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Analysis of Norrin-mediated protective effects on chronic degeneration of photoreceptors

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Zusammenfassung:

Norrin ist ein sezerniertes Protein, das als unkonventioneller Ligand an dem Frizzled-4 Rezeptor binden kann und hierdurch den klassischen Wnt/β-Catenin Signalweg aktiviert. Eine reguläre Entwicklung der Blutgefäße in der Netzhaut hängt stark von der Anwesenheit des Norrin-Proteins ab. Darüber hinaus hat die Forschung gezeigt, dass Norrin neuroprotektive Eigenschaften aufweist und retinale Neuronen schützen kann.

Diese Studie zielt darauf ab, die neuroprotektive Rolle von Norrin bei einer chronischen Degeneration der Photorezeptoren und die molekularen Mechanismen zu untersuchen, die an seinen neuroprotektiven Effekten beteiligt sind. Aus Vorarbeiten ist bekannt, dass Norrin in Mäusen mit einer Netzhautdegeneration Photorezeptoren über die direkte Aktivierung des pAKT-Signalwegs schützen kann. Um den zugrundeliegenden Mechanismus näher zu analysieren wurden Photorezeptoren der Mause (661W Zellen) generiert, die sowohl den FZD4 als auch den IGF1R überexprimieren. Nach der der Behandlung der Zellen mit Norrin konnte eine deutliche stärkere Aktivierung des pAKT-Signalwegs beobachtet werden als bei wildtypischen 661W Zellen ohne Überexpression. In weiterführenden Experimenten konnte gezeigt werden, dass Norrin in doppeltransgenen Zellen nach deren Differenzierung mit Staurosporin eine Apoptose der Zellen vermindern kann. Die anti-apoptotische Wirkung von Norrin konnte durch die gleichzeitige Gabe des pAKT-Inhibitors Triciribin signifikant reduziert werden, was deutlich darauf hinweist, dass der neuroprotektive Effekt von Norrin über die direkte Aktivierung des pAKT-Signalwegs verittelt wird. Homologe Befunde wurden auch nach der zusätzliche Gabe des IGF1R-Inhibitors GSK1904529A beobachtet, die die Hypothese unterstützt, dass Norrin über die Interaktion mit FZD4 und IGF1R den pAKT-Signalweg aktiviert. Um die Interaktion von FZD4 mit dem IGF1R weiter aufzuklären, wurde eine in situ Protein-Protein-

Detektion mittels „rolling circle amplification“ durchgeführt. Diese zeigt deutlich, dass Norrin die Interaktion zwischen dem FZD4 und dem IGF1R induzieren kann.

Zusammenfassend konnte im Rahmen meiner Dissertation gezeigt werden, dass Norrin über die Rekrutierung des IGF1R den AKT/pAKT Signalweg aktiviert und hierdurch Photorezeptoren vor apoptotischem Schaden schützt kann

Abstract:

Norrin is a secreted protein that activates the canonical Wnt/β-catenin signaling pathway via binding to the frizzled-4 receptor (FZD4). In the eye, Norrin is required for normal retinal vascularization and mediates neuroprotective properties on retinal neurons following acute or chronic damage.

The aims of this study were to investigate the neuroprotective role of Norrin in chronic photoreceptor degeneration and to analyse the molecular mechanisms involved in its neuroprotective effects. It is known from preliminary work that Norrin can protect photoreceptors in mice with retinal degeneration via direct activation of the pAKT pathway. To further explore the underlying mechanism, mouse photoreceptor cells (661W) overexpressing both FZD4 and IGF1R were generated. After treatment of these cells with Norrin, significantly stronger activation of the pAKT pathway was observed when compared to wild-type 661W cells without overexpression. In further experiments, Norrin was shown to reduce cell apoptosis in double-transgenic 661W cells after differentiation with staurosporine. The anti-apoptotic effect of Norrin could be significantly reduced by the additional treatment with the pAKT inhibitor Triciribine, clearly indicating that the neuroprotective effect of Norrin is mediated via direct activation of the pAKT pathway. Homologous findings were observed after incubation of double transgenic cells with Norrin and the IGF1R inhibitor GSK1904529A, supporting the hypothesis that Norrin activates the pAKT pathway via interaction with FZD4 and IGF1R. To further elucidate the interaction of FZD4 with IGF1R, in situ protein-protein detection was performed using rolling circle amplification, which clearly demonstrates a Norrin-mediated interaction between FZD4 and IGF1R.

In summary, Norrin can protect photoreceptors from apoptotic damage via pAKT signalling. For this, Norrin recruits the IGF1R to FZD4, which in turn is required for direct activation of AKT pathway.

1. Introduction

1.1 Anatomical, physiological and biochemical characteristics of the retina

1.1.1 The anatomy of the retina

During embryonic development, the neuroectoderm forms the optic cup, which gives rise to the retina[1]. The outer layer of the optic cup becomes the retinal pigment epithelial (RPE) layer, while the inner layer differentiates into the retinal neurosensory layer[1]. There is a potential, anatomical gap between the two layers, which is clinically separated in retinal detachment[2].

RPE is a neatly arranged unilayer of hexagonal cells, which are thicker in the macula and thinner in the periphery[3]. They have many microvilli on the top of the cells, and the outer segment of the photoreceptor is embedded in the mucopolysaccharide interstitium of the RPE[4]. The RPE is arranged polarized and its basal base is closely bound to the Bruch's membrane[4, 5].

The retinal neurosensory layers from the outside to the inside are: (1) The layer of cone and rod, composed of the inner and outer segments of photoreceptor cells; (2) The outer limiting membrane, appearing as a thin web-like structure, results from the combination of adjacent photoreceptors and Müller cells; (3) The outer nuclear layer is composed of photoreceptor cell nuclei; (4) The outer plexiform layer is the site where cone cells, rod cells, bipolar cells, and horizontal cells are connected by synapses; (5) The inner nuclear layer is mainly composed of the cell nuclei of bipolar cells, horizontal cells, amacrine cells, and Müller cells; (6) The inner plexiform layer is primarily composed of

processes and synapses between bipolar cells, amacrine cells, and ganglion cells; (7) the ganglion cell layer is made up of ganglion cell nuclei and displaced amacrine cells; (8) the nerve fiber layer consists of the axons of ganglion cells and astrocytes; (9) The inner limiting membrane is a delicate layer positioned between the retina and the vitreous body[6].

Photoreceptor cells consist of five components: outer segment, cilium, inner segment, nuclear region, and synaptic region[7]. The outer segment of the rod is cylindrical, while that of the cone is conical[8]. The membrane discs are constantly shedding and re-newing[9].

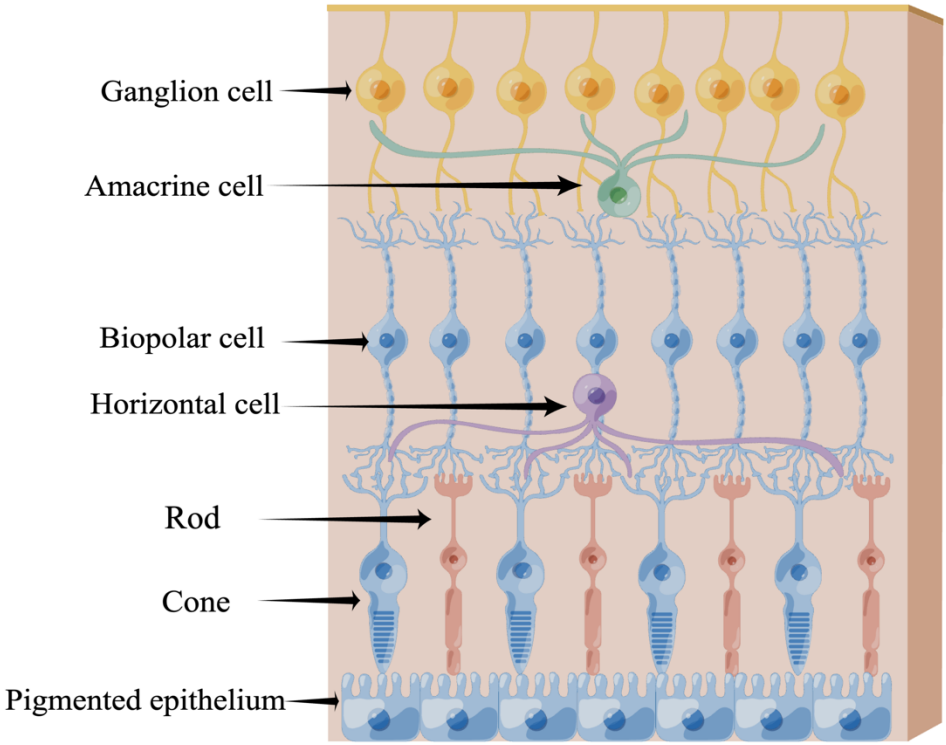


Figure 1: Simple schematic diagram of retinal structure

1.1.2 The physiological and biochemical characteristics of the retina

The RPE not only contains the same organelles as most cells but also melanin granules and phagosomes, which represent its two important functions[10]. The radial glial cells in the retina, the Müller cells, run through the neurosensory layer, with their endfeet

stretching from outer limiting membrane to inner limiting membrane, providing structural support as well as metabolic nutrition for the retina[11].

Visual information forms optic nerve impulses within the retina, which are generated in the three types of retinal neurons, namely photoreceptors-bipolar cells-ganglion cells[12]. Ganglion cell axons transmit visual information along the optic pathway to the lateral geniculate and from there to the primary visual cortex[13]. Photoreceptors are the first level of neurons in the retina, divided into rods and cones[14]. There are millions of cones concentrated in the macular area[15]. In the fovea only cones are located, which are connected in a single line, to bipolar and retinal ganglion cells, to generate very sharp vision[16]. In the optic disc retinal ganglion cell axons converge, penetrate the sclera, and form the optic nerve[17]. No visual function is manifested at the optic disc so that this region imposes as a physiological blind spot in the visual field[18].

The visual pigment contained in the outer segment of rods is rhodopsin[19]. Rhodosin is formed by combining cis-retinal and opsin[19]. In darkness, the resynthesis of rhodopsin improves the sensitivity of the retina to light[20].

Cone cells contain various visual pigments [6]. Color vision is the function of cone cells in well-lit surroundings[21]. The macula has the highest sensitivity to color, while the peripheral retina has minimal color vision[22]. This observation corresponds with the distribution pattern of cone cells in the retina.

1.2 Retinal remodeling

Most retinopathy first involves the photoreceptor layer of the retina or the RPE, and lesions of the photoreceptor layer are usually accompanied by early changes in neural retinal conduction pathway[23]. These alterations further affect retinal neurons and glial

cells at all levels, causing structural rearrangement[24]. The remodeling of Müller cells, horizontal cells, and bipolar cells has been demonstrated to occur in experimental retinal detachment-induced photoreceptor degeneration [23]. This remodeling caused by photoreceptor degeneration follows a pattern that is generally divided into 3 stages[25]. Stage 1 begins with changes caused by the original retinal lesion[26]. In stage 2, after a series of changes, such as the initial photoreceptor stress response to the disintegration of normal structure, an irreversible stress response occurs leading to photoreceptor cell death and gradual ablation of the retinal photoreceptor cell layer[27]. The death of the cones heralds the beginning of stage 3. The residual neural retina is remodeled as a whole, including the death of neuronal cells, the migration of neurons and glial cells, the processing of new axons and synapses, the redistribution of nerve conduction pathways, the hypertrophy of glial cells, and the blockade formed by glial fibrosis, isolating the residual neural retina from the surviving retinal pigment epithelial layer and choroid[28].

1.3 Retinal degenerative disease

Several retinal diseases such as retinitis pigmentosa(RP), age-related macular degeneration(AMD) or diabetic retinopathy(DR) lead to neuronal degeneration of the retina[29-31]. This condition is frequently marked by the degeneration of photoreceptors, resulting in the decline of photoreceptors and/or RPE cells. Ultimately, it leads to gradual reduction of patient's visual field until complete loss of vision [32]. Currently, it is thought that while the genetic mechanism remains the primary contributor to the development of degenerative retinal disease, the significance of non-genetic factors in its pathogenesis should not be overlooked [33].

In every 3000-7000 individuals, one person is impacted by RP which is the most prevalent genetic retinal degenerative disease [34]. In the US, more than half of blind individuals suffer from AMD[35]. The sheer number of patients places a huge burden on the world economy. Given the dire global situation for visual impairment and blindness, research activities into novel treatment strategies have increased, but there is no causative cure for most blinding diseases[36].

Blocking the vascular endothelial growth factor (VEGF) with antibodies can significantly decrease neovascularization in individuals suffering from wet AMD, leading to a delay in destruction of photoreceptors and enhancing and preserving visual function. Nevertheless, the findings from the extensive SEVEN-UP study indicate that over 50% of patients experience a decline in their vision within 7 to 8 years of commencing treatment. The extent of visual impairment is contingent upon the administration pattern of VEGF inhibitors during the initial years of the illness [37]. In 2017, the Leber's Congenital Amaurosis (LCA) gene supplement method received approval from Food and Drug Administration (FDA), marking the introduction of the initial eye gene therapy into the market. This method uses Adeno-Associated Virus (AAV) vectors to introduce the normal RPE65 gene that catalyzes all-trans isomerization to the 11-cis retinal into patient RPE cells and inject it into the subretinal space to improve visual function [38]. Despite the many flaws, the success of RPE65 gene supplementation has inspired researchers to develop gene therapies for a range of other retinal diseases that affect RPE, rods, or cone photoreceptors[39]. By means of this genetic therapy, the functions of RPE and photoreceptors are enhanced to regain visual acuity, instilling optimism for the management of retinal degenerative disorders.

1.3.1 Retinitis Pigmentosa (RP)

Inherited retinal degenerative disease, known as RP, commonly presents with loss of photoreceptor cells and pigment epithelial function[40]. The main clinical manifestations are night blindness, visual field reduction, progressive vision loss, fundus osteocyte-like deposition, and photoreceptor dysfunction[41]. RP is an inherited eye disease that may lead to blindness. The main modes of inheritance of RP are autosomal dominant（ADRP）, autosomal recessive（ARRP）, X-linked （XLRP）and digenic, as well as mitochondrial inheritance and non-genetic sporadic forms[42-44]. There is no strict correspondence between a clinical phenotype and genetic cause leading to genetic heterogeneity and clinical heterogeneity[45]. Apoptosis of photoreceptor cells is the final common pathological event of different genetic types with various pathological mechanisms[29]. At present, there is no specifically efficient therapy for RP. The most studied treatment methods include nerve growth factor therapy, retinal cell transplantation, gene therapy, stem cell transplantation and drug treatment [46-50].

1.3.2 AMD

AMD is a significant contributor to permanent vision loss and a prevalent condition causing blindness among older individuals globally [51]. According to statistics, AMD is currently the first blinding eye disease in elders ($\geqslant$ 60 years old) in Europe and the US. The prevalence of this condition is approximately 11% among individuals aged more than 65, while it rises to about 64% among those aged more than 80 years. AMD can lead to significant impairment of central vision [52]. It affects the quality of life of countless elderly people and brings a huge burden to the whole society. Based on pathologic features, AMD can be divided into 2 types[53]. The first type is wet AMD (neovascular/exudative), which is characterized by choroidal neovascularisation (CNV) of the macula and may

lead to retinal bleeding and edema. Dry AMD (atrophic nature) is another form, distinguished by posterior polar Geographic atrophy (GA), where there is a loss or thinning of the RPE and corresponding photoreceptor degeneration.

1.3.3 Diabetic retinopathy（DR）

With the ongoing growth of the economy, diabetes has emerged as an epidemic illness, leading to a rise in complications like diabetic retinopathy (DR) among individuals with diabetes. Although diabetic patients have a high occurrence of DR, the existing approaches for screening and treating DR primarily concentrate on the later stage of the condition. This is when the compensatory mechanism has ceased to function, leading to significant impairment in retinal structure and vision[54]. In an in-depth study of DR, it was found that DR is not a simple microvascular disease, but a more complex complication of diabetes, and diabetic retinal neurodegeneration (DRN) is a key process in early DR[55]. In human and mouse models of type 1 and type 2 diabetes, the fact that DRN precedes DR microangiopathy was reported in multiple studies[56, 57].

1.4 The cause of retinal degeneration

In most retinal degenerative diseases, histopathological changes in photoreceptors can be observed. The photoreceptor nuclei are reduced in the outer nuclear layer and are arranged disordered. The photoreceptor cells are degenerative, and the cell structure is damaged. Retinal pigment epithelial cells degenerate and partially disappear or shift into the retina.

The cell biological mechanisms of photoreceptor degeneration are complex. There are many inheritance mechanisms, including autosomal dominant, autosomal recessive, X-linked, double gene, or mitochondrial inheritance [58]. Most of these mutations may

lead to a dysfunction of photoreceptors and/or RPE cells which in turn could induce apoptosis of photoreceptor cells. BEST1 gene, also known as VMD2, is located on chromosome 11q12-13, with a size of about 980kb, including 13 exons, and encoding the bestrophin-1 protein. BEST1 is mainly expressed in RPE cells, and small amounts in kidneys, brain, spinal cord, and testes [59]. Mutations in BEST1 may cause dysfunction of retinal structures, resulting in a range of diseases such as best vitelliform macular dystrophy (BVMD), adult-onset vitelliform macular dystrophy (AVMD), RP, LCA and rod-cone dystrophy (CORD)[60]. Gene RPGR, also named RP3, is the most important gene leading to XLRP in Caucasian population, and the incidence of gene mutation is 70%~80%, among which the mutation frequency of exon open reading frame 15 is the highest, about 50%~60% [61]. Meanwhile, the photoreceptor cell-specific ATP-binding transporter gene(ABCR) is defined as the causative gene of Stargardt disease [62]. Besides genetic reasons, reascent studies indicate that intense visible light may also cause the degeneration of photoreceptors [63]. Studies have pointed out that the core of the retinal photodamage hypothesis is that rhodopsin is an important substance that triggers photoreceptor cell damage [64]. Meanwhile, the absorption spectrum of rhodopsin is the same as the spectrum of action of retinal photodamage [65]. Variations in light exposure time may lead to varying degrees of photoreceptor cell degeneration [66]. The pathogenesis of AMD is complex and may involve metabolism, genetics, and environmental factors [67]. In AMD, the degeneration of photoreceptors may be associated with aging [68]. The study reported that in AMD, the photoreceptors, RPE, Bruch's membrane, and choroidal capillaries as a whole are damaged, resulting in the degeneration of RPE and photoreceptors [69].

It has been demonstrated in studies that diabetic retinopathy has the potential to affect retinal neurons [70]. Oxidative stress and other adaptive changes may occur in photoreceptors due to diabetes [71]. Meanwhile, some findings indicated that diabetes alters ion

flux in photoreceptors, and these abnormalities are also associated with oxidative stress [71, 72].

1.5 Therapy of the retinal degeneration disease

1.5.1 Gene therapy

Some genetic diseases in humans are associated with hereditary retinal dysfunction. Gene therapy holds promise to treat a variety of inherited human diseases. The goal of gene therapy is to replace the defective gene part with a native gene by using viral or non-viral vectors. Viral vectors mainly include AAV vectors, lentiviral vectors, adeno-viral vectors, and recombinant AAV vectors [73]. AAV vectors themselves do not integrate into the human genome. It only causes a small immune response and can be detected in a variety of retinal cells for a long time. Therefore, it makes it an important carrier for gene therapy in the eye. In recent years, gene therapy has made great progress. Studies have found that there are more than 140 genes related to the degeneration of retinal photoreceptor cells, of which 32 genes have been cloned [74].

It was found that the introduction of Vwh2 gene in a retinal degeneration (RD) mouse model carrying the Peripherin2 (Prph2) mutant gene could promote the change of different photoreceptor cell subtype function and ultrastructure. It can also increase visual sensitivity. This has a certain effect on the photoreceptor cells of both young mice and adult mice. And the improvement was particularly obvious in young mice. However, with the decrease of the expression of the introduced gene, the apoptosis of photoreceptor cells was not significantly improved [75, 76]. Pang et al. injected β subunit of rod cGMP-phosphodiesterase (PDEβ) into the retina of PDEβ mutation-induced retinal degeneration 10 (RD10) mice using AAV8 as the vector. Optical coherence tomography

(OCT), electroretinogram (ERG) and other methods were observed 6 months later and found to have a protective effect on the mouse retina[77].

Ribozymes are RNA molecules with catalytic functions, which are highly specific for lysing mutant mRNA sequences, which have the effect of reducing the production of mutant proteins. Gorbatyuk et al. used AAV as a carrier and catalytically active ribozyme to treat RP rats caused by the P23H mutation of the rhodopsin gene (RHO). The results showed that it can significantly reduce the loss of photoreceptors, increase the thickness of the outer nuclear layer, and improve the amplitude of ERG dark-adapted b-wave, while the introduction of inactive ribozyme has no therapeutic effect [78].

There has been a suggestion that nerve growth factor may have a protective function in photoreceptors. According to Zhang et al., it was documented that the gene responsible for brain-derived neurotrophic factor was electroporated into RCS rats to postpone apoptosis in cells of the RPE [79]. The introduction of small interfering RNA (small interfering RNA, siRNA) into cells can cause transcriptional silencing of the gene of interest. Chadderton et al. applied RNA interference technology to inject AAV vectors carrying RHO-sir-NA into the subretinal cavity of RP mice caused by RHO mutations, and combined implantation of wild-type RHO genes, which can effectively treat RP caused by RHO mutations [80]. At present, the genes that inhibit apoptosis include B-cell leukemia 2 (B cell leukemia 2, Bcl-2) family. After Nir et al. introduced the Bcl-2 gene into retinal degeneration slow (RDS) mice, the number of apoptotic cells in mice expressing Bcl-2 decreased. They discuss that Bcl-2 expression is one of the effective ways to treat RDS[81].

Several research groups are currently evaluating the effectiveness of solely administering AAV2/2-RPE65 through subretinal injection. After 6 months of treatment for LCA patients, it was found that there were no significant differences in visual electrophysiology, visual acuity and peripheral visual field before and after treatment. However, the visual

function during dark adaptation of visual field testing of one patient was significantly improved [82]. William et al. treated LCA patients with rAAV and found that visual sensitivity was significantly improved after treatment[83]. Voretigene neparvovec-rzyl (Luxturna), the first gene therapy product used for inheritable retinal dystrophies (IRDs), has been approved for therapeutic use by FDA and EMA [84]. The phase III clinical trial data showed that the treatment group got positive result in multi-luminamance mobility test (MLMT) which is a measure of ambulatory navigation. Meanwhile, the trial got a positive result of the secondary endpoint in full field light sensitivity threshold (FST) which was used for evaluating the function of rod photoreceptors [85].

1.5.2 Potential treatment of retinal degenerations

1.5.2.1 Calcium channel blockers

Commonly used calcium channel blockers are diltiazem, nilvadipine, and nicardipine. Individuals diagnosed with RP can possess genetic mutations in the PDEβ gene. Sanges et al. have demonstrated elevated concentrations of Ca^{2+} in degenerating rods [86, 87]. Elevated levels of Ca^{2+} within cells lead to the disturbance of cellular homeostasis and subsequent to apoptosis of cells [88]. Additionally, there was an observed postponement in the deterioration of the central visual field during the administration of nilvadipine treatment [89].

1.5.2.2 Neurotrophic factors

Many neurotrophic factors are known to alleviate photoreceptor death in animal models, such as brain-derived neurotrophic factors, ciliary neurotrophic factor, etc. Studies have found an increase in retinal thickness and reduced loss of photoreceptors in patients using ciliary neurotrophic factor [90].

1.5.2.3 Valproic acid

There has been controversy surrounding the utilization of valproic acid in individuals with RP. A system review showed the positive function of valproic acid on best-corrected visual acuity (BVCA) of RP patients [47]. However, Totan et al. reported that valproic acid showed no improvement in BCVA for RP patients [91]. Valproic acid may function by inducing microglial dysfunction [92].

1.5.3 Cell transplantation therapy

Cell transplantation therapy can replace damaged photoreceptor cells with altered new connections. It can protect damaged retinal cells from death, thereby improving visual function. It is generally used to treat advanced diseases. Retinal cells could replace lost photoreceptor cells and/or RPE cells with embryonic stem cells and pluripotent stem cells, and marrow stromal cells (MSCs) replace RGCs. A phase I clinical trial for AMD patients showed positive result in BCVA by using an embryonic stem cell derived RPE patch [93].

1.6 Wnt and Norrin mediated β-catenin signaling in the brain and retina

The Frizzled family is a class of highly conserved receptors with seven transmembrane domains [94], which belong to the large family of G protein-coupled receptors[95]. Frizzled proteins, as the receptor for the Wingless Integrated (Wnt) signaling pathway, activate downstream signaling pathways following binding of Wnt ligands[96].

Before the discovery of the Frizzled gene, the proto-cancer oncogene Int-1 in mice and the Wingless gene in fruit flies had been discovered. Since the Wingless and the Int-1 gene are homologs, they are collectively named after their ligand the Wnt gene family [97]. The term Wnt is in turn made up of Wg for Wingless and Int-1[98]. Soon after,

researchers confirmed in fruit flies that the seven-transmembrane protein with an extracellular amino terminus was a product of Frizzled gene expression [99]. Since then, the Frizzled gene has been found in different multicellular organisms, including vertebrates, nematodes, sponges, and hydras [100]. In mammals, the Frizzled protein family was found to include 10 Frizzled proteins and 1 smoothened protein [101]. These frizzled receptors possess a cysteine-rich domain, which enables the binding of Wnt glycolipoproteins. Wnt proteins are secreted glycolipoproteins which specifically bind to Frizzled receptors[102]. In mammals, a total number of 19 Wnt family members have been identified [103].

Following binding of Wnt proteins to frizzled receptors and interaction with their co-receptors LRP5 or LRP6 the β-catenin degradation complex is inactivated leading to an accumulation of constitutive expressed β-catenin in the cytosol [104]. In turn, cytosolic β-catenin translocates into the nucleus to induce the transcription of specific target genes following interaction with LEF/TCF transcription factors [105].

Wnt/β-catenin signaling exerts its influence on a wide array of developmental processes including cellular differentiation, proliferation, and migration, while also being essential for the maintenance of tissue homeostasis. Besides its physiological functions, an imbalance in Wnt/β-catenin signaling has been linked to benign and malignant tumors [106].

In the brain, activation of Wnt/β-catenin signaling induces development of central nervous system (CNS) vasculature, formation and maintenance of memory, synaptic plasticity and blood-brain barrier（BBB）formation [107, 108]. Further on, a protective role for Wnt/β-catenin signaling following damage of CNS neurons has been reported several times [26, 109]. In retina, homologous observations were made, but these effects were primarily mediated by the atypical frizzled4/LRP5 ligand Norrin, which has the unique ability to promote canonical Wnt/β-catenin signaling. The gene product of NDP is Norrin,

which is a secreted protein and forms oligomers through disulfide bonds [110]. Structural analyses of Norrin showed a cystine knot motif, which shares homologies with the cystine knot growth factor superfamily, such as transforming growth factor (TGF)-β and other growth factors [111]. Mutations in the *Ndp* are causative for Norrie disease, an X-linked recessive retinopathy, which leads to blindness at birth or in early childhood, and familiar exudative vitreoretinopathy (FEVR), which is characterized by an abnormal or incomplete peripheral retinal vascularization. The predominant localization of Norrin occurs within the retina and brain, indicating limited distribution of its expression [112]. In retinae of reporter mice that express the mRNA of alkaline phosphatase (AP) under the control of the endogenous *Ndp* promoter ($Ndp^{AP/+}$ knock-in), a strong activity of AP was observed in Müller cells [113, 114]. Murine Norrin expression starts in the late foetal period and continues after development in the eye [112, 115].

Compelling evidence suggests that Norrin plays a crucial role in the formation of retinal vasculature, since Norrin-deficient mice ($Ndp^{Y/-}$) have distinct vascular alterations in the posterior eye with a persistence of hyaloid vasculature and a defective retinal vascularization [116, 117]. In detail, the development of the superficial vascular plexus is markedly delayed, and the formation of the intermediate and outer vascular plexus is completely absence. Vascular alterations in $Ndp^{Y/-}$ mice are not solely confined to the retina but have additionally been detected in the stria vascularis of the inner ear, leading to hearing loss, and in the cerebellum [116]. Homologous findings were observed in Frizzled4 (FZD4)-deficient mice ($Fzd4^{-/-}$), observations that motivated Xu et al. to undertake further studies, which showed Norrin's binding to the FZD4/LRP5 receptor complex and thereby activates the canonical Wnt/β-catenin signaling pathway [118]. Subsequent research revealed that Norrin has a strong affinity to the cystine-rich domain (CRD) of FZD4, while it does not exhibit the same binding activity to the CRD of other frizzled receptors found in mammals or secreted frizzled-related proteins [119]. Further studies strongly suggested

an interaction between the TSPAN12 gene and the FZD4/LRP5 receptor complex due to the similarity of the retinal phenotype of mice with disrupted TSPAN12 gene and *Ndp*$^{y/-}$, *Lrp5*$^{-/-}$ and *Fdz4*$^{-/-}$ animals. In these studies, Junge et al. demonstrated that TSPAN12 can enhance Norrin-mediated Wnt/β-catenin signaling in cooperation with FZD4 and LRP5, but not that of Wnt3a [120]. Additionally, Deng et al. revealed in a recent study that Norrin activates Wnt/β-catenin signaling via binding to the leucine-rich repeat-containing G-protein coupled (LGR)-4 receptor after recruitment of LRP5/6 co-receptors [121].

Regarding possible downstream mechanisms, it was demonstrated that the angiogenic impacts of Norrin-induced activation of Wnt/β-catenin signalling are, to some extent, facilitated by the stimulation of the transcription factor SOX17 in microvascular endothelial cells [114]. Furthermore, the inner blood-retinal barrier's development and maintenance necessitate Norrin signalling, effects that are not mediated via an induction of SOX17 [122, 123].

Following an oxygen-induced retinopathy (OIR), the model of retinopathy of prematurity in mice, our group demonstrated that Norrin can promote vascular repair and inhibit preretinal tuft formation, at least in part via the induction of IGF1 [124, 125]. This Norrin-mediated effect following OIR was not observed in mice with a general activation of Wnt/β-catenin signaling [126]. Furthermore, there is strong evidence that Norrin has the ability to counteract the anti-angiogenic effects of TGF-β signaling in the retina by inducing SMAD7 expression, a strong suppressor of the canonical TGF-β pathway.

Besides its angiogenic functions, Norrin has also neuroprotective effects in the retina. In recent years, it has been shown that Norrin, independent of its angiogenic effects, also mediates distinct protective effects on retinal neurons. In immortalized retinal neurons, Lin et al. observed a reduced number of apoptotic cells after incubation with Norrin, which was independent of any influence of Wnt/β-catenin signaling [127]. However,

following an acute excitotoxic injury of RGCs, Norrin decreases the number of apoptotic RGC via activation of Wnt/β-catenin signalling pathway in Müller cells, which in turn results in an upregulation of neuroprotective factors [128, 129]. Homologous results were observed in the retina of transgenic mice with an overexpression of Norrin in the RPE following acute light-induced photoreceptor damage[130]. Further on, in a mouse model of glaucoma, Norrin could protect RGC from degeneration via an enhanced expression of IGF1, which in turn activated pAKT signalling[131].

Overall, the exact cellular and molecular mechanisms via which Norrin mediates its protective effects on retinal neurons are not yet entirely understood.

2. Aim of the thesis

A major current finding was the observation that Norrin could protect photoreceptors against degeneration in a Wnt/β-catenin independent manner via activation of the AKT pathway in mice that had a transgenic overexpression of Norrin in RPE (Rpe65-Norrin) and a chronic degeneration of photoreceptors (VPP mice). Recent publications report that binding of Wnt7 or Wnt3a to frizzled7 can directly activate the phosphatidylinositol-3-kinase (PI3K)/AKT pathway after recruitment of the insulin-like growth factor 1 receptor (IGF1R)[132, 133].

Because of this, we addressed the question of whether Norrin can enhance AKT signaling via a direct frizzled4/IGF1R complex-mediated activation of the AKT pathway.

For this, FZD4 and IGF1R were overexpressed by specific plasmids in immortalized retinal photoreceptors (661W cells). After transfection of 661W cells with one or both plasmids, transfected cells were selected with hygromycin (IGF1R) and/or puromycin (FZD4). The overexpression of FZD4 and IGF1R was confirmed by real-time RT-PCR, western blot analysis and immunohistochemical staining.

After successful generation of 661W cells with an overexpression of FZD4, IGF1R and FZD4/IGF1R, cells were differentiated with staurosporine to induce a growth arrest and a neuronal phenotype. To test if Norrin could enhance survival of neurons via FZD4/IGF1R-mediated activation of the AKT signaling pathway, differentiated 661W cells with an overexpression of FZD4 and/or IGF1R were incubated with Norrin in serum free cell culture medium, since the cultivation of differentiated 661W cell under serum free conditions induces apoptosis. Cell viability was examined using WST-1 analysis after incubating the cells for both 24 and 48 hours.

Further on, the AKT/pAKT signaling pathway was examined through western blot analysis and immunohistochemistry to investigate the activation caused by Norrin in FZD4/IGF1R overexpressing 661W cells.

Finally, to further analyze whether FZD4 and IGF1R bind as a complex after activating by Norrin, the protein interactions of FZD4 and IGF1R were studied by immunocytochemistry and duolink proximity ligation assay.

3. Materials and Methods

3.1 Main Materials and Instruments

3.1.1 Instruments

Instruments	Manufacturer
Balance(ScoutTM Pro)	Wagendienst Winkler GmbH, Germany
Cell counting chamber	Paul Marienfeld GmbH, Germany
Centrifuge(Eppendorf 5417R)	Eppendorf, Germany
Centrifuge(Eppendorf 5424R)	Eppendorf, Germany
Centrifuge(Eppendorf 5810R)	Eppendorf, Germany
Computer(Desktop with control software)	Philips, Netherlands
Computer(Desktop with control software)	LG, Korea
Freezer(4°)	Bosch, Germany
Freezer(-20°)	Bosch, Germany
Freezer(-20°)	Liebherr, Germany
Freezer(-80°)	Heraeus, Germany
Incubator	Heraeus, Germany
Microscope(DMIL)	Leica, Germany
Microscope(DMR)	Leica, Germany
Vortexer(Scientific Industries vortex-genie2)	Fisher Scientific GmbH, USA
Vortexer(L46)	Corning, USA
Water bath	Memmert GmbH, Germany
Water bath(SW21)	Julabo Labortechnik GmbH, Germany
Real time cell analyzer(CFX Connect)	BIO-Rad, Singapore
Laminar Flow	Thermo Scientific, USA
Microwave	Panasonic, Japan

Pipette	Eppendorf, Germany
Shaker(VX7)	Janke and Kunkel, Germany
Shaker(KL2)	Johanna Otto GmbH, Germany
Spectrophotometer	Eppendorf, Germany
Water purification system(MilliQ EQ7000)	Millipore, Germany
Heating Plate(IKA C-MAG HS7)	Ludwig, Empgenzeder, Germany
Thermomixer comfort	Eppendorf, Germany
Stunningly easy western blot imaging system(Ibright CL1000)	Thermo Scientific, USA
Scanning UV-VIS Photometer(Spectramax 190)	Molecular Devices GmbH, USA
Semi-Dry Transfer Cell System	BIO-RAD, USA
Roller mixer(SRT6)	Stuart GmbH, UK
Gel electrophoresis Chamber System	BIO-RAD, USA
Electrophoresis Power Supplies(power pac200)	BIO-RAD, USA

Table 1： Main instruments

3.1.2 Consumables

Consumables	Provider information
Petri dishes	Thermo Scientific, USA
Cell culture plates (6-well)	Cellstar, Germany
Cell culture plates (96-well)	Cellstar, Germany
Cover glass	Paul Marienfeld, Germany
Coverslips	Paul Marienfeld, Germany
Parafilm	Parafilm M, USA
Pipette tips	Sarstedt, Germany
Reaction tubes	Eppendorf, Germany
Nitrile gloves	Meditrade, Germany

Syringes	Becton Dickinson GmbH, Germany
SuperFrost® Plus microscope slides	Epredia, USA
Screw cap tube,15ml	Sarstedt AG&Co.KG, Germany
Screw cap tube,50ml	Sarstedt AG&Co.KG, Germany
Pittman (10µl 100µl 1ml)	Eppendorf, Germany
Culture flask T25	Sarstedt AG&Co.KG, Germany
Culture flask T75	Sarstedt, Germany
96-well plates for luminometer	Sarstedt, Germany

Table 2: Main Consumables

3.2 Cell Culture

3.2.1 Supplies and substances for cell cultivation

Reagent/Medium	Provider information
DMEM	BIO SELL, Munich, Germany
Fetal Calf Serum (FCS)	Life Technologies, Karlsruhe
Hygromycin B Solution	Chem Cruz, Netherlands
Penicillin-Streptomycin	Life Technologies, Karlsruhe
Phosphate Buffered Saline (PBS)	Sigma, UK
Puromycin	Gibco, Germany

Table 3 : Reagents and mediums for cell cultivation

3.2.2 General cell culture protocols

Cells were cultured and treated in a sterile environment. The utilized materials, solutions and buffers were sterilized using an autoclave. Sterile solutions and media were used beneath a sterile workbench, alongside altering the medium and treating the cells. Cell

culture was performed in incubators that maintained a consistent temperature and carbon dioxide level. Cells were observed using an inverted microscope every alternate day to assess the condition of confluence.

When cells reached confluence, the medium was discarded and the monolayer was washed three times with PBS. Then 3 ml of trypsin was added to cover the cell surface. After three minutes, culture medium with 10% FCS was added to stop the digestion of the pancreatic enzymes. Following the transfer of the cell suspension into a culture tube, the cells were centrifuged at 1,000 rpm for 5 min and resuspended in a 5ml cell culture medium. The suspension with cells was then transferred to a fresh cell culture flask containing medium depending on the density of the transplant. When long-term preservation was required, cells were resuspended in cell culture medium containing 10% dimethylthiosulfone (DMSO) and then slowly frozen at -80 °C. For cell quantification, a Neubauer chamber was used in accordance with the manufacturer's instructions.

3.2.3 Cultivation of 661W cells

Composition of culture medium and digest solution

Culture medium	**Digesting solution**
500 ml DMEM	PBS
50 ml FCS	0.25% Trypsin
10ml Penicillin/ Streptomycin	-

Table 4 : Composition of culture medium and digest solution for 661W cells.

The immortalized mouse photoreceptor cell line (661W[134]) was used to explore the neuroprotective effects on photoreceptor cells. Ten microliters of the cell solution were used to count the cells using a Neubauer chamber. A volume equal to $4*10^5$ cells was then pipetted into a 75cm2 flask and left to attach for at least four hours. The cells were then examined under a microscope for signs of adhesion.

3.3 Plasmid construction and cell transfection

3.3.1 Plasmids for FZD4 and IGF1R overexpression

For overexpression of the mammalian FZD4 gene, the pReceiver-M68/pCMV-FZD4 plasmid was used which contains an additional Puromycin resistance gene for cell selection (EX-U0507-M68, GeneCopoeia, USA). For overexpression of the IGF1R gene, the pCMV3-HA-IGF1R with an additional expression of a resistance gene against hygromycin B was purchased from Sino Biological (HG10164-NY pCMV3-HA-IGF1R, Sion Biological).

3.3.2 General protocol of transfection

Lipofectamine™ 3000 Reagents
Lipofectamine™ 3000 (red) P3000™ (yellow)
Other reagents and Materials
Opti-MEM™
1.5ml tubes
Plasmid

Table 5: Reagents and materials for transfection

Prior to transfection, the 661W cells were grown to a confluency of 70% to 90% in a 6-well cell culture dish. Prior to transfection, 3µl Lipofectamine™ 3000 Reagent were diluted in 125µl serum free Opti-Mem medium. Up to 2.5µg plasmid and 5µl of P3000 Reagent were diluted in 125µl Opti-MEM™ medium to produce the master plasmid mixture. Then, the diluted plasmid master mix was introduced gently to the diluted Lipofectamine™ (1:1 ratio). Following incubation of the blend a duration of 15 minutes at room

temperature, 250µl per well was given to the cells and incubated at 37° for 2-4 days. Following transfection 10µg/ml of puromycin and 100µg/ml of hygromycin B were added to the cell culture medium to select pCMV-FZD4 and pCMV3-HA-IGF1R transfected 661W cells, respectively.

3.4 The WST Viability Assay

For viability analysis, the formazan salt WST-1 (Merck) was used, which will be reduced by an NADH dependent mitochondrial reductase system and hence changes its colour from slightly red to dark red. The number of viable cells is proportional to the concentration of the reduced formazan salt.

At the end of the cell incubation, the cell culture medium was discarded and replaced with 70µl of cell culture medium diluted with WST-1 at a 1:10 ratio. The cells were subsequently incubated for up to 30 minutes at 37°C in an incubator. Absorption at 450 nm as well as at 690 nm as reference measurement was performed using an ELISA reader. For quantification the absorption at 690 nm was subtracted from that at 450 nm followed by a normalization to the control group.

3.5 RT-QPCR analysis of FZD4 and IGF1R mRNA

Following transfection 661W cells with overexpression of FZD4 and/or IGF1R were analysed for their mRNA expression by quantitative real-time PCR to identify transgenic cell clones with homologous expression intensity between the groups for further experiments.

3.5.1 Reagents, Materials and Equipment

Reagent	**Company**
Chloroform	Roth, Karlsruhe
iScript™ cDNA Synthesis Kit	BioRad, Germany
Isopropanol	Roth, Karlsruhe
PCR Plates	Biorad, Germany
RNase-free water	Roth, Karlsruhe
SYBR-Green Supermix	BioRad, München
Trifast	VWR, Denmark
Tris-HCl	Roth, Karlsruhe
Equipment	**Company**
CFX Connect™ Real-time PCR Detection System	BioRad, München

Table 6 : Reagents, materials and equipment for RT-QPCR

3.5.2 RNA isolation from cellular samples

For total RNA isolation from transfected 661W cells and wildtype controls, the cells were homogenized in 400µl Trifast with a cell scraper and were transferred from into an RNase-free 1.5ml reaction tube. Subsequently, 80µl of chloroform was added, stirred for 10 seconds and incubated at room temperature for 5 minutes. Following centrifugation at +4 °C for 15 minutes at a speed of 12000g, the liquid phase was cautiously transferred to a fresh RNase-free reaction tube and an equal amount of isopropanol was added. To enhance the formation of precipitation, the specimen was agitated vigorously and stored at a temperature of -20 °C for a duration of 2 hours. After precipitation of the total RNA by centrifugation at 12,000 g for 10 minutes at +4 °C, the pellet was rinsed twice with 500µl

of 75% ethanol, briefly vortexed and centrifuged at 12,000 g for 5 minutes at +4 °C for each washing. The pellet was air-dried and dissolved in 20µl of nuclease-free sterile water. After analysing the concentration and purity of the isolated RNA using spectrophotometry (BioSpectrometer, Eppendorf), it was kept at -80 °C until usage.

3.5.3 cDNA synthesis

For cDNA synthesis, the iScript cDNA Synthesis Kit was utilized for reverse transcription of the RNA in accordance with the manufacturer's instructions (Bio-Rad, Munich).

Up to 1µg total RNA dissolved in 16µl nuclease-free water was added to 4µl of the 5x iScript RT Supermix containing RNase H+ Moloney murine leukemia virus (MMLV) reverse transcriptase, RNase inhibitor, dNTPs, $MgCl_2$ and oligo(dT) as well as random primers. A sample without reverse transcriptase was prepared in parallel to check for DNA contamination (negative control). For reverse transcription, the reactions were incubated in a master cycler (Eppendorf) for 5 min at 25°C for annealing, 20 min at 42 °C for reverse transcription and 1 min at 95 °C for reverse transcriptase inactivation. After cDNA synthesis the cDNA was then frozen at -20 degrees Celsius until usage.

Step:	Temperature	Duration
1. Primer annealing	25 °C	5 mins
2. Reverse transcription	42 °C	20 mins
3. Inactivation of RT	95 °C	1 min
4. Cooling down reaction	4 °C	∞

Table 7: Incubation protocol for cDNA synthesis using the iScriptTM cDNA Synthesis Kit.

3.5.4 RT-qPCR Primer design

For design of qPCR primers, the online program Primer3web was used (https://primer3.ut.ee/). As DNA template the indicated accession numbers were used (Tab. 8). All primers span exon/intron boundaries and were purchased from Thermo Fisher.

Primer	Accession Number	Orientation	Sequence 5' to 3'
hFZD4	NM_012193	fw	TTCACACCGCTCATCCAGTA
		rev	TGCACATTGGCACATAAACA
hIGF1R	NM_000875	fw	TTCAGCGCTGCTGATGTG
		rev	AAGTTCCCGGCTCATGGT
panRPL32	NM_000994	fw	TTGTGAAGCCCAAGATCGTC
		rev	GAACCCTGTTGTCAATGCCT

Table 8: Sequence of primers for quantitative rt-qPCR.

3.5.5 RT-qPCR

For quantitative real-time Polymerase Chain Reaction (RT-qPCR) the CFX Connect Real-Time PCR Detection System (Bio-Rad) with the Bio-Rad CFX Manager software (Version 3.1) was used.

Following cDNA synthesis, the target genes FZD4 and IGF1R as well as the housekeeper gene RPL32 were amplified by specific primers (Table 8), which span exon/intron boundaries to avoid amplification of genomic DNA. After interaction with double-stranded DNA, the fluorescent dye SYBR green emits green light following excitation with blue light[135]. During quantitative rt-PCR, the fluorescent signal will be measured after each amplification cycle and displayed as cycle dependent intensity curve. The cycle-threshold (CT) of target and housekeeper genes will the set in the early exponential phase of the PCR. Following normalization of the target gene to the housekeeper expression by the ΔΔct method[136], the values can be compared between each other.

For rt-PCR, the 2× iTaq Universal SYBR Green SMX mix (Bio-Rad) was used in accordance with the manufacturer's protocol. PCR was performed in a final volume of 15 µL, consisting of 7.5 µL of 2× iTaq Univer SYBR Green SMX mix and 0.16 µL of primer mix (1 µM each, Thermo Fisher). For pipetting, depending on the number of analyses a SYBR Green master mix and the primer mix were prepared (Table 9, 10). The cDNA, -rt control and water control mix were generated as shown in Table 11 and 10µl were pipetted on a 96-well plated (Bio-Rad). Following addition of 5µl of the specific primer mix (Table 10) the 96-well plate was briefly centrifuged and sealed with a sealing film (Bio-Rad) before PCR amplification.

SYBR Green Master Mix	**1x**	**n-times**
2× iTaq Univer SYBR Green SMX mix	7.5 µl	n x 7.5 µl
H2O	2.2 µl	n x 2.2 µl

Table 9 : Generation of the Master mix

	Primer Mix
H2O	122.8 µl
Primer fw	1.1 µl
Primer rv	1.1 µl

Table 10 : Generation of the primer mix

cDNA Mix	**1x**	**n-times**	**-RT control Mix**	**1x**	**n-times**
SYBR Green Master Mix	9.7 µl	n x 9.7µl	Sg MM	9.7 µl	n x 9.7µl
cDNA	0.3 µl	n x 0.3µl	-RT	0.3 µl	n x 0.3µl
H_2O control Mix	**1x**	**n-times**			

SYBR Green Master Mix	9.7 µl	n x 9.7µl
H2O	0.3 µl	n x 0.3µl

Table 11: Generation of cDNA, -rt control and water control mix.

After initial activation at 95 °C for 3 min, the temperature profile was 10 s denaturation at 95 °C and 1 min annealing and extension at 60 °C for 40 cycles (Table 12). As a negative and water control for real-time rt-PCR, RNA that had not been reverse-transcribed and SYBR Green SMX mix which was filled up with nuclease free water used, respectively. For analysis of the specificity of the melting curve and for quantification of the mRNA expression the Bio-Rad CFX Manager software (Version 3.1) was utilized.

Procedure	**Temperature:**	**Duration:**
1. Taq polymerase activation	95 °C	15 mins
2. Denaturation	95 °C	10 s ↔
3. Annealing and extension	60 °C	40 s
4. Step 2 for 39 cycles		
5. Final elongation	95 °C	1 min
6. Transit to melting cycles	55 °C	1 min
7. 81 cycles for melting curve	55 °C + 0.5 °C/cycle	6 s

Table 12: Quantitative real-time polymerase chain reaction protocol.

3.6 Methods in the field of protein biochemistry

3.6.1 Reagents and Materials

Reagent	Company
Acidimetric (NaOH)	Merk, Denmark
Bovine serum albumin (BSA)	Roth, Karlsruhe
Ammonium peroxide di sulfate (APS)	Roth, Karlsruhe
BCA Protein Assay Kit	Thermo Scientific, USA
Coomassie®Brillant blue	Sigma, Taufkirchen
DL-Dithiotreitol (DTT)	Sigma, Taufkirchen
Formaldehyde	Roth, Karlsruhe
Glycerine	Roth, Karlsruhe
Glycine	Roth, Karlsruhe
Potassium chloride (KCl)	Roth, Karlsruhe
Maleic acid	Sigma-Aldrich, USA
Methanol	Roth, Karlsruhe
Skim milk powder	Millopore, Germany
Sodium carbonate (Na2CO3)	Roth, Karlsruhe
Sodium chloride (NaCl)	Roth, Karlsruhe
Prestained Protein Ladder	Biorad, München
Protease Inhibitor Cocktail	Sigma, USA
Rotiphorese® Gel 30	Roth, Karlsruhe
SDS (Sodium Dodecyl Sulphate)	Roth, Karlsruhe
TEMED	Roth, Karlsruhe

Tris	Roth, Karlsruhe
Tween 20	Roth, Karlsruhe
Equipment	**Company**
Spectramax 190 ELISA Reader	Molecular Devices, USA
Empty Columns	Biorad, München
iBright analysis software	Thermo Scientific, USA
Stunningly easy western blot imaging system(Ib-right CL1000)	Thermo Scientific, USA
Micro homogeniser	Roth, Karlsruhe
PVDF Membrane	Roche, Mannheim
Semi-Dry Electrophoretic Transfer Cell	Bio-Rad, USA
Vertica gel electrophoresis chamber	Bio-Rad, USA
Cell Scraper	Sarstedt, Nürnbrecht

Table 13: Reagents and materials for protein biochemistry

3.6.2 Protein isolation for Western Blot

Proteins were obtained from 661W cells for the purpose of conducting Western Blot analysis. To isolate the protein, 500 µl of Ripa buffer was added on ice. Next, the cell suspension was transferred into a 1.5 ml tube and supplemented with 1% of protease inhibitor. After being kept on ice for a duration of 40 minutes and occasionally agitated, the mixture underwent centrifugation at 4°C for 20 minutes at maximum velocity. Eventually, the aqueous phase was gathered for preservation in the freezer set at -80°C until it was needed.

3.6.3 Measuring of protein concentration by BCA-Assay

Protein content in every sample was measured using the bicinchoninic acid (BCA) assay to ensure that equal amounts of each sample could be loaded in immunoblotting. By BCA assay proteins reduce Cu^{2+} in a dose dependent manner to Cu^{1+}. Bicinchoninic acid forms with Cu^{1+} a specific intense purple complex that could be quantified at a wavelength of 560 nm. Standard concentrations of bovine serum albumin (BSA) were obtained by diluting a BSA master solution (2 mg/ml) with the same buffer to a final concentration of 2000, 1500, 1000, 800, 600, 400, 200 and 100 µg/ml BSA. The titrations were carried out in a 96-well plate, utilizing 10µl of every protein sample (both in their original form and diluted by a factor of 1:2), along with the customary BSA concentrations. After addition of 200µl of BCA solution (reagent A + reagent B = 50:1) to each well, the plate was incubated at a temperature of 37°C for 30 min. The absorbance of each well was measured at 560 nm using the Spectramax 190 ELISA reader, and protein concentrations of the samples were determined by extrapolating their absorbance to the reference curve.

BCA Reagents	
Reagent A	1 mg sodium bicinchoninate (BCA) 2 mg sodium carbonate 0.16 mg sodium tartrate 0.4 mg NaOH 0.95 gm sodium bicarbonate
Reagent B	0.4 mg cupric sulfate (5 x hydrated) in 100 ml distilled water.

Table 14: Composition of BCA reagents

3.6.4 SDS PAGE

Proteins were separated using SDS-PAGE, a technique known as SDS-Polyacrylamide gel electrophoresis, in order to conduct Western blot analysis. The gel electrophoresis process and the preparation of the polyacrylamide gel were conducted following the technique procedure[137].

Buffer/solution	**Components**
SDS-solution 10 % (w/v)	100 g SDS 1000 ml distilled H2O
SDS-PAGE-Running buffer, (10x)	30g Tris-Base 144g Glycine 10g SDS 1000ml distilled H_2O
SDS-loading buffer, 4x	250 mM Tris/HCl, pH 6.8 8 % (w/v) SDS 0.2 % (w/v) Bromophenol blue 40%(v/v) Glycerol 20%(v/v) β-Mercaptoethanol
5 x Electrode buffer	15.14g Tris-Base 93.84g Glycine 0.5 % (w/v) SDS
Tris/HCl, 1,0 M, pH 6,8	121,14 g Tris-Basedissolved in distilled H2O
Tris/HCl, 1,5 M, pH 8,8	181,71 g Tris-Base dissolved in distilled H2O

Table 15: Compositions of SDS PAGE buffer

Gel preparation and electrophoresis

Proteins were separated using a separating gel with a concentration of 8% polyacrylamide. In a nutshell, two alcohol-washed glass plates were inserted into a gel caster. The different parts of the resolving gel were mixed and added into the gap between the two glass plates as rapidly as possible followed by an overlay with isopropanol. After polymerization of the running gel, the remaining isopropanol was removed and the stacking gel mix was added onto the resolving gel. To construct sample slots, a comb was placed into the loading gel to a depth of approximately 1.5 cm. After polymerization, the comb was taken off and the polymerized gels were placed into an electrophoresis chamber and the buffer tanks were filled with 1x electrode buffer. The samples, which contained 15–40 µg of protein each, and a molecular weight marker were loaded into the gel slots before the electrophoresis was started with a voltage of 80 V for 20 minutes followed by 180 V for 40 minutes.

Components of the SDS gel

Component	**Stacking gel (7 ml volume)**	**Resolving gel 8 % (20 ml volume)**
Distilled H2O.	4.013 ml	9.467 ml
Rotiphorese® Gel 30	1.167 ml	5.333 ml
Tris/HCl, 1 M, pH 6,8	1.750 ml	-
Tris/HCl, 1,5 M, pH 8,8	-	5.000 ml
10 % SDS	0,07 ml	0,200 ml
10 % APS	0,07 ml	0,200 ml
TEMED	0,004 ml	0,010 ml

Table 16: Components of SDS gel

Buffer/solution	**Components**
10% (w/v) SDS	100 g SDSAdd 1L dH2O
Tris/HCl, 1.0 M, pH 6.8 (for stacking gels)	121.14 g Tris-Base Dissolve in 1l dH2O, adjust pH.
Tris/HCl, 1.5 M, pH 8.8 (for resolving gels)	181.71 g Tris-Base Dissolve in 1L dH2O, adjust pH,
SDS loading dye, 4x	250m M Tris/HCl, pH 6.8 40% Glycerol -8% (w/v) SDS 0.2% (w/v) Bromophenol blue 0.3 M DTT
5 x Electrode buffer	-15.14g Tris-base -93.84g Glycine -50ml 10% SDS -dissolve in 1L dH_2O

Table 17 : Composition of SDS PAGE gel and buffer components

Components of 10x Transfer buffer	
Tris	5.82 g
Glycine	2.93 g
Methanol	200 ml
SDS	3.7 ml 10 % (w/v) dissolved in 1 L dH2O （PH > 8）

Table 18: Composition of transfer buffer

3.6.5 Semi-dry blotting

After polyacrylamide gel electrophoresis, proteins were transferred onto a PVDV membrane by semi-dry blotting. Following a short pre-treatment in methanol, the membrane was rinsed in ultrapure water for 2 minutes and equilibrated in transfer buffer for 5 minutes. In addition, five pieces of filter paper soaked with transfer buffer were needed to arrange the blotting configuration with 3 filter papers at the cathode, followed by the SDS-PADG and PVDF membrane and at the anode two additional filter papers (Table 19). After rolling out the membrane / SDS-PAGE stack to avoid air bubbles, semi-dry blotting was performed at a voltage of 25 V for a duration of 75 minutes.

Semi-dry Blot layout

cathode (-)
3 filter papers (with transfer buffer)
Separating gel with proteins
PVDF-Membrane (5.5cm x 8.5cm)
2 filter papers (with transfer buffer)
Anode (+)

Table 19: Outline Semi-dry blot layout

3.6.6 Western Blot analysis

PVDF membranes were incubated with primary antibodies (diluted 1:1000 5% BSA in TBS-T containing 5% BSA) overnight after being blocked with 5% low-fat milk in TBST. After three washes for 10 minutes each time in TBST, membranes were incubated with specific alkaline phosphatase (AP)-conjugated secondary (diluted 1:2000 in TBST containing 5% BSA) for 1 hour at room temperature to detect the primary antibody-antigen complexes. After additional three washes in maleic acid for ten minutes each, the

membranes were equilibrated once in detection buffer at room temperature for 5 minutes. and incubated with 300µl of Tropix CDP-Star substrate at room temperature for 3 minutes. The membranes were then visualized on an iBright CL1000 imaging system and quantified by the iBright analysis software.

Primary Antibody	**Species**	**Company**
FZD4	Rabbit	R&D system
GAPDH	Mouse	Millipore
IGF1R	Rabbit	Cell signaling
pAkt	Rabbit	Cell signaling
α-Tubulin	Mouse	Calbiochem
Secondary Antibody	**Species**	**Supplier**
Goat anti-Rabbit-AP	Rabbit	Cell signaling
Goat anti-Mouse-AP	Mouse	Cell signaling

Table 20: Antibodies used for western blot analysis.

3.7 Immunohistochemistry

By applying a primary antibody to attach to a specific epitope of a protein and a secondary antibody that is coupled to a fluorescent dye and binds to primary antibodies, this immunohistochemical staining allows for the localization and quantification of particular proteins inside cells.

3.7.1 Reagents and materials

Reagent/material	Supplier

Cold water fish skin gelatin(40%)	Aurin, The Netherlands
Cover lips	Paul Marienfeld, Germany
GSK1904529A	Selleckchem, USA
Hoechst 33342	Invitrogen, USA
Microscope Slides	Epredia, Germany
Mounting Medium	Invitrogen, Germany
Triciribine	Selleckchem, USA

Table 21: Reagents and materials for immunohistochemistry.

3.7.2 Cell fix

For immunohistochemistry, cells were seeded to be 60% confluent on coverslips in a 6-well plate and incubated in cell culture medium overnight to attach. Then the cells were fixed with 4% Paraformaldehyde for 5 minutes at room temperature, washed three times with 0.1M phosphate buffer for 5 minutes each and stored in 0.1M phosphate buffer at 4°C until further use.

Primary Antibody	**Dilution**	**Company**
Rabbit anti human-FZD4	1:50	R&D system
IGF1R	1:100	Cell signaling
pAkt	1:50	Cell signaling
Secondary Antibody	**Dilution**	**Company**
goat anti mouse(H+L)	1:2000	R&D system
goat anti rabbit(H+L)	1:2000	R&D system

Table 22: Antibodies and dilutions used for immunohistochemistry

3.7.3 General protocol of immunohistochemistry

Coverslips with cells were incubated with primary antibody diluted 1:50 or 1:100 in 10% blocking solution in a humid environment at + 4 °C overnight after being rinsed with 0.1 M phosphate buffer for 10 minutes and blocked with blocking solution for 1 hour at room

temperature. After three 5-minute washes with 0.1M phosphate buffer, the slides were incubated with secondary antibodies diluted 1:2000 in 0.1 M phosphate buffer for 1 hour at room temperature protected from light. Following the slides were incubated with Hoechst 33342 (Thermos Fisher) diluted 1:500 in 0.1 M phosphate buffer for 10 minutes and two additional washings with 0.1 M phosphate buffer, coverslips were mounted with ProLong™ Glass Antifade Mountant Medium (Thermo Fisher). The slides were stored at +4 °C in the absence of light prior to examination under a fluorescent microscope.

3.7.4 Fluorescence microscopy

For fluorescent microscopy, an Axio Observer 7 with an Apotom module was used (Carl Zeiss). Images were documented using the ZEN software (Carl Zeiss).

3.8 The Duolink Site Assay

To analyse interacting proteins the proximity ligation assay, DuoLink® In Situ Red Starter Kit Mouse/Rabbit (DUO92101, Sigma-Aldrich, Darmstadt, Germany), was used in accordance with the manufacturer's instructions.

Coverslips were seeded with cells and cultured overnight. After washing with cold 1x PBS, the cells were fixed with 4% paraformaldehyde for 30 minutes. Following pre-treatment of the cells with Duolink blocking solution for 30 min at 37 °C in a humidified chamber, the slides were incubated overnight with primary antibodies against FZD4 (1:50) and IGF1R (1:100) diluted in DouLink antibody dilutant at 4°C. After washing with wash buffer A, the slides were incubated for 1 hour with two PLA probes (1:5 diluted), followed by 30 minutes in Ligation-Ligase solution and additional 100 minutes in Amplification-Polymerase solution in a humidification chamber that has been preheated to 37°C. After

washing with wash buffer B, the coverslips were mounted with Duolink in situ mounting agent with DAPI on slides and analysed by fluorescent microscope.

3.9 Statistics

All Statistics were conducted with students t-test when two groups were compared or One-way ANOVA when three or more groups were analysed via Prism 9. As post hoc test for data that met the assumption of homogeneity of variances, a least significant difference (LSD) test was performed, and for data that did not meet the criteria, a Games–Howell test was performed. p values less than 0.05 were considered statistically significant.

4. Results

4.1 The FZD4 and IGF1R were overexpressed in immortalized retinal photoreceptors (661W cell lines)

To analyze if Norrin could mediate protective effects on retinal neurons via activation of the AKT pathway, immortalized photoreceptor cells (661W cell line), were used. After transfection of 661W cells with one or both plasmids, transfected cells were selected with hygromycin (IGF1R) and/or puromycin (FZD4). The overexpression of FZD4 and IGF1R was confirmed by RT-PCR (Figure 2A, B) and western blot analysis (Figure 2C, D). Cell division into four groups was conducted. The first group was assigned as the control, while groups A, B, and C were all transfected with FZD4 and IGF1R simultaneously and selected with hygromycin and puromycin. Based on the bar graph, it was evident that both FZD4 and IGF1R exhibited the highest expression in Group A when compared to the other groups. Whereas the lowest expression levels were observed in Group B. In contrast, Group C demonstrated a relatively moderate level of expression. Based on the result of RT-PCR, C group was chosen for further study.

By western blot analysis, only a fading signal for FZD4 expression was observed in control and pCMV-IGF1R transfected 661W cells (Figure 2C). In contrast, in pCMV-FZD4 and pCMV-FZD4/IGF1R cells a robust as well as a moderate FZD4 expression was observed when compared to untransfected controls (Figure 2C). In line, following hybridization of control and pCMV-FZD4 transfected 661W cells with IGF1R antibodies, only a weak band for IGF1R was detected (Figure 2D). However, in pCMV-IGF1R and pCMV-FZD4/IGF1R cells, a robust as well as a moderate IGF1R expression was observed when compared to untransfected controls (Figure 2D).

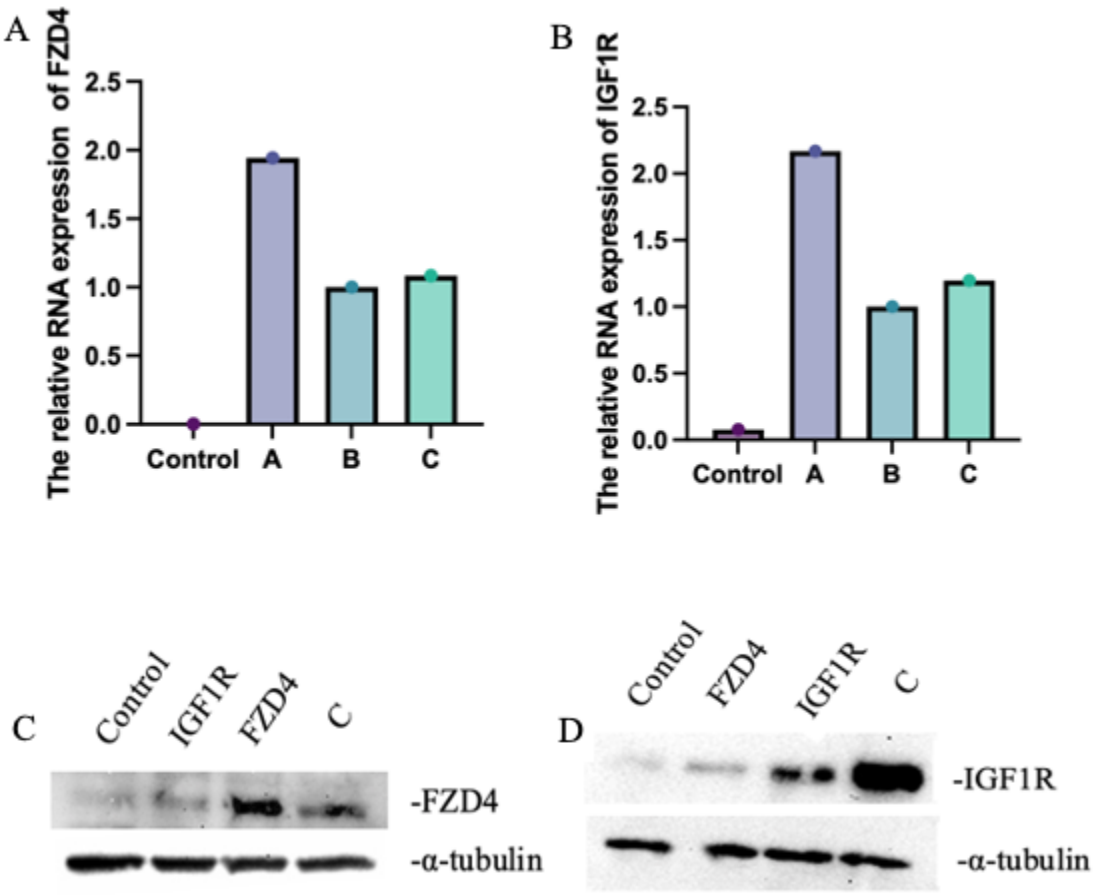


Figure 2: RNA and protein expressions of FZD4 and IGF1R in 661W cells following transfection with overexpression vectors. A, B. RNA expression of FZD4 (A) and IGF1R (B) in the control group and A, B, C group with double transfection of FZD4 and IGF1R. C, D. The western blot analysis for FZD4 (C) and IGF1R (D) overexpression following transfection of 661W cells with pCMV-FZD4 and/or pCMV-IGF1R as well as control cells.

4.2 Norrin activates pAKT signaling in double transgenic 661W cells

To investigate whether Norrin can activate the pAKT signaling pathway via IGF1R and FZD4, double transfected FZD4/IGF1R photoreceptor cells and native controls were incubated with Norrin in cell culture medium for 4 hours. In native 661W cells, only a weak band for pAKT was seen after treatment with Norrin, which was similar to that of untreated controls (Figure 3A). In contrast, in double transgenic FZD4/IGF1R 661W cells, an enhanced signal for pAKT was observed following Norrin treatment when compared to double transgenic control cells (Figure 3A). By densitometry, a 3.0-fold significant increase of pAKT was detected in double transgenic 661W cells after Norrin treatment when compared to untreated control cells (Figure 3B).

By immunohistochemistry, only a weak signal for pAKT could be seen in untreated native and FZD4/IGF1R transfected 661W cells. In contrast, following treatment with Norrin, an intense signal for pAKT was observed in the perinuclear cytoplasm of FZD4/IGF1R transfected 661W cells, which was in line with the western blot analyzes (Figure 3C).

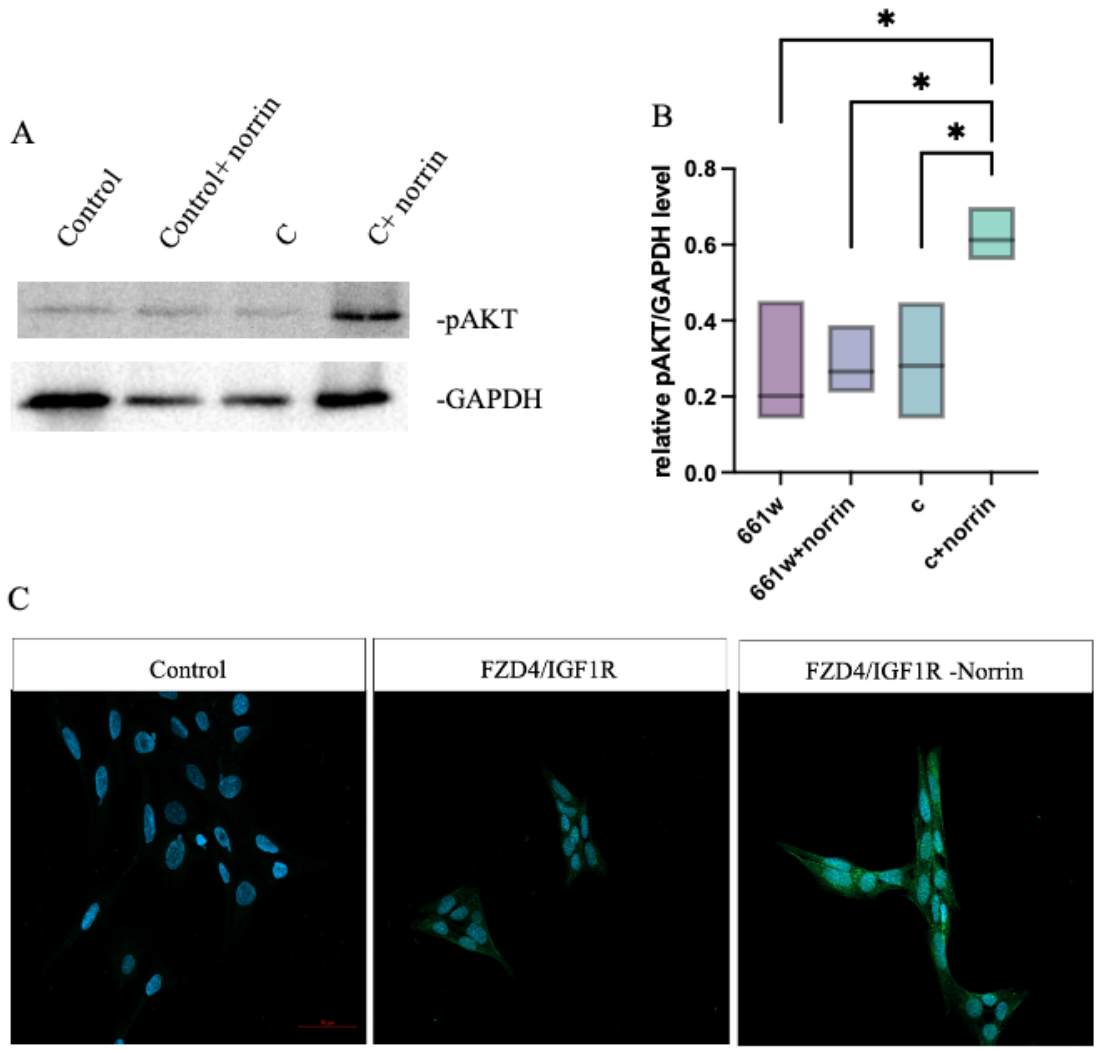
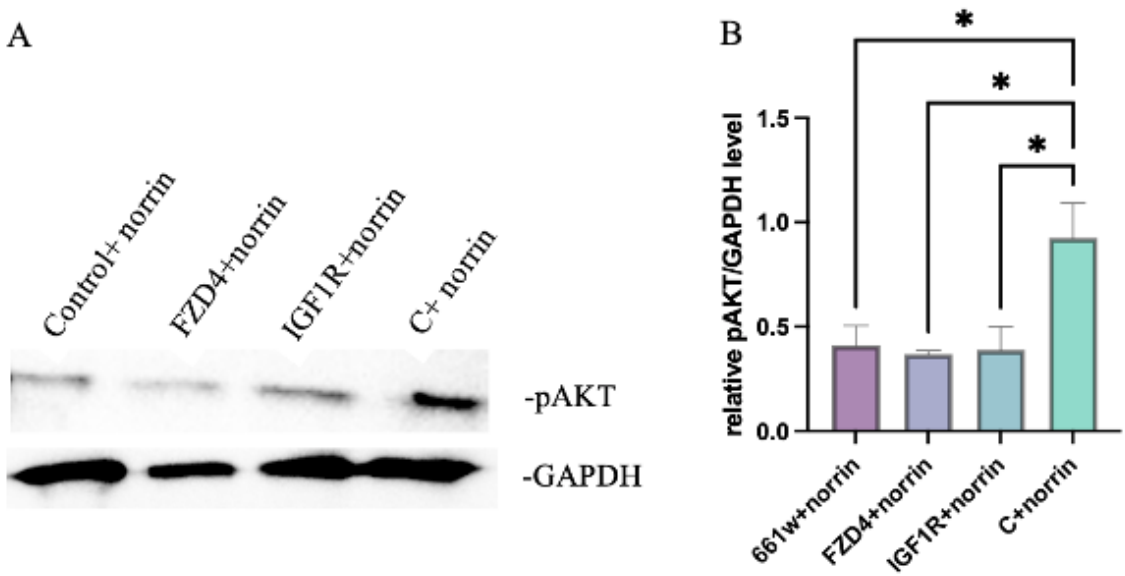


Figure 3: Norrin activates pAKT signalling in double transgenic FZD4/IGF1R 661W cells. A, B. Western blot analysis (A) and densitometry (B) for pAKT of native control (Control) or double-transgenic 661W cells (FZD4/IGF1R) with and without incubation with 200ng/ml Norrin for 4h (A; mean± SEM; *P< 0.05). C. Immunohistochemistry for pAKT of native control and double-transgenic 661W cells with or without Norrin incubation. Blue, DAPI staining; Scale bars:50µm.

To further analyze if Norrin can enhance pAKT signaling solely via FZD4 or IGF1R, FZD4, IGF1R and double transgenic 661W cells were incubated with Norrin for 4h.

Following incubation of FZD4 or IGF1R overexpressing 661W cells with Norrin only a weak but specific band could be seen which was similar in strength to that of the control cells (Figure 4A). In line with previous results, in FZD4/IGF1R 661W cells an intense signal for pAKT was detected following treatment with Norrin, a result that was statistically significant when compared to FZD4, IGF1R and native control 661W cells (Figure 4A, B).

By immunohistochemistry in FZD4, IGF1R and native control 661W cells only a fading signal for pAKT was observed after treatment with Norrin for 4h (Figure 4C-E). By contrast in FZD4/IGF1R transfected 661W cells, a strong perinuclear signal for pAKT was observed following treatment with Norrin for 4h (Figure 4F). Overall, these data strongly suggest that for Norrin-mediated direct pAKT signaling FZD4 and IGF1R are required.



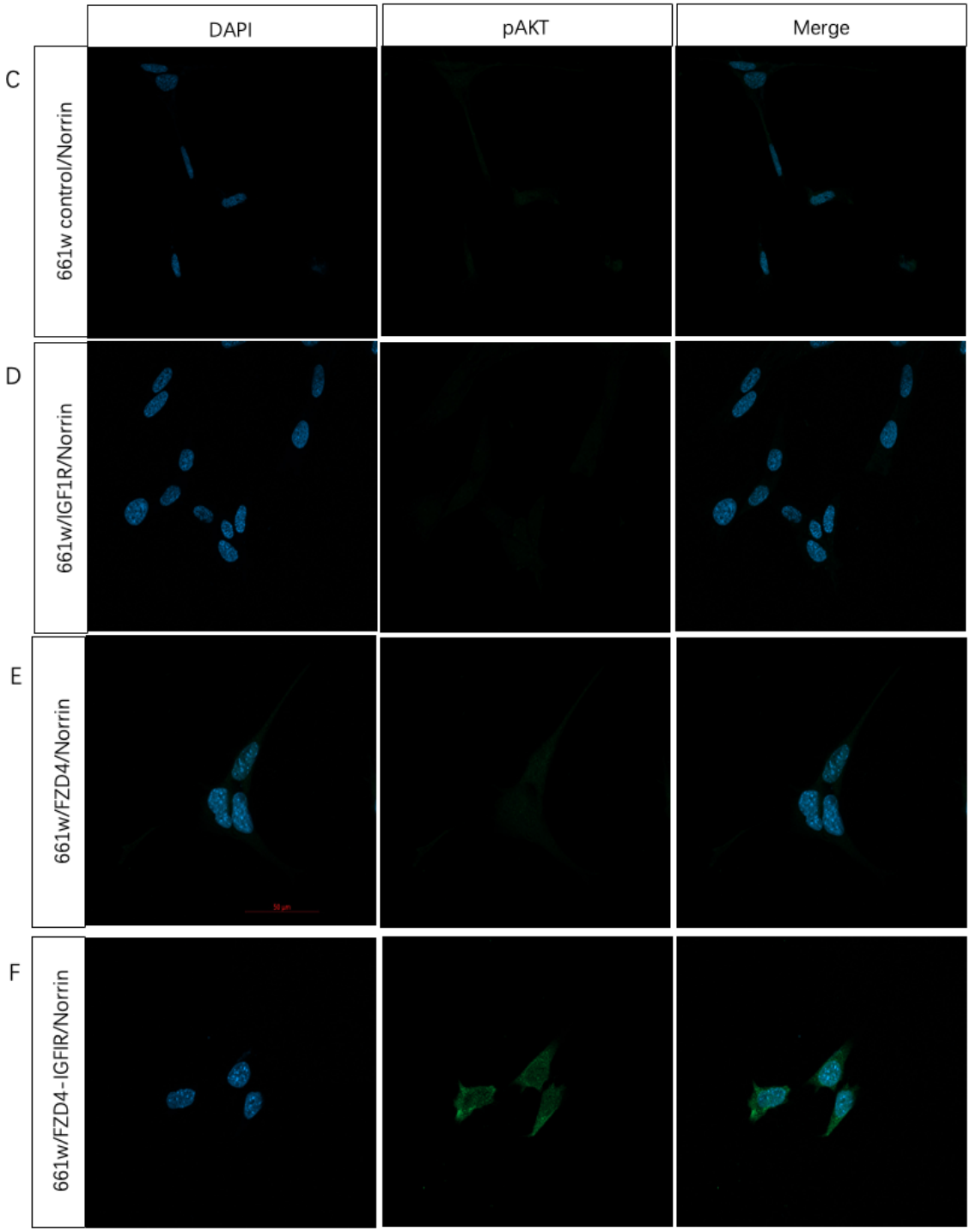


Figure 4: Direct Norrin-mediated pAKT signaling requires the overexpression of FZD4 and the IGF1R in 661W cells. A, B. Western blot analysis (A) and densitometry for pAKT on proteins from FZD4, IGF1R and double transgenic 661W cells after incubation with Norrin for 4h (mean± SEM, *P< 0.05).

4.3 Norrin mediates its neuroprotective effects via pAKT/AKT signaling in 661W cells

4.3.1 Norrin mediates neuroprotective effects in FZD4/IGF1R 661W cells

To examine if Norrin can mediate neuroprotective properties via an enhanced direct pAKT signaling 661W cells were differentiated with staurosporine and cell viability was analysed by measurement of the NADPH turnover using the WST-1 assay.

After treatment with 10 µ/ml staurosporine for 30 minutes, 661W cells underwent morphological changes, exhibiting a more neuronal appearance. Dendrites and axons with synapse-like structures emerged from the cell body.

Following differentiation with staurosporine, the cells were subsequently subjected to serum deprivation to induce apoptosis of the cells and were incubated with Norrin. After treatment for 24h, no differences in WST-1 substrate turnover were observed between native 661W cells with or without additional Norrin incubation (Figure 5). Further on, in FZD4/IGF1R overexpressing 661W cells, a slight increase of WST-1 turnover was detected. In contrast, in FZD4/IGF1R 661W cells with an additional treatment with Norrin, an enhanced WST-1 substrate signal of approximately 1.3-fold was detected when compared to untreated controls and native 661W cells. In summary, these data strongly suggest that Norrin can protect FZD4/IGF1R 661W cells against starving-induced apoptosis.

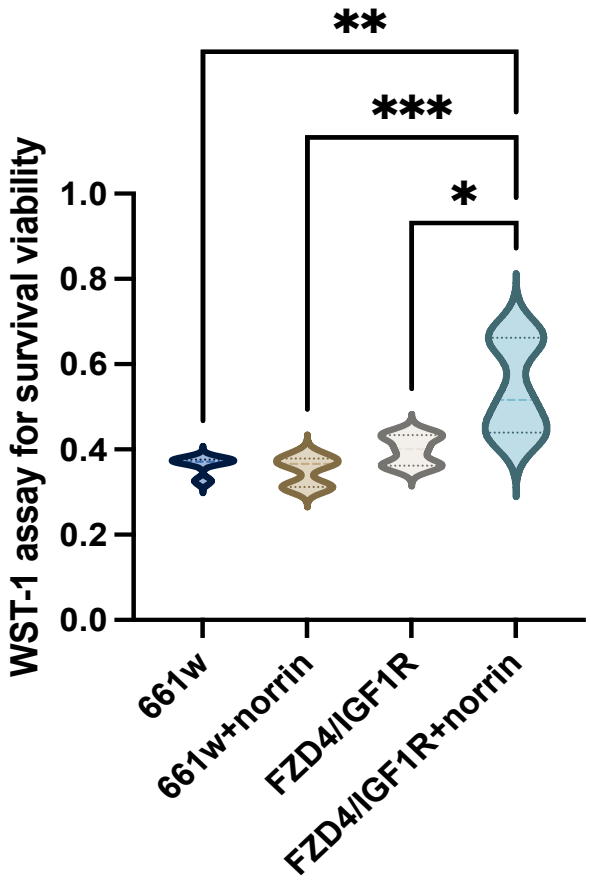


Figure 5: Norrin protects FZD4/IGF1R overexpressing 661W cells against starving-induced apoptosis. WST-1 assay of staurosporine differentiated FZD4/IGF1R and native 661W cells with and without incubation with 200ng/ml Norrin for 24h (One-way ANOVA multiple comparisons, mean± SEM, P*<0.05, P**<0.01, P***<0.001).

4.3.2 Norrin mediates its neuroprotective effects via AKT signaling

In order to examine whether the neuroprotective effects of Norrin are mediated via AKT signalling, FZD4/IGF1R 661W cells were incubated with Norrin and Triciribine, an inhibitor of the AKT pathway.

After treatment of double transgenic 661W cell with Norrin (Figure 6B) an intense cytoplasmic staining for pAKT was detected by immunohistochemistry which was blocked

by the additional treatment of Triciribine (Figure 6C) demonstrating that Triciribine has the distinct potential to block the AKT pathway.

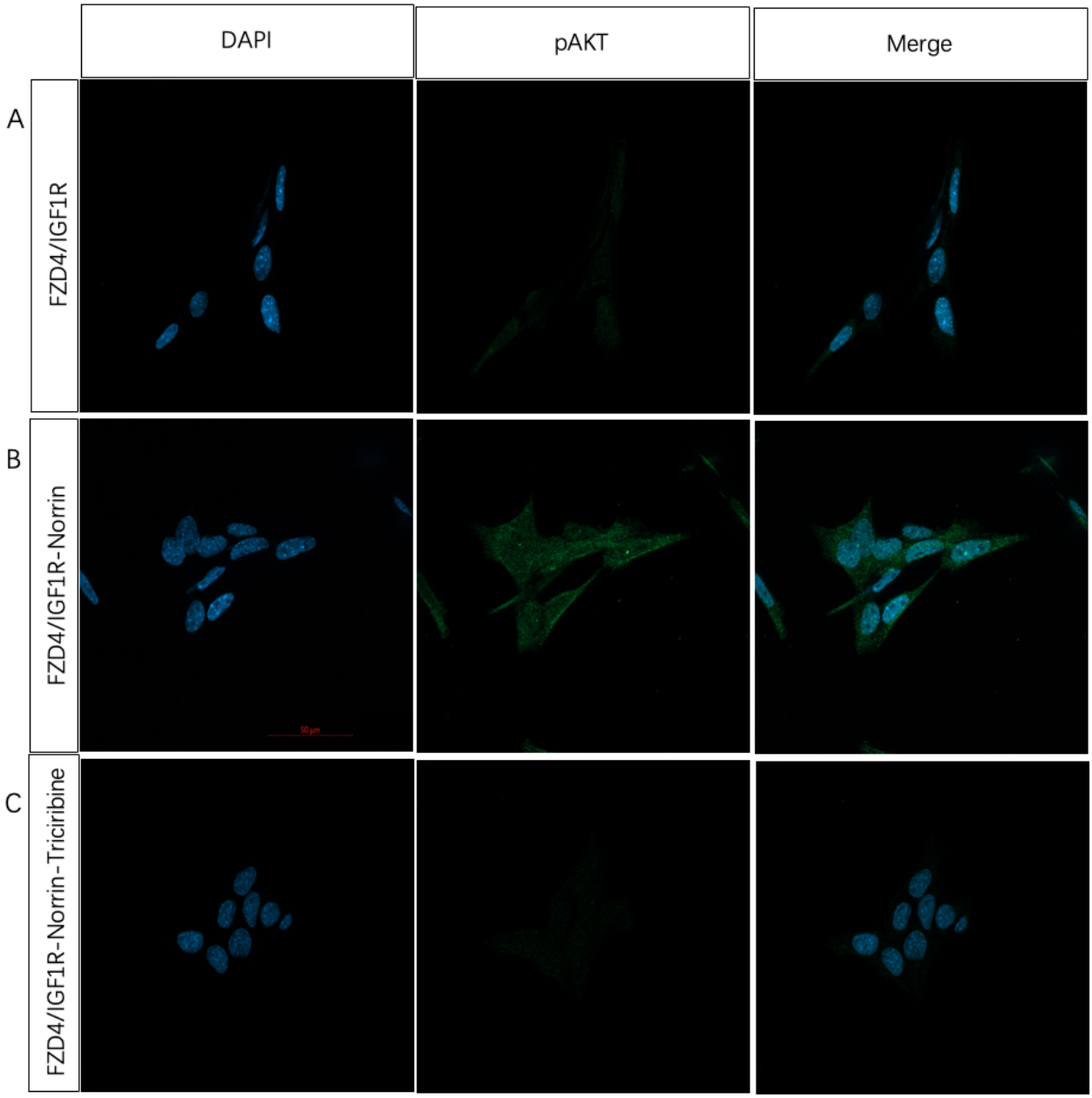


Figure 5: Triciribine blocks Norrin mediated AKT signal in FZD4/IGF1R 661W cells. Immunohistochemical staining of double transgenic 661W cells for pAKT following treatment with 10 µ M Triciribine and/or 200ng/ml Norrin for 4h. Blue, DAPI staining. Scale bar: 50µm.

Since a sufficient inhibition of the AKT pathway could be observed by Triciribine the viability of FZD4/IGF1R 661W cells was investigated by WST-1 assay following treatment with Norrin and Triciribine.

Following differentiation with staurosporine double transgenic 661W cells were incubated with Triciribine to rule out its potential toxic effects. Following incubation for 24h no differences of WST-1 substrate turnover were observed between Triciribine treated FZD4/IGF1R 661W cells and untreated controls (Figure 7A) suggesting that Triciribine does not affect the viability of the cells itself.

As expected, the incubation of double transgenic FZD4/IGF1R cells with Norrin significantly enhances WST-1 substrate turnover when compared to untreated controls (Figure 7B). This effect could be substantially blocked by the additional treatment with Triciribine (Figure 7B) strongly indicating that Norrin mediates its neuroprotective effects on FZD4/IGF1R 661W cells via pAKT signalling (Figure 7B).

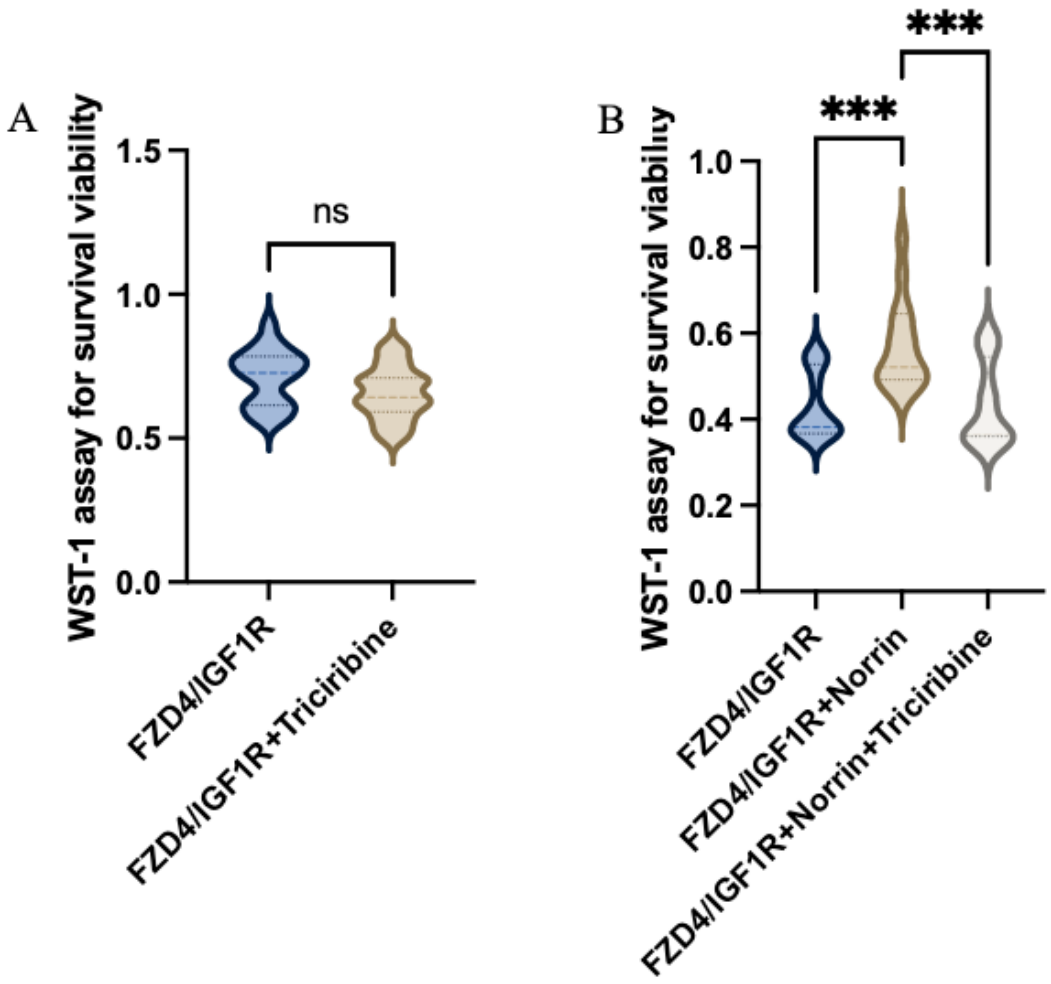


Figure 6: Triciribine blocks Norrin mediated neuroprotective effects on FZD4/IGF1R 661W. WST-1 substrate turnover of differentiated FZD4/IGF1R 661W cells after treatment with 10µM Triciribine and/or 200ng/ml Norrin for 24h (One-way ANOVA multiple comparisons, mean± SEM, P***< 0.001).

4.3.3 Norrin mediates its neuroprotective properties via IGF1R signaling

To analyze if the IGF1R is essential for Norrin-mediated activation of the AKT pathway, the inhibitor GSK1904529A was used to block the IGF1R phosphorylation.

To this end, double transgenic 661W cells were incubated with 10mM GSK1904529A and/or 200ng/ml Norrin. Following incubation for 4h, intense cytoplasmic staining for pAKT was observed by immunohistochemistry when compared to untreated control cells, while the additional treatment with the inhibitor GSK1904529A the Norrin mediated staining for pAKT was blocked, which was homologous to that of untreated control cells (Figure 8).

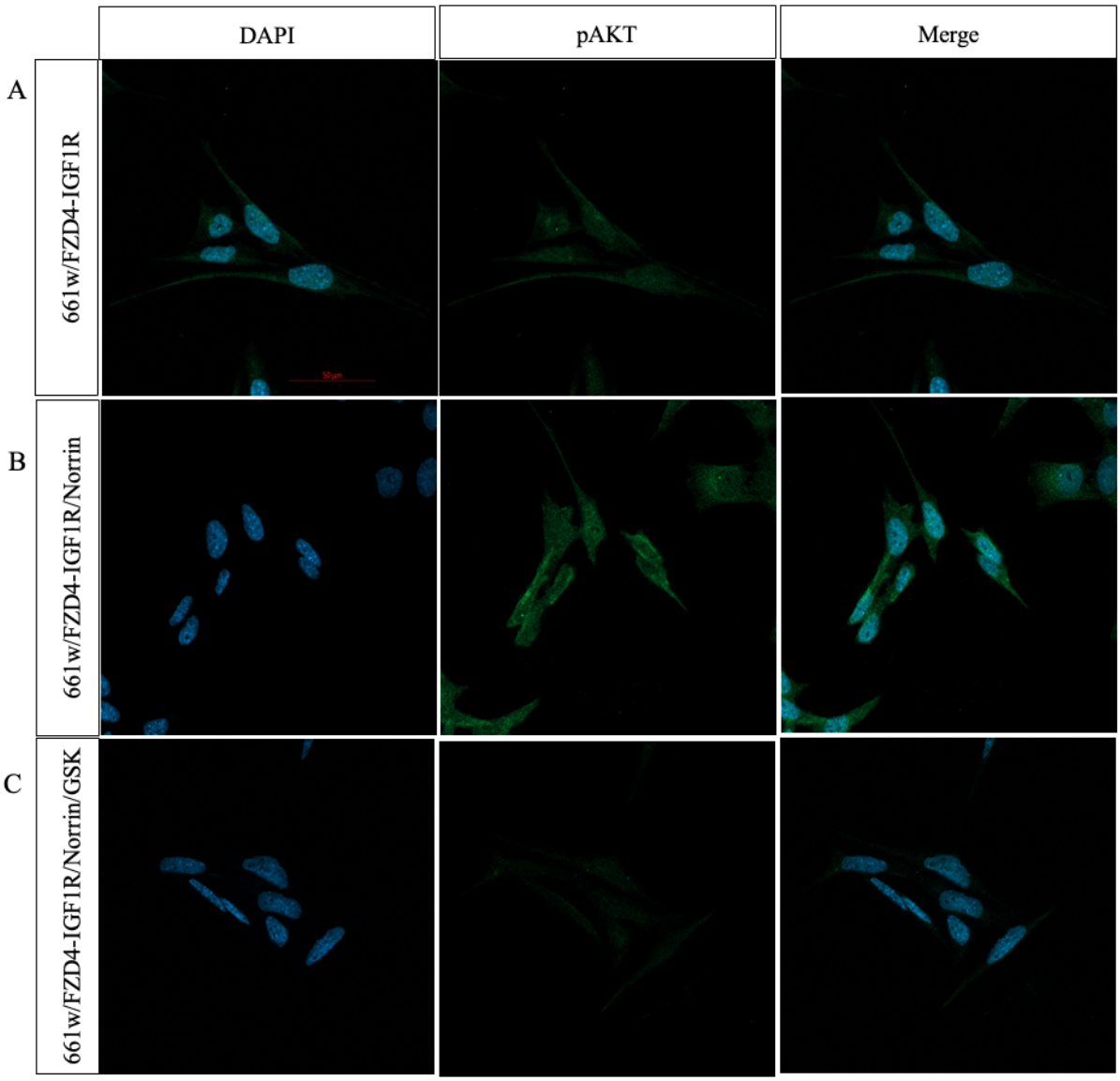


Figure 8: Norrin mediates pAKT signalling in double-transgenic 661W cells via the IGF1R. A-C. Immunohistochemistry for pAKT (green) on FZD4/IGF1R 661W cells after incubation with 10mM GSK1904529A and/or 200n/ml Norrin for 4h. Blue, DAPI staining. Scale bars:50µm.

To further investigate if Norrin mediates its neuroprotective properties on double transgenic 661W cells via the IGF1R, the viability of the cells was analysed after differentiation with staurosporine and IGF1R inhibition.

Following incubation of differentiated FZD4/IGF1R 661W cells with the IGF1R inhibitor GSK1904529A for 24h only a slight decrease of WST-1 substrate turnover was detected when compared to untreated control cells (Figure 9). On the other hand, the treatment of the cells with Norrin led to a substantial increase of reduced WST-1 metabolites, an effect that was completely blocked by the additional incubation with GSK1904529A (Figure 9). Overall, it's most likely that for the Norrin mediated neuroprotective effects via activation of the AKT pathway in double transgenic 661W cells, the IGF1R is essential for signal transduction, since AKT phosphorylation as well as the protective effects could be blocked by the IGF1R inhibitor GSK1904529A.

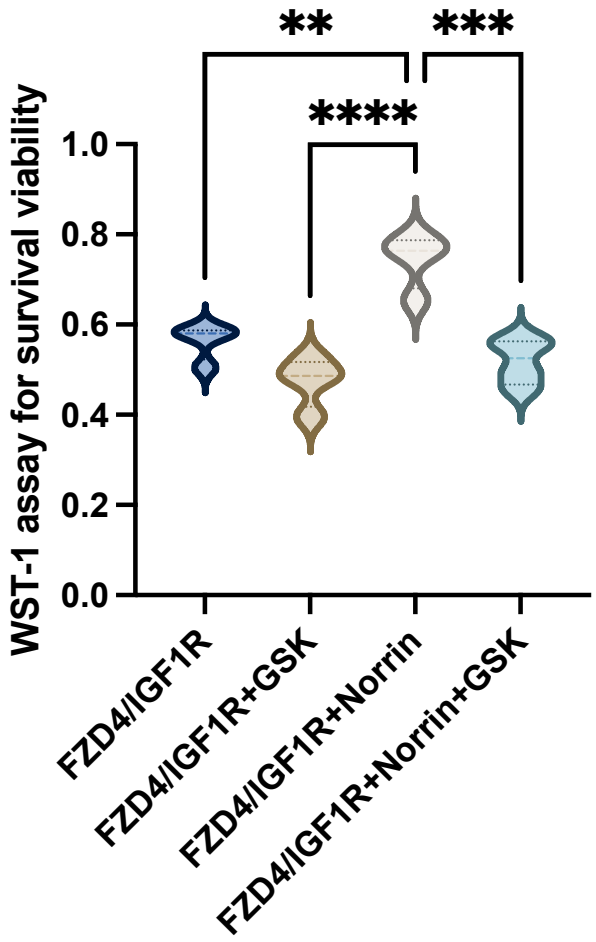


Figure 7: Cell viability assay for double transgenic 661W cells with GSK1904529A and/or Norrin. WST-1 viability assay on staurosporine differentiated double transgenic 661W cells following incubation with 10mM GSK1904529A and/or 200ng/ml Norrin for 24h (One-way ANOVA multiple comparisons, mean± SEM, P*<0.05, P**<0.01, P***<0.001, P****<0.0001).

4.4 Norrin activates pAKT signalling via an interaction between FZD4 and IGF1R

Since Norrin could mediate neuroprotective effects on FZD4/IGF1R 661W cells via the IGF1R it was analysed if the FZD4, after binding of Norrin, recruits the IGF1R which in turn phosphorylates AKT. To this end double transgenic 661W cells were incubated with Norrin for 4h and double immunostaining against FZD4 and IGF1R was performed to detect their co-localization.

In FZD4/IGF1R 661W cells without Norrin treatment a specific signal for FZD4 and IGF1R could be detected in the cytoplasm and on the cell membrane, but only a slight co-localization was observed at the cell membrane (Figure 10). In contrast, following incubation of the cells with Norrin a specific signal for FZD4 and IGF1R was detected as described before, but on the cell surface a co-localization (yellow) between both receptors could be observed (Figure 10) indicating an interaction between both receptors.

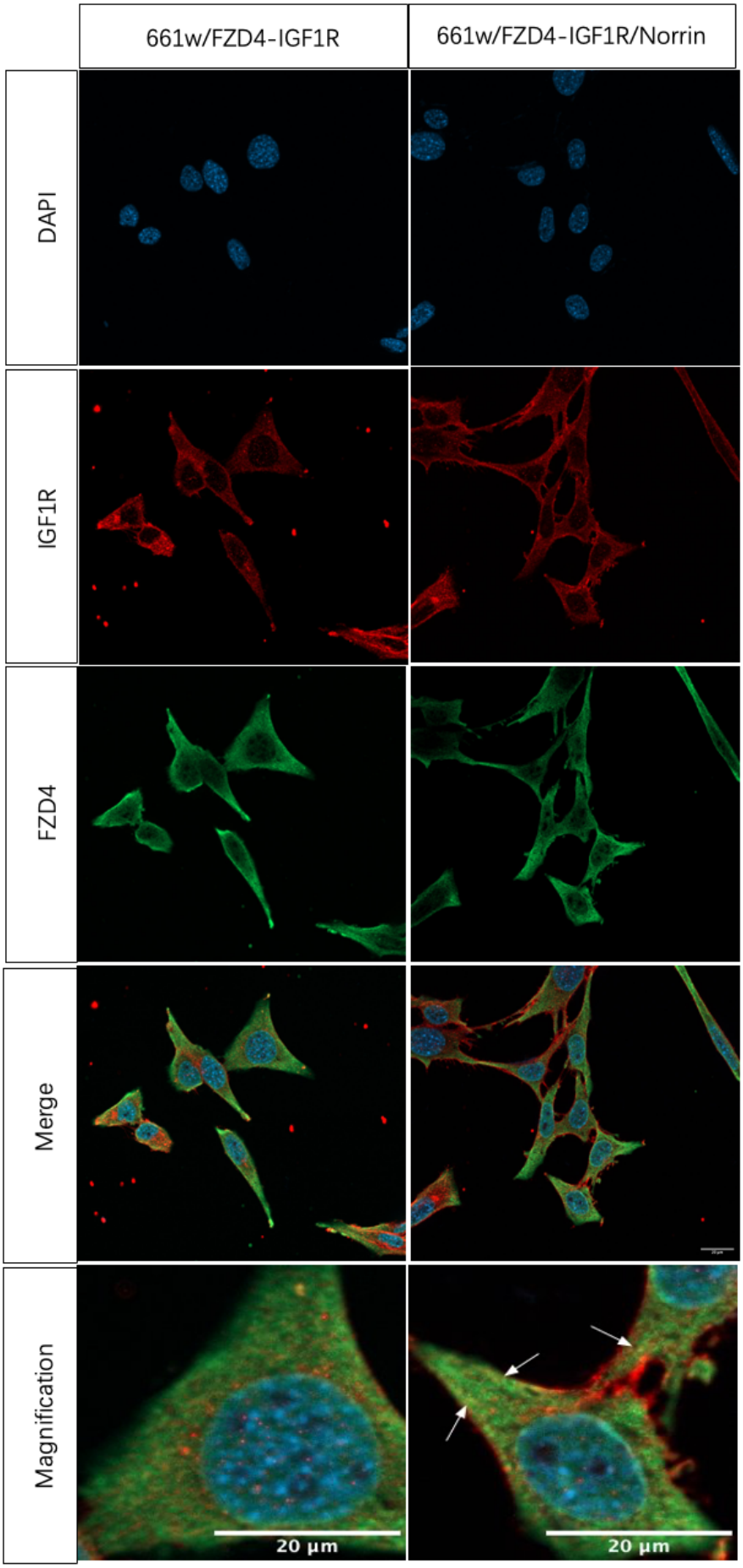

661w/FZD4-IGF1R
661w/FZD4-IGF1R/Norrin
DAPI
IGF1R
FZD4
Merge
Magnification
20 µm
20 µm

Figure 10: Co-localization of IGF1R and FZD4 in double-transgenic 661W cells after incubation with Norrin. Immunohistochemistry for FZD4 (green) and IGF1R (red) on double transgenic 661W cells after treatment with Norrin for 4h. Blue, DAPI staining. Scale bar: 20μm.

4.5 Norrin recruits the IGF1R to FZD4 to mediate direct pAKT signaling

In order to further investigate the direct interaction between FZD4 and IGF1R, a Duolink® Proximity ligation assay was performed. This assay is based on two antibody coupled oligonucleotides which induce a rolling circle amplification, when both are close together within a distance of 40nm, leading to a specific red fluorescence signal and demonstrating a very close interaction between the tested proteins.

In native 661W cells without incubation of Norrin, no specific signal was observed (Figure 11A). In addition, in double transgenic 661W cells without Norrin treatment only a few fluorescent areas were detected on the cell surface (Figure 11C). Further on, in native 661W cells a more pronounced fluorescent labelling was seen following treatment with Norrin when compared to untreated control cells (Figure 11B). In contrast, following treatment with Norrin in FZD4/IGF1R 661W cells an intense fluorescent signal on the cell membrane could be detected, demonstrating a close interaction between both proteins and supporting the hypothesis that Norrin mediates its direct activation of AKT pathway via the IGF1R.

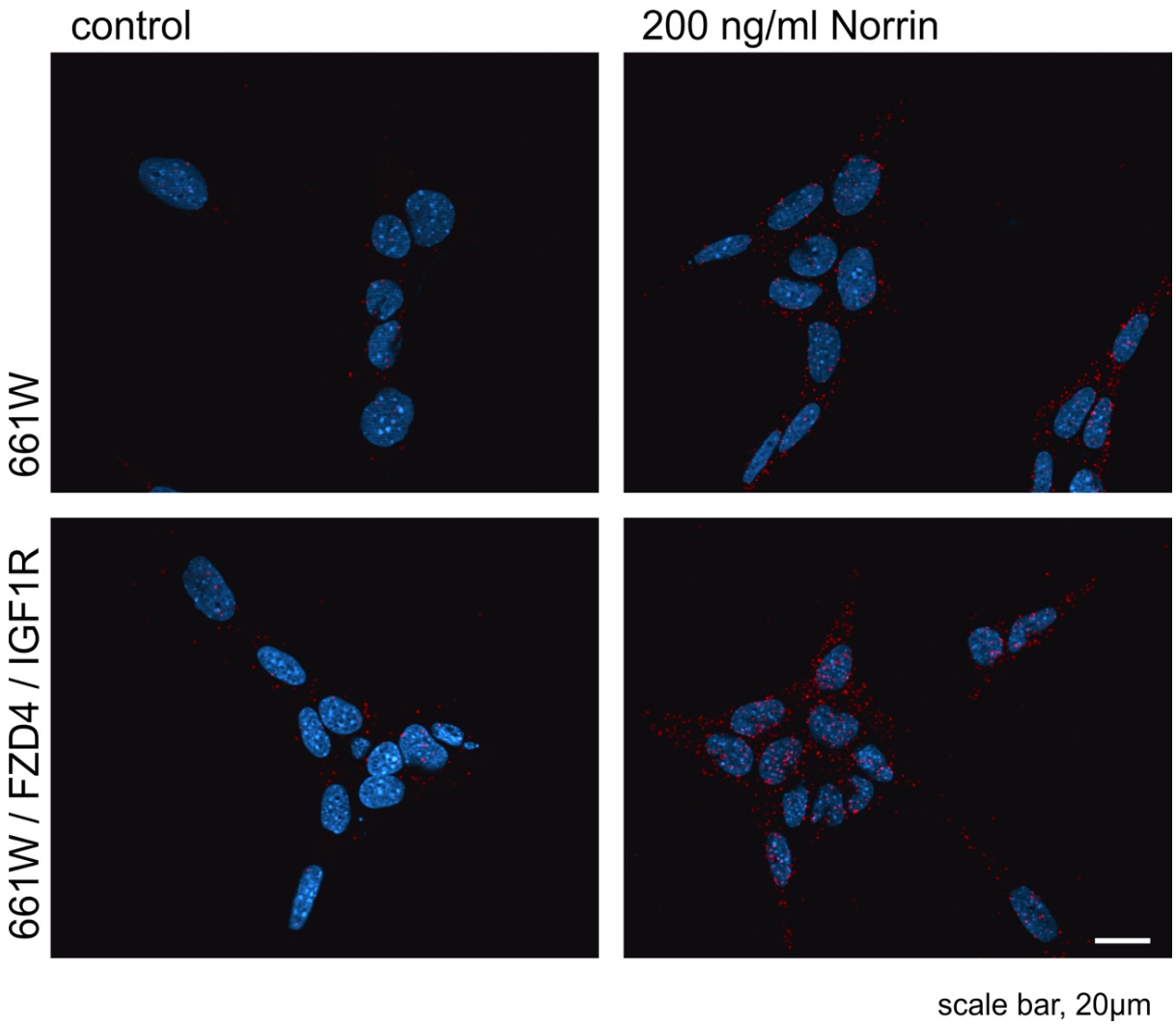


Figure 11: Norrin recruits the IGF1R after binding to FZD4 to mediate direct pAKT signalling. Duolink® Proximity ligation assay (red) of double transgenic and native 661W cells with and without incubation of 200ng/ml Norrin for 4 h. Blue, DAPI staining, scale bar, 20µm.

5. Discussion

Conclusively, the results from my investigation demonstrate that Norrin is capable of activating the pAKT/AKT signalling pathway through the FZD4/IGF1R receptor complex, and thereby could exert a neuroprotective effect on photoreceptors.

This conclusion is substantiated by the following findings: (1) the observation that treatment with Norrin leads to the activation of pAKT signalling in double-transgenic 661W cells, (2) the finding that treatment with Norrin facilitates the interaction between FZD4 and IGF1R, resulting in the formation of a complex, (3) the results that activation of pAKT signalling by Norrin prevents double-transgenic 661W cells from undergoing apoptosis, (4) the observation that the IGF1R inhibitor GSK1904529A blocks Norrin-induced activation of pAKT signalling and reducing photoreceptor apoptosis in double-transgenic 661W cells, and (5) the results that triciribine exhibits potential in blocking Norrin-induced activation of pAKT signalling and reducing photoreceptor apoptosis in double-transgenic 661W cells.

Norrin acts as an atypical FZD4 ligand, propelling the protein-protein interaction with FZD4 via specific binding [111, 138]. Previous reports showed that Norrin exerts its protective effects on RGCs by activating the Wnt/β-catenin pathway and modulating LRP-1 phosphorylation [139]. Based on an in vivo study, it has been determined that Norrin possesses specific neuroprotective properties via induction of specific neuroprotective factors and hence acts as a safeguard for retinal neurons against excitotoxic damage [140]. Further on, in a previous work our group could demonstrate that in mice with a chronic loss of RGC, a transgenic overexpression of Norrin could protect these cells via an IGF1-mediated activation of the AKT pathway[131]. In line, unpublished data of our group of mice with RP leading to a chronic loss of photoreceptors and an additional transgenic

overexpression of Norrin in the retina strongly suggest a protective effect on these retinal cells. In contrast to previous observations, these effects were not mediated via an enhanced expression of neuroprotective factors but were triggered by a potential direct activation of AKT signalling (unpublished data).

Following damage of the retina, activation of pAKT signalling is known to mediate neuroprotective effects on retinal neurons such as RGC or photoreceptors[130, 131, 141]. Several studies indicate an expression of the IGF1R, a key receptor for the activation of the AKT pathway[142], in the retina, including both the outer and inner segments of photoreceptors[143, 144]. Intriguingly, conditional deficiency of IGF1R in photoreceptors and other retinal cells can lead to a loss of photoreceptors, of age-related visual function and subsequent to retinal degeneration[145]. Besides the expression of the IGF1R, a robust expression for FZD4 was reported in the retinal vasculature and the outer nuclear layer of the retina where the photoreceptors are located[146].

5.1 Cell culture model

Since in a previous study an activation of pAKT signalling was observed via an interaction between Wnt3a, FZD7 and the IGF1R[133], it was tempting to speculate if Norrin could mediate homologous effects via binding to FZD4 and recruitment of IGF1R. To prove this hypothesis murine 661W cells with a substantial transgenic overexpression of human IGF1R and human FZD4 were generated. Following incubation of these cells with Norrin, a substantial activation of the AKT pathway could be observed in double transgenic 661W cells while in wild-type controls human recombinant Norrin did not influence pAKT level. However, the strong overexpression of the IGF1R and FZD4 could pretend an interaction of both proteins due to their high concentration, which could not be the case under normal conditions. On the other hand, several studies use a transgenic

overexpression of two proteins to analyse their potential interaction[118]. Since pAKT signalling was only activated by Norrin in double transgenic 661W cells, we could rule out that strong overexpression of FZD4 or IGF1R could activate pAKT signalling itself without incubation with Norrin. In addition, the sole Norrin-mediated activation of the pAKT signaling pathway in double transgenic 661W cells strongly suggests a specific effect of Norrin. Even the transgenic overexpression of FZD4 and IGF1R has disadvantages such as the high expression level of both receptors, it is a suitable tool to analyse the Norrin mediated activation of AKT signalling via the interaction of FZD4 and IGF1R.

Unpublished data from our group strongly suggest a Norrin mediated protection of photoreceptor cells via AKT signalling. Therefore, the protective effect of Norrin was tested on 661W cells with an overexpression of FZD4 and IGF1R. Since 661W cells are derived from retinal precursor cells and are proliferative[147], a differentiation of the cells with staurosporine was performed to analyse the neuroprotective effects of Norrin mediated pAKT signalling on 661W. Differentiation of neuronal precursor cells with staurosporine is a common treatment to induce a postmitotic and neuronal phenotype of the cells[148]. In 661W cells staurosporine induced a neuronal phenotype with extended neurites with branching and a blocked proliferation[149]. Since staurosporine blocks a broad spectrum of kinases the mechanism of mediating the neuronal phenotype remains unclear so far[150]. Following the treatment of 661W cells we observed a homologous phenotype with neurites and block proliferation strongly suggesting that our staurosporine treated 661W cells develop a neuronal phenotype as well.

5.2 Norrin mediates neuroprotective effects on 661W cells via AKT signalling

Since in mice with retinal degeneration, an additional transgenic overexpression of Norrin protects photoreceptors against apoptosis via activation of AKT signalling cell survival of differentiated 661W cells with an overexpression of the IGF1R and FZD4 was analysed. Following incubation with Norrin significantly more 661W double transgenic cells survived and the activation of AKT signalling was enhanced when compared to Norrin treated native 661W cells or untreated controls. AKT signalling can be activated by various growth factors and is a common neuroprotective signalling pathway in neurons of the brain and the retina[151]. For instance, in the retina the activation of AKT signalling can protect neurons against oxidative stress mediated apoptosis, ischemia induced degeneration and light induced apoptosis[130, 152-154]. However, besides AKT signalling in the retina several other neuroprotective pathways such as β-catenin, Janus kinases/signal transducer and activator of transcription (JAK/STAT) or mitogen-activated protein kinase (MAPK/ERK) have been described as well[155-157]. Since the neuroprotective effects of Norrin could be blocked by triciribine, an inhibitor of AKT signalling, in double transgenic 661W cells it is most likely that Norrin mediates its protective effects via the AKT pathway.

5.3 Norrin induces FZD4 / IGF1R interaction for direct AKT signalling

The IGF1R is a receptor tyrosine kinase, which is involved in several physiological and pathological processes including cell proliferation, growth, metabolism, tumour progression and cell survival[158, 159]. Binding of IGF1 to the IGF1R can lead to an activation of the phosphatidylinositol 3-kinase (PI3K)-AKT and the Ras-mitogen-activated protein kinase (MAPK) pathway[158]. Intriguingly, in several studies, an interaction of the IGF1R with other Receptors had been reported. For instance, in squamous cell

carcinomas, the IGF1R can interact with the epidermal growth factor receptor (EGFR) with the consequence of an enhanced phosphorylation of the EGFR[160]. In line, Bernis et al. reported an interaction of frizzled-7 with the IGF1R following binding of Wnt3a to activate pAKT signalling[133] , an observation that encouraged us to perform this study. By immunocytochemistry and proximity ligation assay (PLA) we observed a colocalization of the IGF1R and FZD4 in transgenic 661W cells. Even a technology for a high resolution of the focal plane, the Apotome of Zeiss, was used for visualization of colocalization of IGF1R and FZD4 by immunocytochemistry this method could be defective because of the display method and the investigator's perception[161]. To rule this deficiency out, a proximity ligation assay for the potential interaction between the IGF1R and FZD4 was performed. This analysis is based on an immunoreaction of primary antibodies detecting the two proteins of interest. Instead of a conventional immunohistochemical staining the secondary antibodies are coupled with a specific DNA strand for each species. If the proteins of interest are close together the two DNA strands hybridise and form a circular DNA [162]. Following ligation, the DNA will be amplified and visualized by incorporation of fluorescent complementary oligonucleotide probes[162]. In contrast to biochemical immunoprecipitation methods, which are preferred for the analysis of stable protein-protein interactions, PLA is suitable for investigating more transient interactions and allows a subcellular localization of the protein complex [163]. After incubation of double transgenic 661W cells with Norrin, a specific signal for the IGF1R and FZD4 was detected by PLA. Since this assay can only be positive if both proteins of interest are closer together than 40nm [162] it appears most likely that the IGF1R and FZD4 interact with each other after binding of Norrin. The hypothesis of an interaction of the IGF1R and FZD4 will be further supported by the observation that only in double transgenic 661W cells Norrin activates AKT signalling, but not in cells with a solitary overexpression of FZD4 or IGF1R. In line, following treatment of double transgenic 661W cells

with the IGF1R blocker GSK1904529A the Norrin mediated activation of AKT signalling and its neuroprotective effects on the cells were lost suggesting that both receptors are required. Overall, our results strongly suggest that for the Norrin mediated effects on FZD4/IGF1R 661W cells, the IGF1R is essential and a direct interaction of both receptors is very most likely.

5.4 Crosstalk of the Wnt/β-catenin signalling with other pathways

For frizzled mediated β-catenin signalling several interactions between other pathways have been proposed to modify its signal intensity via transcriptional regulation of components of the Wnt pathway or non-coding RNAs. For instance, leptin or insulin can modify the expression of canonical Wnt signalling components in chondrocytes or intestinal cells, respectively, leading to an enhanced β-catenin signalling [164, 165]. In line, non-coding RNAs play a critical role in fine tuning of the expression of components of the Wnt pathway under physiological and pathological conditions [166]. Complex crosstalk involving transcriptional regulation as well as molecular interaction takes place between Notch and Wnt signalling which could lead to activation or inhibition of the other pathway[167].

Vice versa, canonical Wnt signalling or the interaction of frizzled proteins with other membrane receptors can modify several intracellular pathways. For instance, in the retina transgenic expressed Norrin could induce the expression of Smad7 which in turn partially rescued the TGF-β mediated retinal phenotype by a diminished TGF-β signalling[168]. A direct interaction of frizzled-5 with the epidermal growth factor receptor (EGFR) following incubation with Wnt5a was observed in cardiomyocytes, which induces an

increased phosphorylation of ERK and subsequently leads to enhanced cardiac fibrosis [169].

For pAKT signalling an activation of the Wnt/β-catenin pathway has been observed in various studies. Following phosphorylation activated AKT can phosphorylate and hereby inactivate the glycogen synthase kinase (GSK)-3β in neurons[170]. Since the GSK-3β is an essential component of the β-catenin destruction complex, β-catenin accumulates in the cytoplasm, translocates into the nucleus and induces the transcription of specific target genes following GSK-3β phosphorylation by pAKT[170]. Vice versa, as mentioned before, following binding of Wnt3a frizzled-7 can recruit the IGF1R to enhance the phosphorylation of AKT and hereby activates pAKT signalling[133]. Since Wnt and AKT signalling pathways are closely regulated by each other, frizzled receptors have the distinct potential to interact with receptor tyrosine kinases and the observation that other frizzled proteins could activate pAKT signalling via IGF1R recruitment, it is most likely that Norrin mediates its protective effects on retinal neurons via an activation of the AKT pathway through an FZD4 / IGF1R receptor complex.

6. Conclusion

In summary, the data presented provide strong evidence of an additional Norrin-mediated, previously unknown neuroprotective signalling network, which directly enhances AKT signalling after the formation of a Norrin-FZD4-IGF1R receptor complex in retinal neurons. The activation of this novel Norrin mediated mechanism could be a promising target for prospective therapeutic strategies to prevent chronic photoreceptor degeneration, such as retinitis pigmentosa, in humans.

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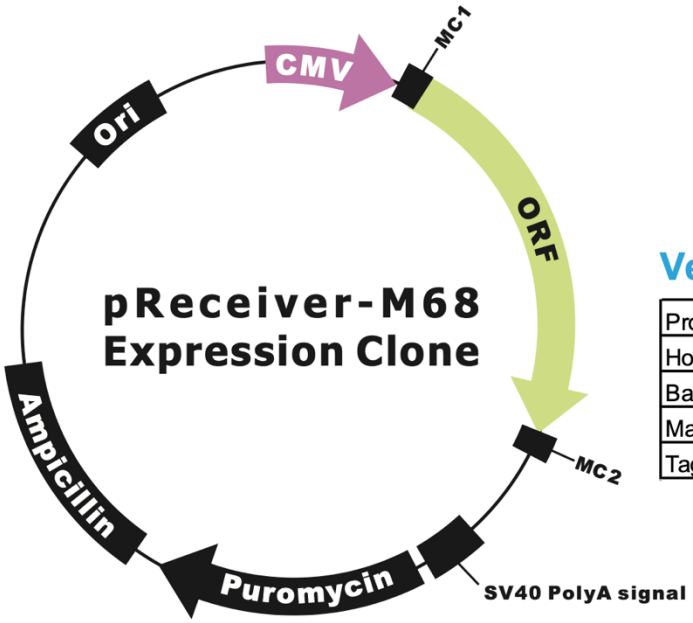
Appendix:

Plasmid map of FZD4

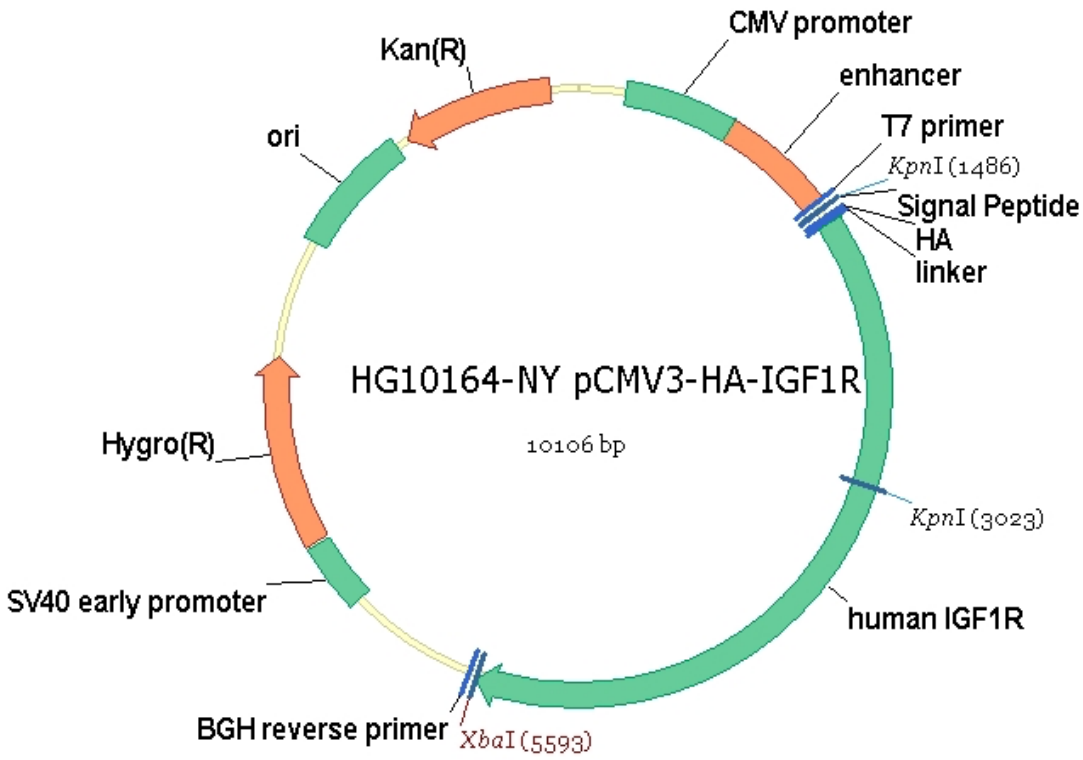


Vector Features

Promoter	CMV
Host Cell	Mammalian
Bacterial selection antibiotic	Ampicillin
Mammalian selection marker	Yes
Tag	N/A

Appendix:

Plasmid map of IGF1R



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List of Abbreviations

AAV	Adeno-Associated Virus
ADRP	autosomal dominant retinitis pigmentosa
AMD	age-related macular degeneration
AP	alkaline phosphatase
ARRP	autosomal recessive retinitis pigmentosa
AVMD	adult-onset vitelliform macular dystrophy
BCA	bicinchoninic acid
BSA	Bovine serum albumin values
BVMD	best vitelliform macular dystrophy
CNS	Central nervous system
CNV	choroidal neovascularisation
CORD	rod-cone dystrophy
CRD	cystine-rich domain
CT	cycle-threshold
Cu^{1+}	cuprous cation
DMSO	Dimethyl sulfoxide
DR	diabetic retinopathy
DRN	diabetic retinal neurodegeneration
EMA	European Medicines Agency

ERG	electroretinogram
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FEVR	familiar exudative vitreoretinopathy
FZD4	Frizzled 4
GA	geographic atrophy
GT	gene therapy
IGF1R	insulin-like growth factor 1 receptor
LCA	Leber's Congenital Amaurosis
LGR	leucine-rich repeat-containing G-protein coupled
LRP	lipoprotein receptor-related protein
MSCs	marrow stromal cells
OCT	optical coherence tomography
OIR	oxygen-induced retinopathy
ORF	open reading frame
PBS	Phosphate Buffered Saline
PI3K	phosphatidylinositol-3-kinase
Prph2	Peripherin2
PS	Penicillin-Streptomycin
RCS	Royal College of Surgeons
RD	retinal degeneration

Rd10	retinal degeneration 10
RDS	retinal degeneration slow
RGCs	retinal ganglion cells
RHO	rhodopsin gene
RP	retinitis pigmentosa
RPE	retinal pigment epithelial
RT	Room temperature
RT-QPCR	Quantitative real-time Polymerase Chain Reaction
TGF	transforming growth factor
VEGF	Vascular endothelial growth factor
Wnt	Wingless Integrated
XLRP	X-linked retinitis pigmentosa

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Affidavit

Affidavit

Ma, Wenxiu

Surname, first name

I hereby declare, that the submitted thesis entitled:

Analysis of Norrin-mediated protective effects on chronic degeneration of photoreceptors.

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the dissertation presented here has not been submitted in the same or similar form to any other institution for the purpose of obtaining an academic degree.

Munich, 03. 02. 2025

Place, Date

Ma Wenxiu

Signature doctoral candidate

List of publications

Yang T, Chen C, Ma W, Duan Y, Zhu Q, Yao J. Effect of bilateral inferior oblique partial myectomy on V pattern exotropia with inferior oblique overaction. BMC Ophthalmol. 2022 May 21;22(1):230. doi: 10.1186/s12886-022-02456-1.PMID: 35597903; PMCID: PMC9123781.

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