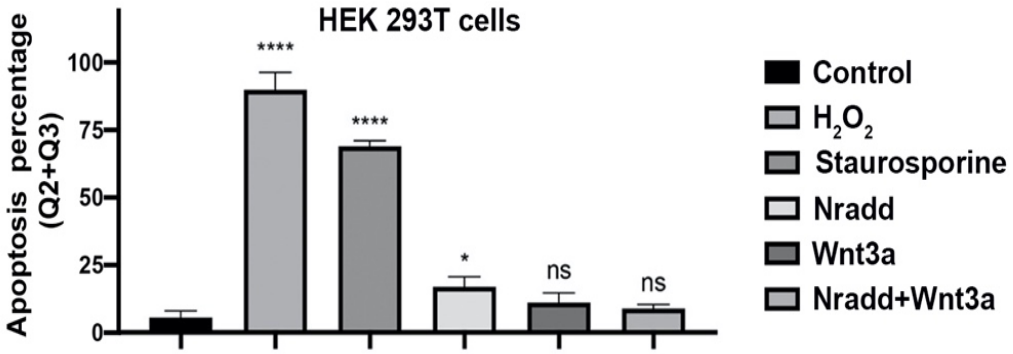



**Figure 30:** Percentage of apoptotic cells in HEK293T cells. * indicates p <0.05, **** p <0.0001 and ns is not significant. All experiments were performed in five replicates. Three independent experiments were conducted.

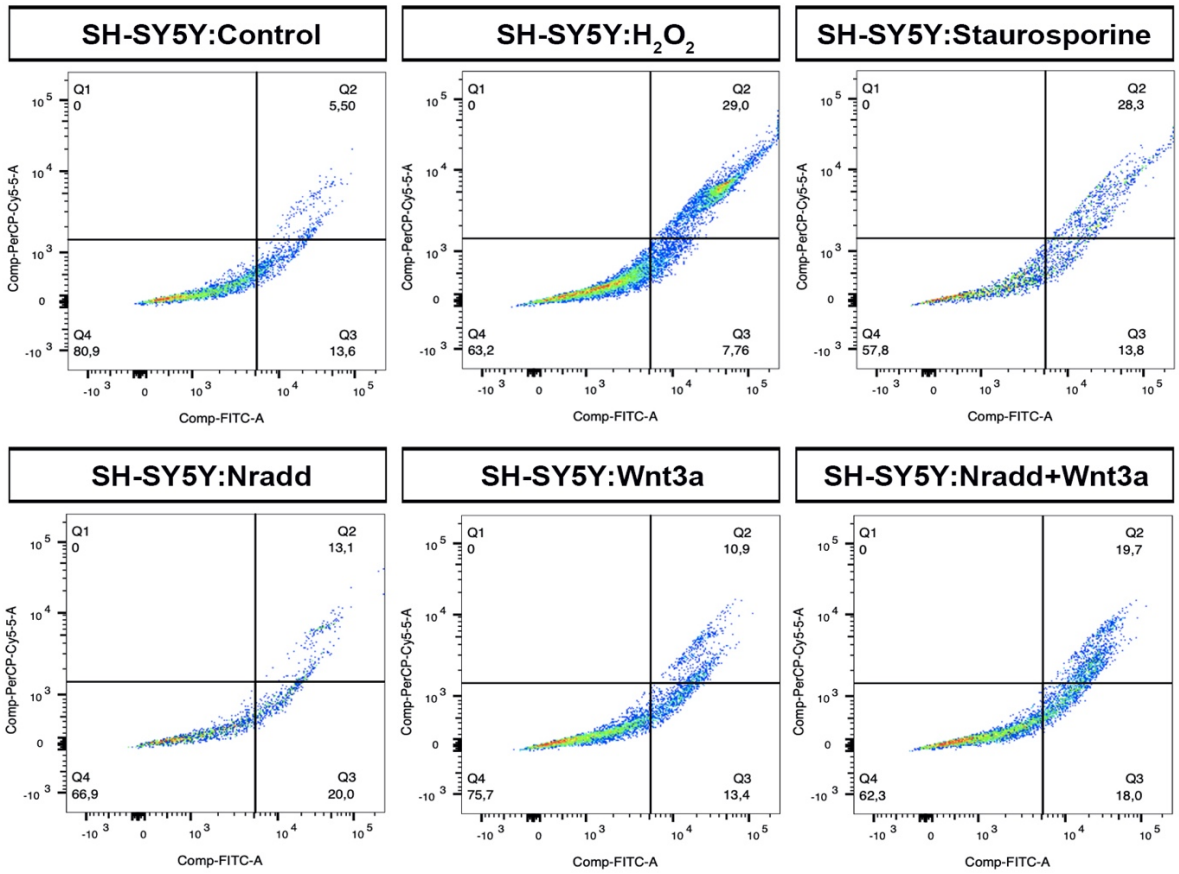



**Figure 31:** Analysis of apoptosis in SH-SY5Y cells using flow cytometry after staining with Annexin V-FITC and 7AAD. Representative scatter plots between 7AAD (y-axis) and annexin V-FITC (x-axis).

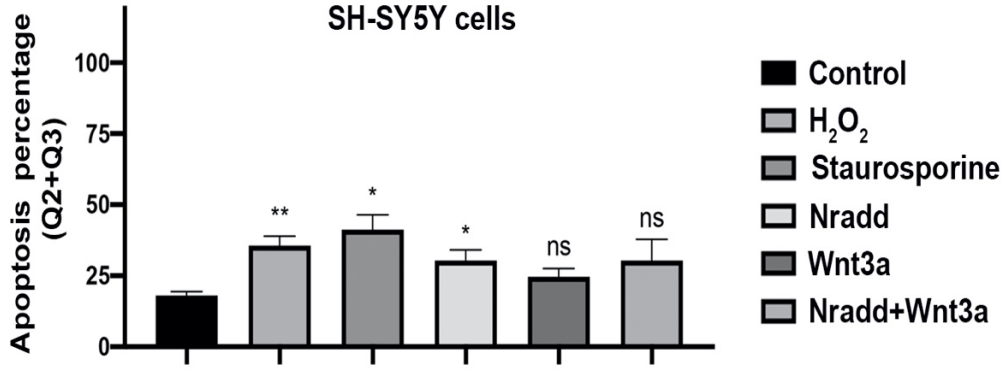



**Figure 32:** Percentage of apoptotic cells in SH-SY5Y cells. * indicates p <0.05, **** p <0.0001 and ns is not significant. All experiments were performed in five replicates. Three independent experiments were conducted.

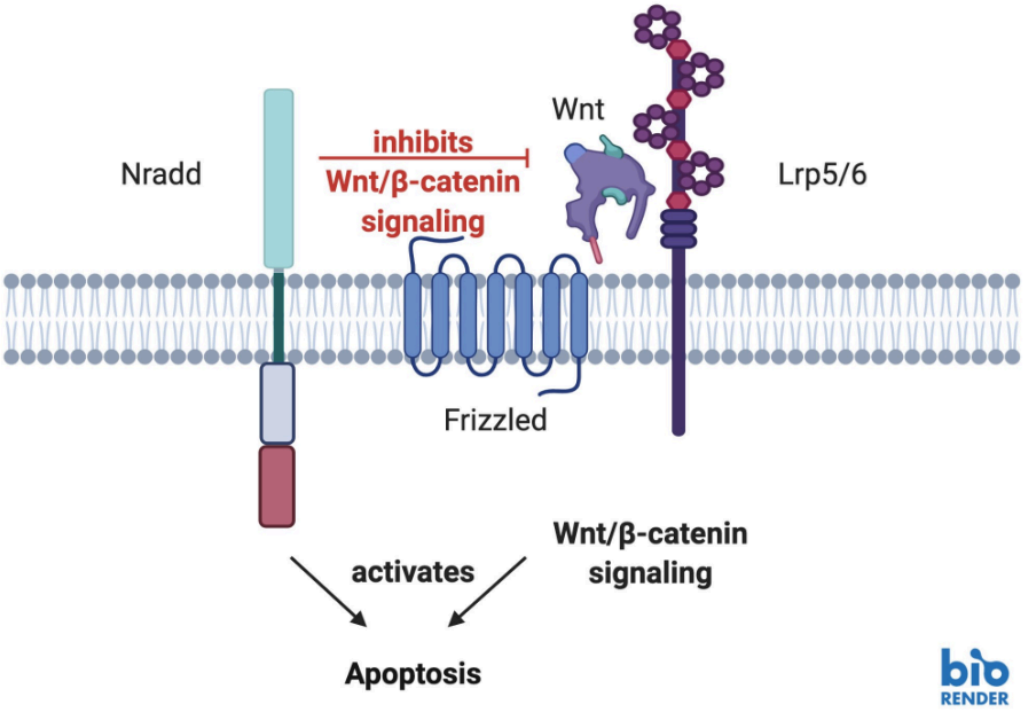



**Figure 33:** The dual role of Nradd in Wnt/β-catenin signaling to promote apoptosis during development (Created with BioRender.com)

Based on our cellular and molecular data, we describe a novel dual role of Nradd in Wnt/β-catenin signaling to enhance apoptosis during development (Figure 33).

## 5. DISCUSSION

The role of the Wnt/β-catenin signaling pathway has been underlined in crucial biological processes including homeostasis, embryonic development and cell death. (Steinhart and Angers, 2018; Klaus and Birchmeier, 2008; Cabello et al., 2010). Therefore, regulation of this pathway is of great importance in the proper accomplishment of cellular functions. The proper regulation of the Wnt/β-catenin signaling relies on the control of several modulators in the pathway (Seldin et al., 2005; Jho et al., 2002; Ueno et al., 2011; Kagermeier-Schenk et al., 2011; Özhan et al., 2013).

Signaling pathways modulated by trophic factors are responsible for the accurate regulation of neuronal survival (Tovar-y-Romo et al., 2014; Tovar-y-Romo et al., 2016). In addition, neuronal cell death mechanisms display a well-established removal process of damaged cells to maintain the durability of the nervous system (Cavallaro, 2015; Dekkers and Barde, 2013). The death receptor pathway, which is also called as extrinsic apoptosis, constitutes a crucial cell death mechanism type by recruiting extracellular ligands (Fricker et al., 2018; Kumar, 2007).

The apoptotic role of $p75^{NTR}$ has been implicated in several vital functions including the activation of cell death via various signaling pathways (Chao, 2003; Kraemer et al., 2014; Pathak and Carter, 2017; Bamji et al., 1998). Previous studies have shown that Nradd is able to induce apoptosis via its intracellular death domain (Wang et al., 2003; Frankowski et al., 2002). However, it is the first time we have unraveled that Nradd, a $p75^{NTR}$ homolog, can trigger cell death synergistically with the Wnt-β-catenin signalling pathway (Ozalp et al., 2021).

Wnt signaling acts as a mediator of the early and late stages of apoptosis during the development of several organs, including the brain (Ellies et al., 2000; Grotewold et al., 2002; Pećina-Šlaus et al., 2010). Depending on the molecular context in the cell, the role of Wnt signaling might be activatory or inhibitory in apoptosis process during the development of nervous system (Yeo and Gautier, 2004; Das et al., 2006; Masuda and Ishitani, 2017). Moreover, the association between Wnt signaling and neurotrophin signaling in neuronal development has been reported previously (Hiester et al., 2013;

Tsai et al., 2014). A relevant study showed that Wnt/β-catenin signaling pathway is able to modulate the activity of the death receptors DR6 and TROY, which are involved in blood–brain barrier development (Tam et al., 2012). Based on our results, to investigate what kind of cells are programmed to death after Nradd overexpression would improve our knowledge about the apoptotic potential of Nradd during brain development in zebrafish.

Many studies have exhibited that $p75^{NTR}$ has important domains to activate apoptotic signaling. For instance, the cytoplasmic part of $p75^{NTR}$ includes a functional death domain to induce neuronal apoptosis (Lin et al., 2015; Yuan et al., 2019; Vilar et al., 2009). Moreover, an elegant study has unraveled that an additional modification such as N-glycosylation is also involved in the elevation of apoptosis (Wang et al., 2003).

Our results has revealed that these regions containing N-glycosylated extracellular domain and intracellular death domain are essential for inhibition of Wnt/β-catenin signaling and activation of apoptosis. Therefore, we propose that these important domains might be useful for further manipulations of novel interactions in apoptotic pathways.

Previous studies imply that mouse Nradd is able to trigger apoptosis in neuronal cells and neuroblastoma cell lines (Wang et al., 2003; Frankowski et al., 2002; Kim and Hempstead, 2009). Interestingly, we indicate that zebrafish Nradd, which is a mutated as a pseudogene in human, can activate early apoptosis particularly in healthy and cancerous human cell lines. Therefore, we consider that Nradd might possess an dual role conserved during the evolution in the scope of the regulation of Wnt signaling and apoptosis. Furthermore, we speculate that human $p75^{NTR}$ homologs might replace for the Nradd in humans. However, we determined that Nradd can not lead to a significant increase of apoptosis rate in human cells along with Wnt induction, in contradiction to their synergistic apoptotic role in development.

Importantly, Wnt signaling might stimulate modulations in oncogenesis and embryogenesis in a contrary manner (Peifer and Polakis, 2000). For example; the elevation of Wnt/β-catenin signaling might contribute to apoptosis in human melanoma cells by inducing the TNF-related apoptosis-inducing ligand (Zimmerman et al, 2013).

In contrast, another study has shown that Dkk3 acts as a proapoptotic besides being a Wnt inhibitor in human ovarian cancer cells. (Takata et al., 2015). Moreover, various inhibitors of NGF and NGF receptors have been reported to raise β-catenin expression in ovarian cancer (Li et al., 2016). Hence, we suggest that the results of interaction between Nradd and Wnt signaling in the scope of apoptosis depends on the different cellular responses.

As a result, many studies have revealed that targeting the Wnt signaling could provide new treatment approaches for neurological disorders (McCord et al., 2017; Palomer et al., 2019; Berwick and Harvey, 2012; Xie et al., 2014; Fuenzalida et al., 2016; Yu et al., 2013; Gao et al., 2019). In addition, the dysregulation of pseudogene expression has been associated with different types of cancer (Hayashi et al., 2015; Lou et al., 2019; Chan et al., 2018). Therefore, we suggest that Nradd might have a possibility to be evalueated in targeted therapy in Wnt-related neurodegenerative diseases.

## 6. CONCLUSION AND FUTURE DIRECTIONS

The comprehensive results of this thesis reveal that *nradd* acts as a novel Wnt target during development and a negative feedback regulator of the Wnt/β-catenin signaling pathway in zebrafish embryos, as well as mammalian cells. Furthermore, Nradd functions along with Wnt/β-catenin signaling to enhance apoptosis by physically interacting with the Wnt signaling complex at the plasma membrane during in embryonic development of zebrafish. In addition, *nradd* overexpression triggers the apoptosis rate in neuroblastoma cells, besides healthy human embryonic cells. As a result, we propose that Nradd may become as a promising target for the development of new therapeutics for treatment of Wnt-related disorders, since it is able to modulate Wnt signaling and cell death.

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T.C.
DOKUZ EYLÜL ÜNİVERSİTESİ
İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ
HAYVAN DENEYLERİ YEREL ETİK KURULU (İBG-HADYEK)
KARARI

İBG-Enstitü

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| TOPLANTI TARİHİ | 26/07/2017 | TOPLANTI GÜNÜ | Çarşamba |
| TOPLANTI SAYISI | 11 | TOPLANTI SAATİ | 15.00 |

**Sayın Doç.Dr. Güneş ÖZHAN,**

**12/2017 Protokol No'lu;** yürütücüsü olduğunuz "Nörotrofin reseptörü ile ilişkili ölüm bölgesi proteininin Wnt/ß-katenin sinyal iletiminin düzenlenmesindeki rolünün zebrabalığı modelinde araştırılması" başlıklı projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.



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## Scientific Technological Activity Fields

**Keywords**

Beyin Kanseri

Zebra Balığı

Wnt Sinyal Yolağı

Glioblastoma

Beyin Türevli Nörotrofik Faktör

Programlı Hücre Ölümü

Embriyonik Kök Hücre

Kanser Kök Hücreleri

İndüklenmiş Pluripotent Kök Hücreler

## R&D Competency

**Articles**

O. OZALP, O. CARK, Y. AZBAZDAR, B. HAYKİR, G. CUCUN, I. KUCUKAYLAK, G. ALKAN-YESİLYURT, E. SEZGİN & G. OZHAN, Nradd Acts as a Negative Feedback Regulator of Wnt/beta-Catenin Signaling and Promotes Apoptosis, BIOMOLECULES, 2021, 2218-273X, 11, 1.

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Ö. ÖZALP, Ö. ÇARK, A. Y. AZBAZDAR, B. HAYKIR & H. G. ÖZHAN, Investigation of the role of a novel death domain protein in regulation of Wnt/ß-catenin signaling by using the zebrafish model, Poster Sunumu, 6th International Congress of the Molecular Biology Association of Turkey, 05 September 2018, 08 September 2018.

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**Awards**

Üniversite, Kurum veya Kuruluşların Verdiği Ödüller, Uluslararası, EMBO Short-Term Fellowship, Kısa Süreli Araştırma Projesi Bursu , Awarded Institution: European Molecular Biology Organization (Sistemde kayıtlı olmayan kuruluş), ALMANYA, 10 March 2020.

Üniversite, Kurum veya Kuruluşların Verdiği Ödüller, Uluslararası, EMBL Corporate Partnership Programme Registration Fee Waiver, Uygulamalı Kursa Katılım Bursu , Awarded Institution: European Molecular Biology Organization (Sistemde kayıtlı olmayan kuruluş), ALMANYA, 20 November 2018.

Üniversite, Kurum veya Kuruluşların Verdiği Ödüller, Ulusal, Gönül Külahçıoğlu Kanser Araştırma Bursu , 41. FEBS Kongresi için Katılım Bursu , Awarded Institution: EGE ÜNİVERSİTESİ, TÜRKİYE, 03 September 2016.

Üniversite, Kurum veya Kuruluşların Verdiği Ödüller, Uluslararası, Travel Fellowship , EMBO Conference on Cell Therapy Today: Achievements, hopes and hypes, Awarded Institution: European Molecular Biology Organization (Sistemde kayıtlı olmayan kuruluş), ALMANYA, 09 September 2015.

Üniversite, Kurum veya Kuruluşların Verdiği Ödüller, Uluslararası, Erasmus Internship Mobility Programme Fellowship, Erasmus Projesi Bursu , Awarded Institution: Avrupa Birliği Erasmus Programı (Sistemde kayıtlı olmayan kuruluş), AVUSTURYA, 02 July 2012.

## TÜBİTAK Scholarships and Fundings

**Project Info**

217Z123, Nörotrofin Reseptörü ile İlişkili Ölüm Bölgesi Proteininin Wnt/ß-Katenin Sinyal Iletiminin Düzenlenmesindeki Rolünün Zebrabalığı Modelinde Araştırılması, 1001 - Araştırma, Burslu, Sonuçlandı, ARDEB, KBAG - Kimya Biyoloji Araştırma Destek Grubu, Project Participation/Leave Dates: 6/1/18 - 6/1/21, Project Start/End Dates: 6/1/18 - 6/1/21.
219Z040, Melanosit Rejenerasyonunun ve Melanomun Moleküler Mekanizmalarının Zebrabalığı Modelinde Karşılaştırmalı Analizi ve Melanom Hücrelerinde Gen İfadesi Profillerinin CRISPR/Cas9 Yöntemi ile Düzenlenerek Rejenerasyon Benzeri Bir İlerleyişin İndüklenmesi, 1001 - Araştırma, Burslu, Yürürlükte, ARDEB, KBAG - Kimya Biyoloji Araştırma Destek Grubu, Project Participation/Leave Dates: 6/1/21 - 9/1/21, Project Start/End Dates: 7/15/20 - 7/15/23.
219Z040, Melanosit Rejenerasyonunun ve Melanomun Moleküler Mekanizmalarının Zebrabalığı Modelinde Karşılaştırmalı Analizi ve Melanom Hücrelerinde Gen İfadesi Profillerinin CRISPR/Cas9 Yöntemi ile Düzenlenerek Rejenerasyon Benzeri Bir İlerleyişin İndüklenmesi, 1001 - Araştırma, Burslu, Yürürlükte, ARDEB, KBAG - Kimya Biyoloji Araştırma Destek Grubu, Project Participation/Leave Dates: 9/1/21 - 5/1/22, Project Start/End Dates: 7/15/20 - 7/15/23.

**Number of Panelist/Observer/Reporter Jobs**

| | | | |
|---|---|---|---|
| Refereeing/Panelist/Outer Consultant Count | ARDEB/BİDEB 0 | TEYDEB 0 | Total 0 |
| Number of Observer/Consultant Jobs | ARDEB/BİDEB 0 | TEYDEB 0 | Total 0 |
| Number of Reporter Jobs | ARDEB/BİDEB 0 | TEYDEB 0 | Total 0 |

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*Article*

# Nradd Acts as a Negative Feedback Regulator of Wnt/β-Catenin Signaling and Promotes Apoptosis

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**Abstract:** Wnt/β-catenin signaling controls many biological processes for the generation and sustainability of proper tissue size, organization and function during development and homeostasis. Consequently, mutations in the Wnt pathway components and modulators cause diseases, including genetic disorders and cancers. Targeted treatment of pathway-associated diseases entails detailed understanding of the regulatory mechanisms that fine-tune Wnt signaling. Here, we identify the neurotrophin receptor-associated death domain (Nradd), a homolog of p75 neurotrophin receptor ($p75^{NTR}$), as a negative regulator of Wnt/β-catenin signaling in zebrafish embryos and in mammalian cells. Nradd significantly suppresses Wnt8-mediated patterning of the mesoderm and neuroectoderm during zebrafish gastrulation. Nradd is localized at the plasma membrane, physically interacts with the Wnt receptor complex and enhances apoptosis in cooperation with Wnt/β-catenin signaling. Our functional analyses indicate that the N-glycosylated N-terminus and the death domain-containing C-terminus regions are necessary for both the inhibition of Wnt signaling and apoptosis. Finally, Nradd can induce apoptosis in mammalian cells. Thus, Nradd regulates cell death as a modifier of Wnt/β-catenin signaling during development.

**Keywords:** Nradd; p75 neurotrophin receptor; Wnt/β-catenin signaling; apoptosis; death receptor

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## 1. Introduction

Wnt/β-catenin signaling is an evolutionarily conserved signaling pathway that controls a plethora of cellular processes including proliferation, migration, apoptosis, cell fate determination and tissue patterning during development and adult homeostasis [1–3]. Due to these essential roles, misregulation of the signaling pathway has been associated with various human diseases, including cancer [1,4–7]. Pathway misregulation in cancer is triggered via either mutational alterations, such as gain-of-function mutations in β-catenin and Tcf transcription factors and loss-of-function mutations in the β-catenin destruction complex components, or nonmutational alterations such as silencing of extracellular Wnt antagonists through epigenetic mechanisms [8–11].

Wnt/β-catenin signaling is thought to operate with its core components as follows: If there exist no active Wnt ligands in the environment, the so-called Wnt-off state, the

β-catenin destruction complex containing Axin, adenomatous polyposis coli (Apc), glycogen synthase kinase 3 (Gsk3) and casein kinase 1 (Ck1) bind to β-catenin and leads to its proteasomal degradation after phosphorylation [12,13]. The cell switches to the Wnt-on state when the Wnt ligand triggers the formation of a complex between itself, the receptor Frizzled (Fz) and the co-receptor low-density lipoprotein receptor-related protein (Lrp) 5/6 at the plasma membrane. This interaction appears to recruit cytoplasmic proteins Dishevelled and Axin to the (co)receptors at the membrane. Further receptor clustering in turn causes Lrp6 phosphorylation and subsequent internalization of the receptor complex by endocytosis [14,15]. The removal of Axin from the destruction complex, direct inhibition of Gsk3 kinase activity by the phosphorylated cytoplasmic portion of Lrp6 and sequestration of Gsk3 from the cytoplasm into multivesicular bodies are collectively assumed to prevent the degradation of β-catenin [16–18]. β-catenin then enters the nucleus and interacts with the transcription factors of the T cell factor (Tcf)/lymphoid enhancer factor (Lef) family to regulate the expression of its target genes [19].

Due to the deleterious consequences of aberrant signal transduction, it is not surprising that Wnt/β-catenin signaling is controlled by a large number of positive and negative regulators. Tight regulation of the pathway at the plasma membrane is achieved by a complex network of extracellular or membrane-bound modulators [14,20,21]. Many of these pathway modulators, including Axin2, Dkk1, Waif1/5T4 and Lypd6, are also Wnt targets and act in feedback regulation, a key feature of Wnt pathway regulation [22–26]. Since these modulators are usually not required for vital cellular activities and are highly specialized for the signaling pathway, they constitute a worthwhile option for targeted therapeutic interventions that can dampen abnormal Wnt pathway activity.

Here, we identify the *neurotrophin receptor-associated death domain* (*nradd*) gene, encoding for a single-pass transmembrane protein with a C-terminal death domain, as a transcriptional target of Wnt/β-catenin signaling during the embryonic development of zebrafish. Nradd exhibits a high degree of homology with the p75 neurotrophin receptor ($p75^{NTR}$), which has been characterized as a receptor for members of the neurotrophin family [27,28]. The neurotrophins are a family of growth factors that are essential for growth, differentiation, survival, apoptosis and regeneration of the neurons in vertebrates [29,30]. There are four types of neurotrophins in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin 4 (NT-4) [31]. Neurotrophins bind to and signal through two main types of receptors: tropomyosin receptor kinase (Trk) family receptors, also known as neurotrophic tyrosine kinase receptors, and $p75^{NTR}$, a member of the tumor necrosis factor receptor superfamily (TNFRSF) [32–34]. Being produced as larger precursors called proneurotrophins that turn into mature forms via proteolytic cleavage, neurotrophins exhibit distinct binding affinities for their receptors [29,35]. Proneurotrophins, for example, show high affinity to a complex composed of $p75^{NTR}$ and sortilin [36,37].

Neurotrophins induce neuronal survival or cell death by activating Trk receptors and $p75^{NTR}$, respectively [38]. The binding of mature neurotrophins with Trk results in the activation of phosphoinositide 3-kinase (PI3K), phospholipase Cγ1 (PLCγ1) and mitogen-activated protein kinase (MAPK) pathways and promotes cell growth, differentiation and survival [39]. On the other hand, mature neurotrophins that bind to $p75^{NTR}$ can enhance neurotrophin binding to Trk receptors and Trk signal transduction through protein kinase B (PKB or AKT) and MAPKs, depending on the circumstances. They can either enhance survival by the nuclear factor-κB (NF-κB) pathway or antagonize the action of Trk through the activation of the JUN N-terminal kinase (JNK) and Rhoa pathways [39–44]. This ultimately generates a paradoxical paradigm that harbors a matter of life and death depending on Trk receptor and $p75^{NTR}$ signaling [45]. Mouse NRADD has been found to induce apoptosis in neuroblastoma cell lines through the activation of caspase 8 and independently of the mitochondrial pathway, or so-called intrinsic apoptosis [27]. However, little is known about its mechanism of action and potential interaction with a signaling pathway.

We describe Nradd as a feedback inhibitor of Wnt/β-catenin signaling during zebrafish development and in mammalian cells. Nradd localizes to the plasma membrane and interacts with the Fz8 and Lrp6 receptors. Our functional analyses show that Nradd acts together with Wnt/β-catenin signaling to promote apoptosis during development and that both the death domain and N-glycosylated N-terminus are essential for its apoptotic function. Finally, Nradd can also enhance apoptosis in human embryonic kidney and neuroblastoma cell lines. Overall, Nradd acts as a negative modulator of Wnt/β-catenin signaling and a key player in the regulation of apoptosis during development.

## 2. Materials and Methods

### *2.1. Transgenic Fish Lines*

Transgenic zebrafish (*Danio rerio*) lines Tg(hsp70l:wnt8a-GFP)$^{w34}$ and Tg(hsp70l:Mmu.Axin1-YFP)$^{w35}$ were outcrossed to wild type (wt) AB zebrafish [22,46]. The embryos were heat shocked and identified as described previously [22]. The Tg(7XTcf-Xla.Siam:nlsmCherry)$^{ia}$ transgenic line was used as a reporter of Wnt/β-catenin signaling, outcrossed to wt zebrafish and embryos were sorted as described previously [47]. 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.

### *2.2. Cloning*

RNA was isolated from whole embryos at 24 h post-fertilization (hpf) using a Direct-Zol RNA kit (Zymo Research, Irvine, CA, USA) and cDNA was synthesized with an iScript reverse transcription kit (Biorad, Hercules, CA, USA) using a 1:1 mixture of oligo (dT) and random primers. iScript reverse transcriptase (RT) was replaced with water for (-RT) controls. The following products were amplified with the corresponding primer pairs using 1 µL of cDNA. Zebrafish wt Nradd-EGFP: forward primer 5′-AAAAAGGATCCCCACCATGAAAGGAGCAACTGAAGC-3′ and reverse primer 5′-AAAAAGAATTCGACCACTGATACTCCTTGCG-3′; Nradd death domain deleted (DDD)-EGFP: forward primer 5′-AAAAAGGATCCCCACCATGAAAGGAGCAACTGAAGC-3′ and reverse primer 5′-AAAGAATTCATCCTGTTTGCTGTCCCGTTTA-3′. Both PCR products were digested with BamHI and EcoRI and ligated into a pCS2P+ vector that has EGFP. The Nradd DDD construct was generated through the deletion of a 95 amino acid region between the 318$^{th}$ and 413$^{th}$ amino acids at the C-terminal region. Nradd glycosylation site deleted (GSD)-EGFP was generated by site-directed mutagenesis at wt Nradd-GFP using overlap extension PCR. The Nradd GSD construct was generated through the deletion of a 101 amino acid region between the 22nd and 123rd amino acids at the N-terminal region. The first round of PCR was conducted with the two primer pairs forward1 5′-AAAAAGGATCCCCACCATGAAAGGAGCAACTGAAGC-3′, reverse1 5′-ACACTCCCCATCCTGGCCCACAGCCAAAGGCCATCTT-3′ and forward2 5′-AAGATGGCCTTTGGCTGTGGGCCAGGATGGGGAGTGT-3′, reverse2 5′-AAAAAGAATTCGACCACTGATACTCCTTGCG-3′. The second round of PCR was performed by using the purified PCR product of the first round as the template and the primers forward1 and reverse2. The purified PCR product of the second round of PCR was again digested with BamHI and EcoRI and ligated into a pCS2P+ vector that has EGFP. For Fz8a-EGFP, zebrafish Fz8a was amplified with the forward primer 5′-AGAATTCAACCACCATGGAGTGCTACCT-3′ and the reverse primer 5′- GGATCCTCAGACTTGGGACAAAGGC-3′. The PCR product was digested with EcoRI and BamHI and ligated into a pCS2P+ vector that has EGFP. For Fz8a-mRuby3, mRuby was amplified with the forward primer 5′-AAGGCGCGCCTATGGTGTCTAAGG-3′ and the reverse primer 5′- AATCTAGATTACTTGTACAGCTCGTCCATGCCAC-3′ from the mRuby3 plasmid. The PCR product was digested with AscI and XbaI and ligated into an Fzd8a-EGFP plasmid after excising EGFP with the same restriction enzymes. For Nradd-mRuby3, mRuby3 was amplified with the forward primer 5′-AAATCTAGAATGGTGTCTAAGGGCGAAGA-3′ and the reverse primer 5′- AAAGATATC

TTACTTGTACAGCTCGTCCAT-3′ from the mRuby3 plasmid. The PCR product was digested with XbaI and BsaBI ligated into a wt Nradd-EFGP plasmid after excising EGFP with the same restriction enzymes. Successful cloning was verified by sequencing, restriction digestion and agarose gel electrophoresis.

### *2.3. Capped Sense mRNA Synthesis, Microinjection and Whole-Mount in situ Hybridization (WMISH)*

Capped sense RNAs of GFP, wt Nradd, Nradd-GFP, Nradd DDD-EGFP and Nradd GSD-EGFP were synthesized using an mMessage mMachine Kit (Thermo Fisher Scientific, Waltham, MA, USA). mRNAs were injected into 1-cell zebrafish embryos as 250 pg for *nradd* and 20 pg for *Wnt8*. Embryos were fixed at indicated stages (30% epiboly, 50% epiboly, 60% epiboly, 80% epiboly, 100% epiboly, 3-somite, 5-somite, 10-somite or 24 hpf) in 4% paraformaldehyde (PFA) overnight. WMISH was performed with *nradd*, *mCherry*, *goosecoid, otx2, hoxb1b, foxg1a, her5* and *krox20* antisense RNA probes as described previously [48].

### *2.4. Quantitative PCR (qPCR)*

cDNA was synthesized from RNA using a ProtoScript II First Strand cDNA Synthesis Kit (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's instructions. Zebrafish *rpl13a* or *β-actin* were used as the reference genes for normalization to determine the relative gene expression levels. qPCR was performed in triplicate using GoTaq qPCR Master Mix (Promega, Madison, WI, USA) in an Applied Biosystems 7500 Fast Real Time PCR machine (Foster City, CA, USA). The data were analyzed using GraphPad Prism 8 software (Graphpad Software Inc., San Diego, CA, USA). The values are mean ± standard deviation (SD) of three samples. The following primers were used: *mCherry* forward 5′-GAACGGCCACGAGTTCGAGA-3′ and reverse 5′-CTTGGAGCCGTACATGAACTGAGG-3′, zebrafish *sp5l* forward 5′-GCTTCACGCAGGTGTGGAT-3′ and reverse 5′-TTCTGGAGA TGAGCTGGGAGT-3′, zebrafish *axin2* forward 5′-TAGTTTTGCCCCTGCCACG-3′ and reverse 5′-TCCCAGCTTGTAAGGAGGAATG-3′, zebrafish *cdx4* forward 5′-CCAGAGAAAA TCAGAGCTGGCA-3′ and reverse 5′-TTGCACCGAGCCTCCACTATT-3′, zebrafish *nradd* forward 5′-AAGTGTCAGCCATGCCAAGA-3′ and reverse 5′-AGGAATACCGATGGGGC AATG-3′, zebrafish *rpl13a* forward 5′-TCTGGAGGACTGTAAGAGGTATGC-3′ and reverse 5′-AGACGCACAATCTTGAGAGCAG-3′, zebrafish *β-actin* forward 5′-GAAGGAGATCAC CTCTCTTGCTC-3′ and reverse 5′-GTTCTGTTTAGAAGCACTTCCTGTG-3′.

### *2.5. Cell Culture*

Human embryonic kidney 293T (HEK293T), neuroblastoma SH-SY5Y and bone osteosarcoma U2OS cell lines were purchased from ATCC (Manassas, VA, USA). HEK293T and U2OS cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) and SH-SY5Y cells in DMEM F-12 at 37 °C in a 5% (v/v) $CO_2$ humidified environment. All media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin.

### *2.6. Transfection and Luciferase Assay*

HEK293T were seeded on 24-well plates and transfected in triplicate with 20 ng of Wnt8, with and without 300 ng of Nradd. SH-SY5Y cells were seeded on 24-well plates and transfected in triplicate with 250 ng of Nradd and stimulated with Wnt3a conditioned media (CM) for 6h. All cells were co-transfected with 20 ng of firefly luciferase reporter pGL3 BAR [49] and 5 ng of renilla luciferase reporter pGL4.73 hRLuc/SV40 (Promega, Madison, WI, USA) using Fugene HD Transfection Reagent (1 µg/1 µL, Promega, Madison, WI, USA). Twenty-four hours after transfection, reporter activity was measured with the dual luciferase reporter assay kit (Promega, Madison, WI, USA) in a Varioskan Flash multimode reader (Thermo Fisher Scientific, Waltham, MA, USA). Statistical analysis was performed using a Student's *t*-test. Error bars represent SD, where * and ** indicate $p < 0.05$ and $p < 0.01$, respectively.

### *2.7. Subcellular Localization*

Zebrafish embryos were injected with 250 pg Nradd-EGFP and 250 pg RFP-GPI at the 1-cell stage, dechorionated at 3 hpf, mounted in 3% methylcellulose, and imaged using a Zeiss LSM 880 confocal microscope (Carl Zeiss AG, Oberkochen, Germany). U2OS cells were seeded in a 6-well plate on a 35 mm glass-bottom dish with a 10 mm micro-well #1.5 cover glass (0.16–0.19 mm) and transfected with Fz8a-EGFP and Nradd-mRuby3 plasmids (500 ng each) using Lipofectamine 3000 reagent at 24 h. The next day, cell membranes were stained with CellMask Deep Red at a concentration of 5 µg/mL for 5 min. The cells were imaged in Leibovitz's L-15 medium without phenol red using a Zeiss LSM 780 confocal microscope.

### *2.8. Isolation of Giant Plasma Membrane Vesicles (GPMVs)*

U2OS cells were seeded in a 6-well plate on a 35 mm glass-bottom dish with a 10 mm micro-well #1.5 cover glass and transfected with 500 ng Nradd-EGFP using Lipofectamine 3000 reagent at 48 h. At ~70% confluence, the cells were washed twice with GPMV buffer (10 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4) and incubated for 1 h at 37 °C with GPMV buffer including 2 mM *N*-ethyl maleimide (NEM). After 1 h, the supernatant containing vesicles was collected, incubated with Atto647N-labeled 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (Atto647N-PE) for membrane staining and imaged using a Zeiss LSM 780 confocal microscope.

### *2.9. Co-Immunoprecipitation and Western Blotting*

HEK293T cells were seeded in 6-well plates and transfected with 500 ng Nradd-GFP and 500 ng LRP6-HA. At 48 h, Wnt induction was performed for 6h and cells were washed with ice-cold PBS and lysed with NOP buffer (10 mM HEPES KOH pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP40 (Igepal CA-630, Sigma-Aldrich, St. Louis, MO, USA). Lysate was centrifuged at 300 g for 5 min at 4 °C. Supernatant was precipitated with a SureBeads™ Protein G Magnetic Beads Co-Immunoprecipitation Kit (BioRad, Hercules, CA, USA) according to the kit protocol. For western blotting, samples were dissolved in 5X loading dye and separated by SDS gel electrophoresis on a 10% acrylamide-bis acrylamide gel. Proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare Life Science, Chicago, IL, USA). Membranes were blocked in 5% milk powder for 45 min at room temperature (RT) and incubated with the following antibodies at the corresponding dilutions. Primary antibodies: rabbit anti-GFP ((D5.1) XP, 1:1000; Cell Signaling Technology, Danvers, MA, USA) and mouse anti-HA (1: 2000; OriGene Technologies, Rockville, MD, USA). Secondary antibodies: anti-rabbit IgG, horseradish peroxidase (HRP)-linked and anti-mouse IgG, HRP-linked (both 1:2500; Cell Signaling Technology, Danvers, MA, USA).

### *2.10. Fluorescence Cross-Correlation Spectroscopy (FCCS)*

The molecular interaction between Nradd protein and the components of the Wnt–receptor complex was examined by comparing the diffusion characters on the membrane using a fluorescence cross-correlation spectroscopy (FCCS) technique. FCCS measurements were performed on a Zeiss LSM 780 confocal microscope equipped with 63X oil immersion objective (1.4 NA). GFP- and mCherry-labeled proteins were excited with 488 and 561 nm lasers, respectively. Detection intervals of 500–550 nm and 600–690 nm were used to detect green and red signals, respectively. Curves were analyzed using the FoCuS-point software package [50]. Cross-correlation percentages were calculated by the amplitudes of autocorrelation and cross-correlation curves of molecules tagged with two different fluorophores at the plasma membrane. Calculations were performed by using the GNO values obtained from the FoCuS-point program. Among the values of GFP- and mCherry-labeled molecules, the one which was closer to the cross-correlation GNO value was considered for comparisons. This value was normalized using the positive control. The experiment was performed in U2OS cells that were seeded on 0.17 mm cover slides. The next day,

cells were transfected with 500 ng of each plasmid using Lipofectamine 3000 reagent. The diffusion rates were measured at 24 h. The following combinations of molecules were used: (1) Fz8a-EGFP + Lrp6-mCherry, (2) Fz8a-EGFP + Lrp6-mCherry + Wnt3a CM, (3) Fz8a-mRuby3 + Nradd-EGFP, (4) Fz8a-mRuby3 + Nradd-EGFP + Wnt3a CM, (5) Lrp6-mCherry + Nradd-EGFP, (6) Lrp6-mCherry + Nradd-EGFP + Wnt3a CM. All values were normalized to the positive control EGFP-mCherry. GPI-EGFP + Fzd8a-mRuby3 + Wnt CM was used as a negative control. Wnt3a CM was produced from murine L cells. Cells were grown in a 10 cm plate in DMEM supplemented with 10% FBS. At 90% confluence, cells were diluted 1:10 and seeded in new 10 cm plates. Wnt3a CM was collected at 2 days, 4 days and 6 days, and added to the cells 20 min prior to measurement.

### *2.11. Immunofluorescence Staining*

Zebrafish embryos produced by crossing wt AB and Tg(*hsp70l:*wnt8a-GFP)$^{w34}$ were injected with the capped sense RNAs of control GFP (100 pg), wt Nradd-GFP (250 pg), Nradd-DDD (250 pg) or Nradd-GSD (250 pg) at the 1-cell stage. Injected embryos were heat shocked at 60% epiboly, dechorionated at 24 hpf by adding 100 µL of 10 mg/mL pronase in 10 mL E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) and fixed in 4% PFA in PBST (1× PBS, 0.1% Tween-20) overnight at 4 °C. Embryos were incubated in ice-cold methanol at −20 °C for 24 h. Methanol was removed and embryos were washed twice with 1 mL 1× PDT (1× PBST, 0.3% Triton-X, 1% DMSO) for 30 min by rocking gently at RT. Next, embryos were incubated with 500 µL blocking buffer (1× PBST, 10% heat-inactivated fetal bovine serum, 2% bovine serum albumin) at RT for 1 h 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 1× PDT, incubated with rhodamine (TRITC)-AffiniPure donkey anti-rabbit IgG (1:400; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h at RT, re-washed with 1× PDT, mounted in 50% glycerol in PBS and imaged using a Zeiss LSM 880 confocal microscope.

### *2.12. Annexin V-FITC Apoptosis Assay*

HEK293T or SH-SY5Y cells were seeded in 6-well plates. For the control and Nradd, cells were transfected with firefly luciferase reporter pGL3 BAR (100 ng) and the plasmid containing Nradd without GFP tags (250 ng), respectively, according to the manufacturer's protocol. For the positive controls, cells were treated with 0.3 mM $H_2O_2$ and 1 µM staurosporine for 18 h. Relevant cells were treated with Wnt3a CM overnight to induce Wnt signaling. Twenty-four hours after transfection, supernatants and trypsinized cells were collected. Cells were washed and stained with annexin V-FITC and 7AAD using an FITC Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend, San Diego, CA, USA). Flow cytometry was performed using a BD Fortessa and data were analyzed with FlowJo 8.8.6 software (Tree Star Inc., Ashland, OR, USA). Experiments were performed in three biological replicates and data are representative of at least three independent experiments.

## 3. Results

### *3.1. Nradd is Transcriptionally Regulated by Wnt/β-Catenin Signaling during Development*

To identify new modulators of Wnt/β-catenin signaling, we exploited the fact that various pathway components are also transcriptional targets of the pathway. To identify Wnt targets, we conditionally activated or inhibited Wnt/β-catenin signaling by giving transgenic zebrafish embryos a single heat shock at three independent developmental stages, namely, gastrula, somitogenesis and organogenesis, and performed RNA sequencing. By screening for genes that are upregulated upon activation and downregulated upon inhibition, i.e., positively regulated by the pathway, during all three developmental stages, we identified neurotrophin receptor-associated death domain (*nradd*) that has no previously defined function in Wnt/β-catenin signaling and in vertebrate embryonic development. At late gastrula stages, *nradd* was expressed at the marginal zone and later became concen-

trated at the posterior sites of the embryo (Figure 1A). During organogenesis, its expression was downregulated in the posterior regions and observed at a weaker level throughout the body (Figure 1A). 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. In all three developmental stages, the inhibition of Wnt/β-catenin signaling significantly suppressed *nradd* expression, while its activation upregulated *nradd* (Figure 1A). To support these data quantitatively, we performed qPCR in the same conditions and found that the expression of both *nradd* and the direct Wnt/β-catenin target gene *sp5l* increased with activated Wnt signaling in hs:Wnt8 embryos and decreased with inhibited signaling in hs:Axin1 embryos at all developmental stages tested (Figure 1B–D). These results confirm that *nradd* is a transcriptional target of Wnt/β-catenin signaling and its expression is broadly regulated by the pathway during development.

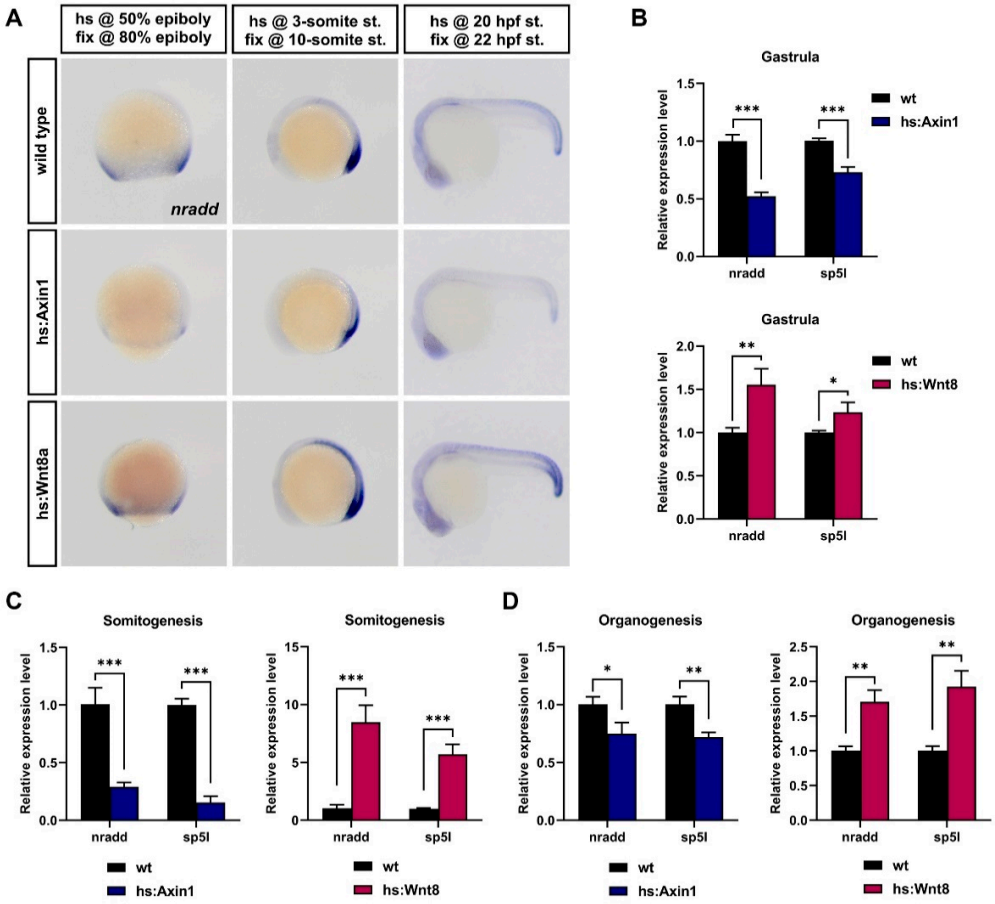



**Figure 1.** *nradd* is transcriptionally regulated by Wnt/β-catenin signaling during development. (**A**) Whole-mount in situ hybridization (WMISH) shows that *nradd* expression is downregulated in transgenic embryos expressing Axin1 (hs:Axin1) and upregulated in transgenic embryos expressing Wnt8a (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. (**B**–**D**) *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 ± standard deviation (SD, $n = 3$). Three independent experiments were conducted.

### *3.2. Nradd Acts as an Inhibitor of Wnt/β-Catenin Signaling*

To test whether *nradd* has an influence on Wnt/β-catenin signaling, we first cloned wild-type zebrafish *nradd* into a pCS2P+ expression vector with or without EGFP, termed Nradd and Nradd-EGFP, respectively. Next, we overexpressed *nradd* mRNA in a transgenic reporter of Tcf/Lef-mediated transcription, Tg(7XTcf-Xla.Siam:nlsmCherry)$^{ia}$ (7xTcf:mCherry). Reporter expression dramatically decreased in *nradd*-overexpressing embryos at late gastrula, somitogenesis and organogenesis stages (Figure 2A). Activation of Wnt8-mediated β-catenin signaling generates a range of phenotypes from mild neuroectodermal posteriorization to loss of forebrain and notochord in zebrafish embryos (classes 2 to 4, Figure 2B) [23,51]. To test whether *nradd* overexpression can rescue these phenotypes, we activated Wnt/β-catenin signaling in hs:Wnt8 transgenic embryos by giving a heat shock at early gastrula (shield stage), which generated class 3 and 4 phenotypes at 24 hpf (Figure 2C). *nradd* overexpression indeed significantly restored the phenotypes to class 2 and 3 (Figure 2C). Moreover, *nradd* significantly downregulated the expression of *axin2*, *cdx4* and *sp5l*, three direct Wnt/β-catenin target genes in these embryos [25,46,52]. Strikingly, *nradd* overexpression was also able to reduce the expression of these genes in wild-type embryos (Figure 2D–F). Finally, zebrafish *nradd* efficiently suppressed Wnt/β-catenin signaling in HEK293T and SH-SY5Y cells, as evidenced by the activation of the firefly luciferase pBAR reporter of Tcf/Lef-mediated transcription (Figure 2G–H) [49]. These results strongly suggest that *nradd* acts as a negative feedback regulator of Wnt/β-catenin signaling during zebrafish development and can suppress Wnt signaling in mammalian cells.

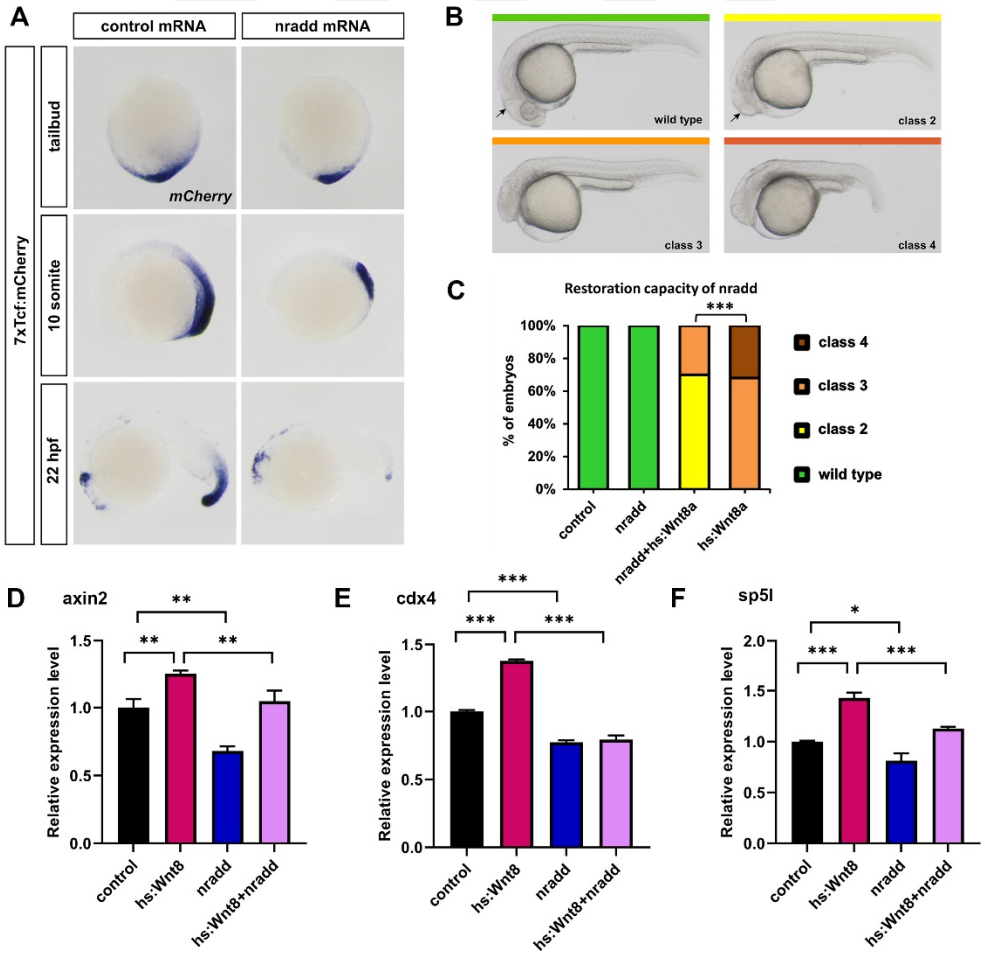



**Figure 2.** *Cont.*

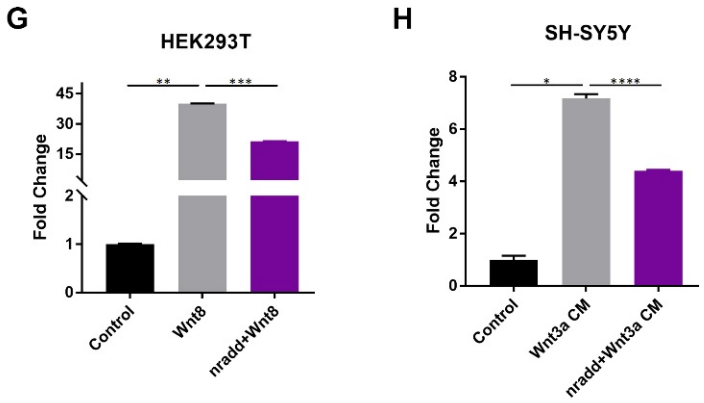



**Figure 2.** *nradd* acts as an inhibitor of Wnt/β-catenin signaling. (**A**) Wnt/β-catenin reporter activity is reduced in 7xTcf:mCherry embryos injected with 250 pg *nradd* mRNA. (n (tailbud): control 26/26 embryos, nradd 27/31 embryos; n (10-somite): control 26/26 embryos, nradd 25/26 embryos, n (22 hpf): control 21/21 embryos, nradd 29/30 embryos). Three independent experiments were conducted. (**B**) 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. (**C**) Phenotypes in wild-type (wt) or hs:Wnt8 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. (**D**–**F**) 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 ± standard deviation (SD, n = 3). Three independent experiments were conducted. (**G**–**H**) Average and SD of the mean (error bars) values of pBAR luciferase reporter activity monitoring Wnt/β-catenin signaling activity (normalized to renilla luciferase activity) in (**G**) HEK293T and (**H**) SH-SY5Y cells where Wnt/β-catenin signaling is activated by Wnt8 or Wnt3a conditioned media (CM) and transfected with Nradd. Nradd significantly inhibited Wnt signaling in both cell lines. Statistical significance was evaluated using an unpaired *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. Error bars represent SD. Three independent experiments were performed.

### *3.3. Nradd Suppresses Wnt-Mediated Patterning of the Mesoderm and the Neuroectoderm*

During gastrulation, wnt8-mediated β-catenin signaling has essential roles in the specification of the ventrolateral mesoderm and the repression of the dorsal organizer [51]. To test whether *nradd* affects mesodermal patterning of the embryo, we examined how the organizer reacts to *nradd* overexpression during early development. *nradd* caused a significant enlargement of the dorsal organizer region marked by goosecoid (gsc) expression [53], not at the late blastula (30% epiboly) stage, but at the early gastrula (60% epiboly) stage (Figure 3A,B). This suggests that *nradd* can relieve wnt8-mediated repression of the dorsal organizer. Wnt/β-catenin signaling is also required for the induction of posterior neural fates during gastrulation. *nradd* overexpression caused an extension of the early forebrain and midbrain region marked by otx2 [54] and a concomitant restriction of the posterior neuroectodermal region marked by hoxb1b [55] at late gastrula (100% epiboly, Figure 3C). To further investigate the role of *nradd* in later patterning of the neuroectodermal fates, we tested whether it can rescue wnt8-induced posteriorization of the zebrafish embryo brain. *nradd* overexpression rescued the anterior neuroectodermal fates, shown by the forebrain marker foxg1a, which completely disappeared in wnt8-overexpressing embryos during mid-somitogenesis (10–13 somites (Figure 3D). Thus, we conclude that *nradd* acts as a feedback inhibitor of wnt8-mediated patterning of mesoderm and neuroectoderm during zebrafish gastrulation.

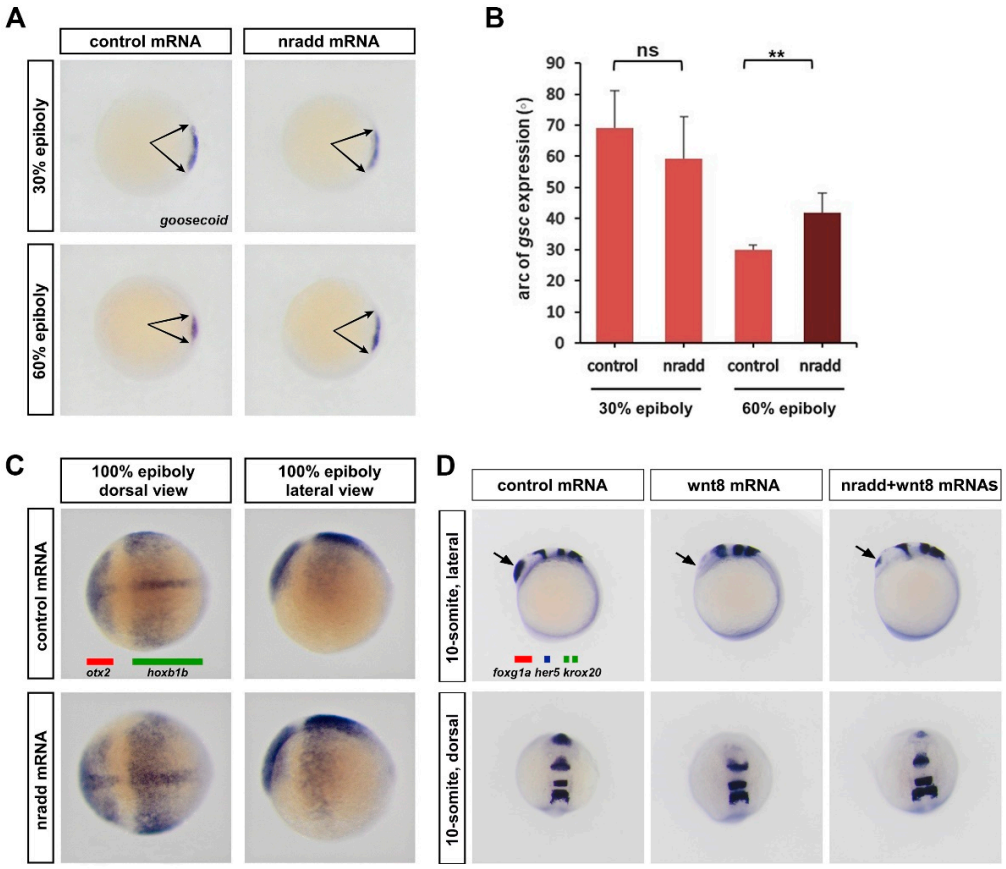



**Figure 3.** *nradd* suppresses Wnt-mediated patterning of the mesoderm and the neuroectoderm. (**A**) *nradd* mRNA (250 pg) causes expansion of the dorsal organizer domain (arrows) marked by *goosecoid* (gsc) WMISH, not at late blastula (30% epiboly, n: control 23/23 embryos, nradd 26/29 embryos), 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. (**C**) *nradd* mRNA (250 pg) results in expansion of the anterior neuroectodermal marker *otx2* (red bar) and a complementary reduction of the posterior neuroectodermal marker *hoxb1b* (green bar) defined by WMISH at the 100% epiboly stage (n: control 35/35 embryos, nradd 38/41 embryos). Three independent experiments were conducted. (**D**) *nradd* mRNA (250 pg) restores the telencephalon, marked by *foxg1a* (arrowhead), that is completely abolished by *wnt8* mRNA (20 pg). WMISH is performed to detect expression of three independent RNAs, the forebrain marker *foxg1a* (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. Nradd Localizes to the Plasma Membrane and Interacts with the Wnt–Receptor Complex*

Since *nradd* encodes for a transmembrane protein, we next aimed to examine its subcellular localization and test whether it interacts with the Wnt–receptor complex. Nradd-EGFP localized to the plasma membrane and co-localized with the membrane bound RFP-GPI (glycosylphosphatidylinositol) in zebrafish embryos during the early blastula stage (Figure 4A). When expressed in the human osteosarcoma epithelial cell line U2OS, we observed that Nradd tagged with mRuby partially localized to the plasma membrane along with the canonical Wnt receptor Fz8a tagged with EGFP (Figure 4B). Fractions of both proteins, Nradd to a larger extent, were detected in the intracellular space (mostly endoplasmic reticulum [ER]), presumably due to overexpression. To further confirm the plasma membrane localization of Nradd-EGFP, we prepared giant plasma membrane vesicles (GPMVs) from Nradd-EGFP-expressing U2OS cells. GPMVs are pure plasma membrane

encapsulating soluble cytoplasmic content while being devoid of any organelles [56]. In GPMVs, Nradd-EGFP was clearly detectable at the surface of the vesicles, confirming its plasma membrane localization (Figure 4C) [56]. Next, we asked whether there is a physical interaction between Nradd and components of the Wnt–receptor complex at the plasma membrane. First, Nradd coimmunoprecipitated with the canonical Wnt co-receptor Lrp6, showing a potential interaction between these two proteins (Figure 4D). Second, we applied fluorescence cross-correlation spectroscopy (FCCS) that can be used to analyze the synchronous movement (co-diffusion) and binding behavior of two fluorescently labeled proteins with single molecule detection sensitivity in live cells [57]. Representative graphics for low cross-correlation (negative control), high cross-correlation (positive control and Lrp6+Nradd+Wnt3a) are provided (Figure S1). In addition, Wnt3 stimulation increased the interaction of all three combinations of molecules at the membrane, defined by percentages of cross-correlation (Figure 4E). Thus, Nradd appears to diffuse together with both the Fz8a receptor and Lrp6 co-receptor at the membrane and this diffusion is further enhanced by canonical Wnt stimulation. These results together show that Nradd localizes at the plasma membrane and physically interacts with the components of the Wnt–receptor complex.

### *3.5. Nradd Acts Together with Wnt/β-Catenin Signaling to Promote Apoptosis during Development*

Although Nradd has been shown to promote apoptosis in neuroblastoma cell lines [27], its potential role in embryonic development has not been described. Nradd has four amino-terminal cysteine-rich domains (CRDs) conserved among TNFRSF members and located between the 23rd and 184th amino acids and a carboxy-terminal death domain located between the 326th and 404th amino acids. In addition to wt Nradd, we thus generated two other constructs, Nradd-DDD and Nradd-GSD, in which the C-terminal region including the death domain and the N-terminal region including the N-glycosylation site and the two CRD domains were deleted, respectively (Figure 5A). Initially, to address whether Nradd can induce apoptosis during development, we overexpressed wt *nradd* mRNA in zebrafish embryos and performed immunostaining for activated caspase 3, a marker of apoptosis. Since Nradd can suppress wnt8-mediated patterning of the neuroectoderm during development, we focused on the brain regions of embryos. Nradd was sufficient to significantly enhance apoptosis on its own and also acted together with wnt8-mediated β-catenin signaling, which is activated in hs:Wnt8 transgenic embryos by giving a heat shock at 60% epiboly (Figure 5B,C). In contrast to Nradd, Nradd-DDD and Nradd-GSD could neither inhibit canonical Wnt signaling in the 7xTcf:mCherry Wnt reporter line (Figure 5D) nor induce apoptosis in zebrafish embryos (Figure 5E,F). Thus, Nradd appears to promote apoptosis together with Wnt/β-catenin signaling and both the C-terminal death domain and the N-glycosylated N-terminal domain are essential for its apoptotic function.

### *3.6. Zebrafish Nradd Promotes Apoptosis in Human Embryonic and Neuroblastoma Cell Lines*

The *nradd* gene has been inactivated by mutation and is nonfunctional in humans (NRADDP, Gene ID: 100129354). To test whether zebrafish Nradd is functional in humans, first we transfected human embryonic kidney 293T (HEK293T) cells with the zebrafish Nradd. Nradd significantly induced apoptosis but did not act together with Wnt3a (Figure 6A,B). Moreover, zebrafish Nradd was able to significantly enhance apoptosis in human neuroblastoma SH-SY5Y cells, again independently of Wnt3a (Figure 7A,B). These results suggest that while the apoptotic role of Nradd is conserved across vertebrates, its additive effect with Wnt signaling is not conserved.

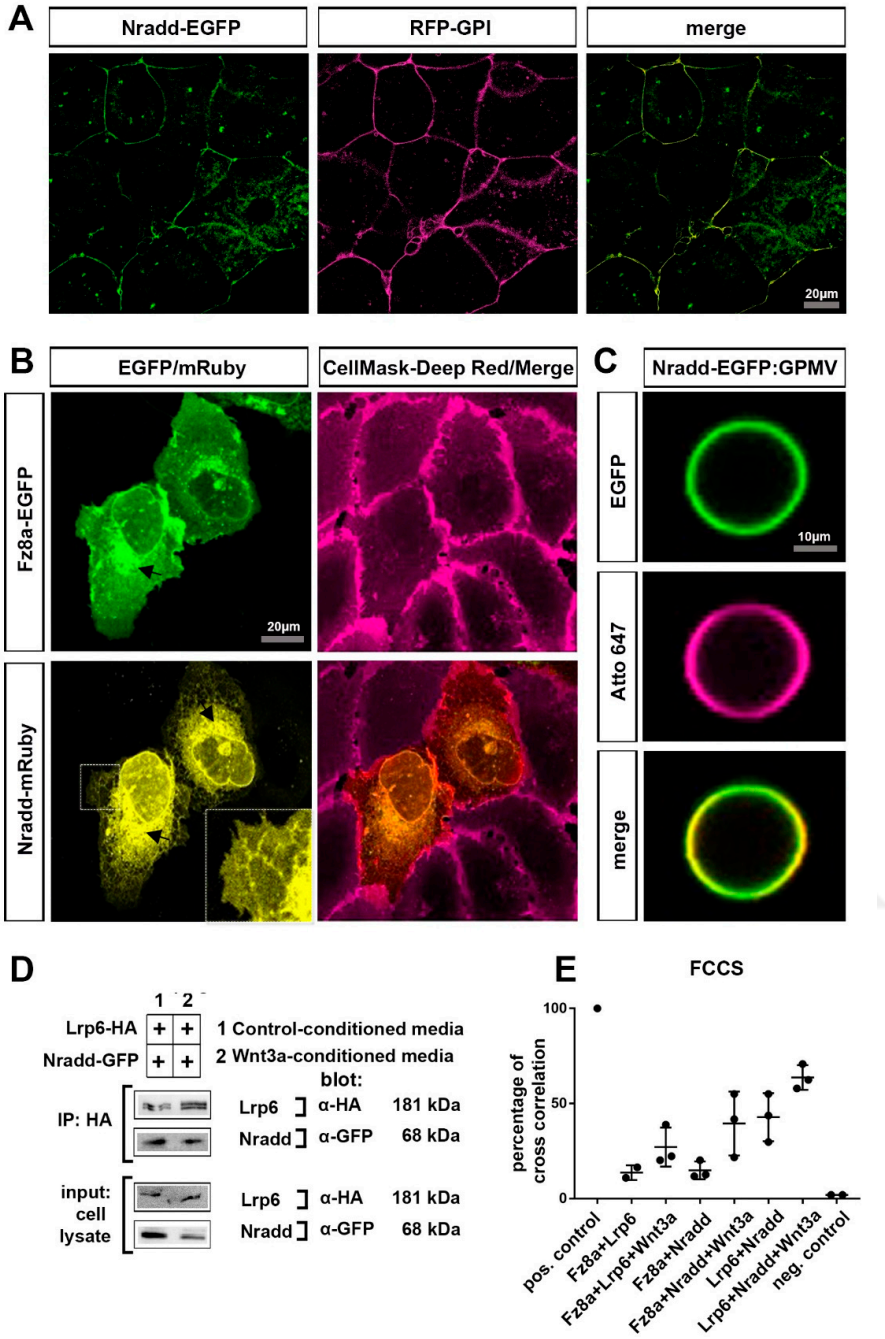



**Figure 4.** Nradd localizes to the plasma membrane and interacts with the Wnt–receptor complex (**A**) Nradd-EGFP (green) and RFP-GPI (red) localize at the plasma membrane of the enveloping layer cells of zebrafish embryos at 4 hpf (dome stage). (**B**) In U2OS cells, Fz8a-EGFP (green) co-localizes with the CellMask Deep Red (red) at the plasma membrane while Nradd-mRuby (yellow) localizes to the ER, but a significant fraction of it is in the plasma membrane, as seen from the zoom-in inset image. Arrows indicate protein transport from the ER to the plasma membrane. (**C**) Nradd-EGFP (green) localizes to the surface of cell-derived giant plasma membrane vesicles (GPMVs) and overlaps with the plasma membrane marker Atto647N-PE (red). (**D**) Nradd-GFP co-immunoprecipitates with Lrp6-HA in HEK293T cells. Three independent experiments were performed. (**E**) Fluorescence cross-correlation spectroscopy (FCCS) measurements show cross-correlation percentages between Fz8a-Lrp6, Fz8a-Nradd and Lrp6-Nradd, with/without Wnt3a stimulation at the plasma membrane. Nradd co-diffuses with Fz8a and Lrp6 at the membrane and this co-diffusion is enhanced by Wnt3 stimulation. EGFP and mCherry proteins were used as negative controls and their cross-correlation percentage is 1.70%.

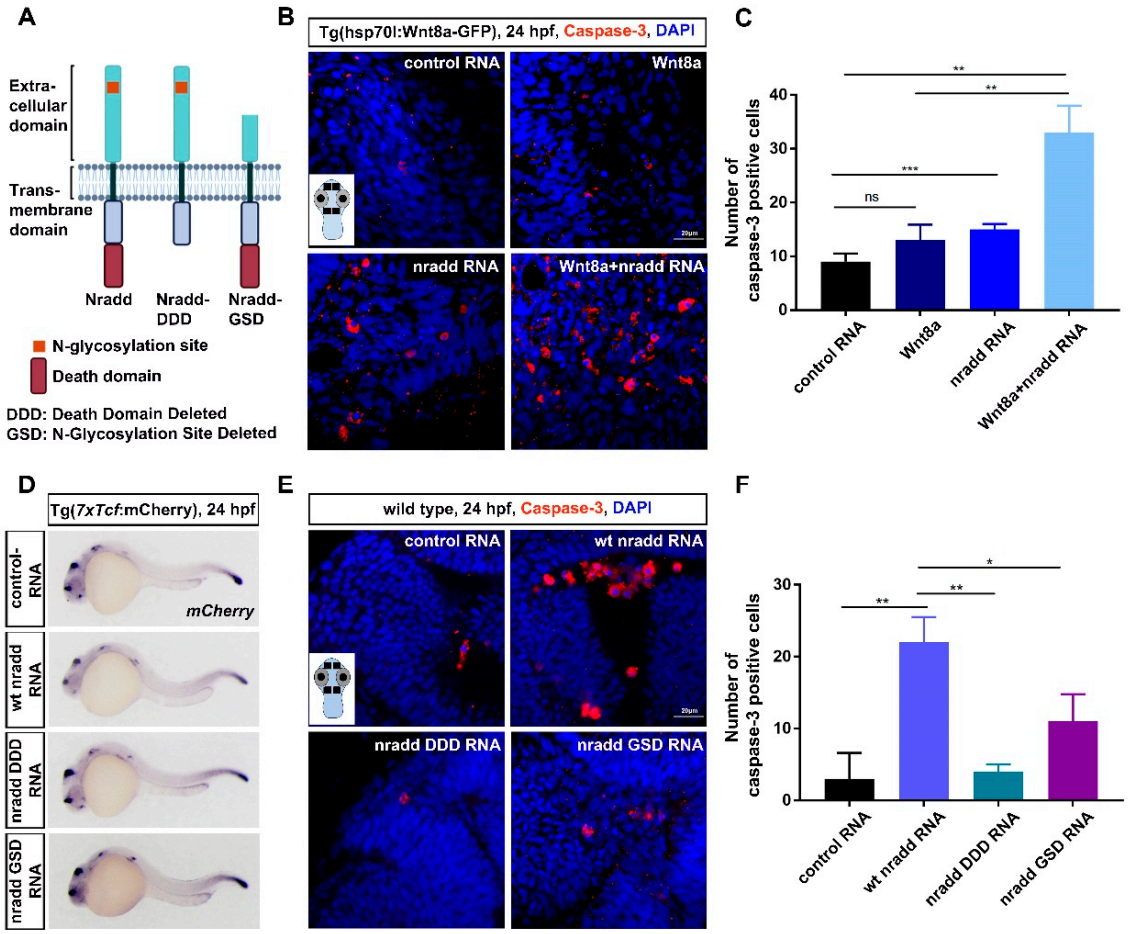



**Figure 5.** Nradd acts together with Wnt/β-catenin signaling to promote apoptosis during development. (**A**) Schematic representation shows domain structures of wt Nradd, Nradd without the death domain (Nradd death domain deleted (DDD)) and Nradd without the N-glycosylated N-terminal region (Nradd glycosylation site deleted (GSD)). (**B**) Anti-cleaved caspase 3 staining of control, Wnt8a activated, *nradd* mRNA-injected and Wnt8a activated+*nradd* mRNA-injected zebrafish embryos at 24 hpf. Sections are counterstained for DAPI. The inset shows a representative embryo with four different regions indicated with black rectangles that are used for counting the cleaved caspase 3-positive cells. The embryo image is created with BioRender.com. Scale bar: 20 μm. *nradd* mRNA (250 pg) induces apoptosis in zebrafish embryos compared to control group and also acts together with Wnt8a to further enhance apoptosis. The experiment was performed in hs:Wnt8 transgenic embryos where Wnt8a expression is induced by giving a heat shock at 60% epiboly. Four independent experiments were conducted. (**C**) Quantification of cleaved caspase 3-positive apoptotic cells shown in (B). Numbers represent average number of apoptotic cells counted from four different regions of 20 embryos for each group. Error bars are SD, ** indicates $p < 0.01$ and *** indicates $p < 0.001$. (**D**) WMISH showing the loss of capacity in *nradd DDD* (250 pg) and *nradd GSD* (250 pg) mRNA to inhibit canonical Wnt signaling. mCherry WMISH shows downregulation of signaling in the transgenic 7xTcf:mCherry Wnt/β-catenin reporter embryos by wt *nradd* (58/61). Overexpression of *nradd DDD* (44/46) or *nradd GSD* (51/52) cannot inhibit Wnt/β-catenin signaling. Three independent experiments were conducted. (**E**) Anti-cleaved caspase 3 staining of control (100 pg), wt *nradd* (250 pg), *nradd DDD* (250 pg) and *nradd GSD* (250 pg) mRNA-injected zebrafish embryos at 24 hpf. Sections are counterstained for DAPI. The inset shows a representative embryo with four different regions indicated with black rectangles that are used for counting the cleaved caspase 3-positive cells. Scale bar: 20 μm. *nradd* mRNA (250 pg) induces apoptosis in zebrafish embryos compared to control group, while *nradd DDD* or *nradd GSD* cannot. Three independent experiments were conducted. (**F**) Quantification of cleaved caspase 3-positive apoptotic cells shown in (**E**). Numbers represent average number of apoptotic cells counted from four different regions of 20 embryos for each group. Error bars are SD, * indicates $p < 0.05$ and ** indicates $p < 0.01$.

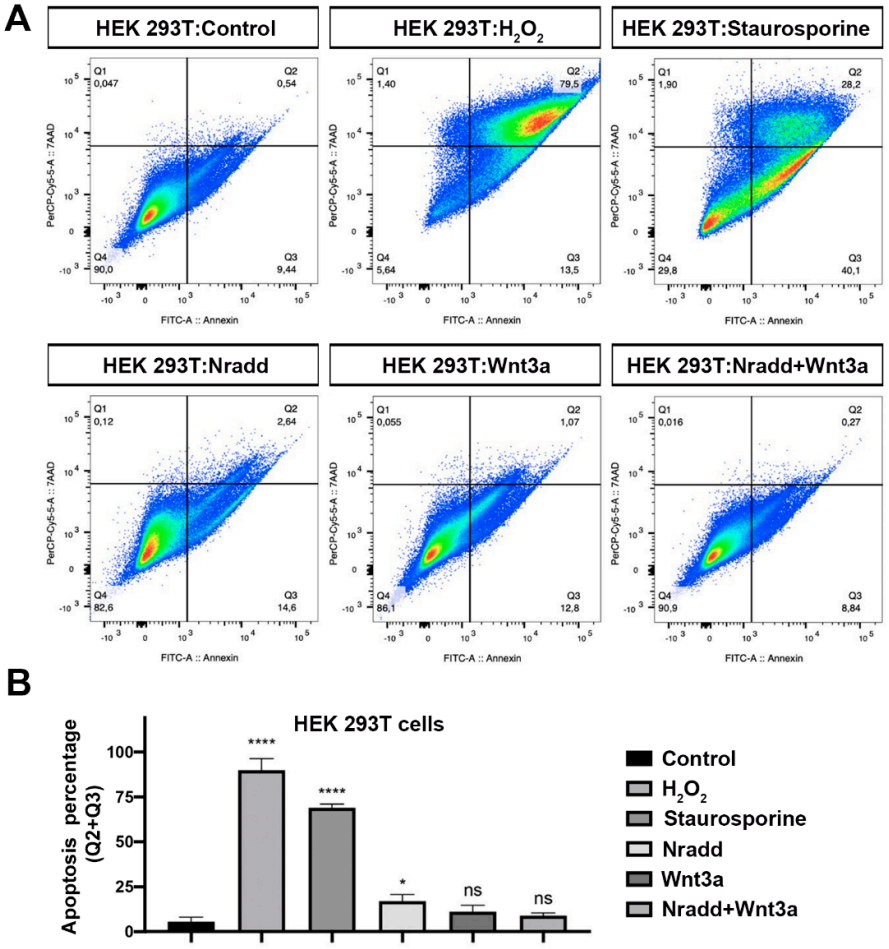



**Figure 6.** Zebrafish Nradd promotes apoptosis in human embryonic kidney cells. (**A**) Apoptosis assay on HEK293T cells using flow cytometry after staining with annexin V-FITC and 7AAD. Representative scatter plots of 7AAD (y-axis) vs. annexin V-FITC (x-axis). Nradd significantly enhances apoptosis. (**B**) Percentage of apoptotic cells, * indicates $p < 0.05$, **** is $p < 0.0001$ and ns is non-significant. All experiments were performed in five replicates. Three independent experiments were conducted.

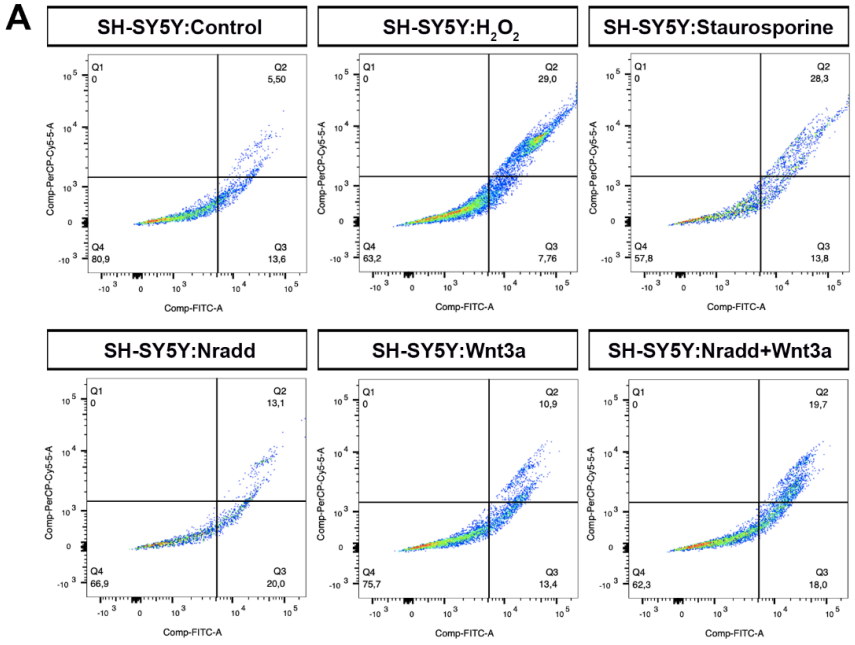



**Figure 7.** *Cont.*

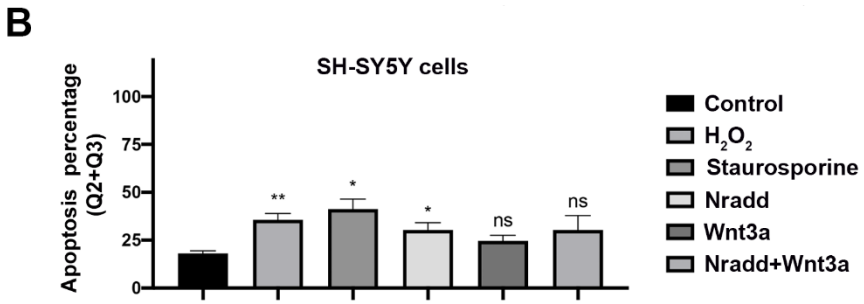



**Figure 7.** Zebrafish Nradd enhances apoptosis in neuroblastoma cells. (**A**) Apoptosis assay on SH-SY5Y cells using flow cytometry after staining with annexin V-FITC and 7AAD. Representative scatter plots of 7AAD (y-axis) vs. annexin V-FITC (x-axis). Nradd significantly induces apoptosis. (**B**) Percentage of apoptotic cells, * indicates $p < 0.05$, ** is $p < 0.01$ and ns is non-significant. All experiments were performed in five replicates. Three independent experiments were conducted.

## 4. Discussion

Owing to its essential roles in embryonic development and the maintenance of tissue homoeostasis, Wnt/β-catenin signaling is tightly regulated by various pathway modulators. Here, we characterize the functional role of Nradd, a poorly characterized homologue of $p75^{NTR}$, in pathway regulation during zebrafish development. Our data suggest that Nradd i) is a Wnt/β-catenin target during zebrafish development, ii) can suppress Wnt/β-catenin signaling during development and in mammalian cells, iii) efficiently inhibits Wnt8-mediated ventralization of the mesoderm and posteriorization of the neuroectoderm, iv) localizes at the plasma membrane and physically interacts with the Wnt–receptor complex, v) promotes apoptosis by acting together with Wnt/β-catenin signaling during development and vi) can also enhance apoptosis in mammalian cells.

Neuronal survival and innervation rely on the presence of developing neuronal populations that receive accurate trophic factors from neighboring cells and activate intracellular signaling pathways [58]. Nevertheless, neuronal cell death exhibits an essential cell death program by which a neuron directs its own destruction and is essential for the proper development and functioning of the nervous system [59]. The death receptor, or so-called extrinsic apoptosis, pathway is one of the essential mechanisms of neuronal cell death [60]. The pathway is mediated by the interaction of death ligands with TNFRSF death receptors at the cell surface and further recruitment of Fas-associated death domain protein (FADD), which in turn leads to autoproteolytic cleavage and the activation of initiator caspases *and downstream executioner caspases including caspase 3 [61]. Aberrant regulation of the death receptor signaling pathway has been associated with neuronal death in* neurodevelopmental disorders, *neurodegenerative conditions such as traumatic brain injury, amyotrophic lateral sclerosis and stroke and psychiatric disorders [62,63].* $p75^{NTR}$, a pleiotropic signaling molecule, can act as a co-receptor for various receptors, including Trk, sortilin and Nogo, and mediate a variety of cellular functions from survival and axonal growth to apoptosis through several signaling pathways [29,64–67]. Our results unravel Wnt/β-catenin signaling as a novel interaction partner of Nradd, a $p75^{NTR}$ homolog, in the regulation of apoptosis during embryonic development. Wnt signaling regulates the early and late stages of apoptosis during the development of various organs, including the brain, the limbs and the heart [68–71]. During neural development, the ability of Wnt signaling to facilitate or prevent apoptosis is highly dependent on the cellular context and other signaling pathways that integrate apoptosis with other cellular processes [72]. Wnt signaling *has been associated with neurotrophin signaling* in the coordination of neuronal development and differentiation [73,74]. Wnt/β-catenin signaling has previously been shown to regulate expression of the death receptors DR6 and TROY in the brain epithelium to mediate blood–brain barrier development in the central nervous system [75]. An elegant work has recently shown that cells with abnormal Wnt/β-catenin activity are eliminated via apoptosis with the involvement of cadherin proteins and Smad signaling and this elimination is required for proper anterior–posterior

patterning [76]. Our results support this finding that Wnt/β-catenin signaling can induce apoptosis during development and further unravel that Nradd can potently enhance the apoptosis-promoting role. Since Nradd is both a feedback inhibitor of Wnt/β-catenin signaling and can potently induce apoptosis in the zebrafish brain during development, it will be very interesting to further analyze which specific cells of the brain undergo apoptosis upon Nradd expression.

$p75^{NTR}$ has been shown to promote the apoptosis of neurons and oligodendrocytes in development, regeneration and pathological conditions [67,77–80]. Several studies have unraveled the roles of $p75^{NTR}$ structural domains in the activation of death receptor signaling. The extracellular domain of $p75^{NTR}$ appears to be responsible for the conformational changes that propagate the signal to its death domain, which further recruits the interactors of various signaling pathways [81–83]. The death domain is also necessary for $p75^{NTR}$-induced neuronal apoptosis [83,84]. The induction of apoptosis is dependent on the N-terminal domain that was shown to be modified by N-glycosylation, i.e., the attachment of an oligosaccharide to the protein in the ER [27]. Our functional analysis with the Nradd constructs has demonstrated that both the N-glycosylated N-terminal region and the death domain-containing C-terminal region are necessary for the suppression of Wnt/β-catenin signaling and the induction of apoptosis. Thus, we suggest that the N-terminal region undergoing N-glycosylation and the C-terminal death domain are key regions that can be exploited for the control of cell death mechanisms.

Mouse NRADD has been reported to induce cell death in primary neuronal cells and neuroblastoma cell lines [27,85,86]. Our data unravel the apoptotic role of zebrafish Nradd in both human embryonic and neuroblastoma cell lines and further suggest that Nradd induces early apoptosis characterized mainly by alterations occurring at the plasma membrane. Unlike encoding for a transmembrane protein in mice and zebrafish, the human *NRADD* gene carries inactivating mutations that have converted it into a pseudogene in humans. Strikingly, our data show that zebrafish Nradd can not only inhibit Wnt/β-catenin signaling but also promote apoptosis in human embryonic and cancer cell lines, indicating that Nradd has an evolutionarily conserved function in the regulation of Wnt signaling and apoptosis. It is possible that human p75NTR homologs might compensate for the absence of Nradd in humans. While Nradd appears to act together with Wnt signaling to enhance apoptosis during development, Wnt activation does not further enhance Nradd-mediated apoptosis in human cells. The activation of Wnt/β-catenin signaling could, however, sensitize human melanoma cells to apoptosis induced by the TNF-related apoptosis-inducing ligand (TRAIL/APO2L) [87]. In contrast, the secreted Wnt inhibitor Dkk3 has been found to induce Fas death receptor signaling in human ovarian cancer cells, suggesting a proapoptotic role for Dkk3 [88]. Furthermore, death receptor signaling can contrariwise regulate Wnt signaling. Several inhibitors of NGF and NGF receptors, such as Ro 08-2750 (targets NGF), K252a (targets TrkA) and LM11A-31 (targets P75), have been shown to increase β-catenin expression and cell migration in ovarian cancer cells [89]. Thus, we believe that the relationship between Nradd and Wnt signaling in the regulation of apoptosis mechanisms is context dependent. Future studies on understanding the relationship between Nradd and Wnt signaling regulation in different contexts, such as neurodevelopment, neurodegeneration and cancer, are very likely to constitute a promising approach for the development of disease-specific therapies.

The role of Wnt/β-catenin signaling in neural development starts at the early induction of the neural plate and continues throughout subsequent patterning of the neuroectoderm during CNS development [90]. Our results reveal Nradd as a new player in this process with a robust capacity to modify canonical Wnt-mediated posteriorization of the neuroectoderm. Moreover, the pathway is known to be actively involved in the modulation of the synaptic function to maintain the basal neural activity [91]. Consequently, alterations in Wnt signaling have been implicated in many neurodegenerative and neuro-oncological diseases, raising the importance of the specific pathway targeting for efficient treatment strategies [92–97]. Besides, the misexpression of pseudogenes has been linked to various

human diseases, including several cancers [98–103]. For example, deletion of the PTENP1 pseudogene locus in melanoma enhances miRNA-mediated suppression of PTEN and tumor progression [104]. Overexpression of a PTENP1 transgene could restore its tumor suppressor activity and inhibit tumor growth [105]. Therefore, the ability of Nradd to modulate Wnt signaling in human cells offers a promising target for therapeutic interventions in neurological disorders.

## 5. Conclusions

Our study reveals the death receptor-encoding nradd, a homolog of p75NTR, as a Wnt target during development and an inhibitor of the Wnt/β-catenin signaling pathway in zebrafish embryos and mammalian cells. Nradd can potently suppress canonical Wnt-mediated phenotypes in mesodermal and neuroectodermal patterning during zebrafish gastrulation. By physically interacting with the Wnt–receptor complex at the plasma membrane, Nradd acts together with Wnt/β-catenin signaling to promote apoptosis during development. Moreover, human embryonic and neuroblastoma cells undergo apoptosis upon Nradd overexpression. Thus, by both fine-tuning pathway activation at the plasma membrane and regulating cell death, the Wnt modulator Nradd might serve as an attractive target for the discovery of therapeutic interventions against Wnt-related human diseases.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/2218-273X/11/1/100/s1, Figure S1: Autocorrelation and cross-correlation analysis of EGFP and mCherry/mRuby3 tagged proteins in live U2OS cells.

**Author Contributions:** Conceptualization, G.O.; methodology, O.O., O.C., Y.A., B.H., G.C., I.K., G.A.-Y. and E.S.; writing—original draft preparation, G.O., O.C. and O.O.; visualization, G.O.; funding acquisition, G.O. All authors have read and agreed to the published version of the manuscript."

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Animal Experiments Local Ethics Committee of Izmir International Biomedicine and Genome Institute (protocol code 12/2017, 26.07.2017).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

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**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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