THE ROLE OF *NBS1* IN THE INSULIN-LIKE GROWTH FACTOR-1 SIGNALING

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The role of *NBS1* in the insulin-like growth factor-1 signaling

ABSTRACT

The Nbs1 protein (nibrin, p95) is a member of the DNA repair/checkpoint complex Mre11/Rad50/Nbs1 (MRN), which plays a critical role in the cellular responses to DNA damage, cell cycle checkpoints, and telomere and genome stability. Many transgenic models in mice and clinical symptoms of NBS patients have clearly shown that Nbs1 exerts pleiotropic actions in growth and development of mammals. However, the molecular role of Nbs1 in mitogenic signaling pathways which could explain the growth retardation, developmental defects and impaired proliferation capacity of NBS patient cells has not been demonstrated, so far.

This study shows that after repression of endogenous Nbs1 levels using short interference RNA, hTERT-immortalized RPE cells exhibit decreased proliferation ability and poor response to IGF-1 stimulation. After release from G1 arrest, *NBS1* siRNA-transfected cells display disturbances in periodical oscillations of cyclin E and A, and delayed cell cycle progression. Remarkably, lower phosphorylation levels of c-Raf, and diminished activity of ERK1/2 in response to IGF-1 suggest a link between *NBS1*, IGF-1 signaling, and Ras/Raf/MEK/ERK cascade. The functional relevance of *NBS1* in mitogenic signaling and initiation of cell cycle progression are demonstrated in *NBS1* siRNA-transfected cells where IGF-1 has a limited capacity to induce expressions of *FOS* and *CCND1*. The impact of *NBS1* on the IGF-1 signaling cascade is finally identified by the reduction of IGF1R, *SOS1* and *SOS2* expression in *NBS1* siRNA-transfected cells. The disturbed IGF-1 signaling, a consequence of diminished expression of the key components of the cascade, results in a failure of IGF-1 to rescue *NBS1* siRNA-transfected cells from gamma radiation-induced cell death.

In conclusion, this study provides the first evidence that, by modulating the IGF-1 signaling cascade, *NBS1* has a functional role in the promotion of cell cycle progression, cell proliferation, and cellular radio-resistance in addition to its well known function for proper DNA double strand break signaling.

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ABBREVIATIONS

A	adenine
a.a.	amino acid
ATP	adenosine 5'-triphosphate
°C	degree celsius
C	cytosine
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DSB	DNA double strand break
Fig.	figure
g	relative centrifuge force
G	guanine
GDP	guanosine diphosphate
GTP	guanosine-5'-triphosphate
Gy	gray
h	hour
IGF-1	Insulin-like growth factor-1
IgG	immunoglobulin G
kb	kilobase
kD	kilodalton
min	minute
ml	milliliter
mm	millimeter
mRNA	messenger ribonucleic acid
NBS	Nijmegen breakage syndrome
ng	nanogram
nM	nanomolar
nm	nanometer
PCR	polymerase chain reaction
ref.	reference
RNA	ribonucleic acid
T	thymine
µg	microgram
µM	micromolar
µl	microliter

1. INTRODUCTION

1.1 Statement and significance of the problem

The Nbs1 protein plays an important role in cellular responses related to DNA damage, such as cell cycle control, DNA double strand break (DSB) repair, maintenance of telomeres and genomic stability [1-4]. Mutations of the gene *NBS1* encoding Nbs1 may cause the Nijmegen breakage syndrome (NBS), which is characterized by microcephaly, developmental defects, growth retardation, immunodeficiency, radiation hypersensitivity, and increased cancer risk [4,5]. Based on the clinical symptoms of NBS patients and cellular defects of NBS cells, the functional roles of *NBS1* in DNA damage repair processes and cell cycle checkpoint signaling have been investigated intensively. However, the molecular role of Nbs1 in mitogenic signaling pathways which could explain growth retardation and developmental defects of NBS patients and impaired proliferation capacity of NBS patient cells has not been demonstrated.

Insulin like growth factor 1 (IGF-1) is a potent mitogenic factor which plays a critical role in both fetal and postnatal growth in mammals, and the IGF-1 signaling system also controls tissue homeostasis throughout life via regulation of cell proliferation and apoptosis [6-10]. Mutations in the IGF-1 gene result in severe pre- and post-natal growth and developmental defects and mental retardation in humans [11]. Decreased IGF-1 signaling is associated with increased incidence of cancer and enhanced cellular radio-sensitivity [12], while increased IGF-1 signaling stimulates proliferation, promotes metastasis, and causes radio-resistance of cancer cells [13,14]. IGF-1 has been shown to regulate both expression and activity of various molecules involved in cell cycle progression especially during the G1 phase, like cyclin D1 and cyclin E [15] . Furthermore, the IGF-1 signaling system is reported to confer a strong anti-apoptotic activity in response to a variety of stimuli inducing cell death, including ionizing radiation [16].

Deregulation of *NBS1* and IGF-1 system share some common phenotypic characteristics, therefore it can be expected that there are some functional links between them. Elucidation of the link between Nbs1 and IGF-1 is an ideal research topic for providing further aspects to functions of Nbs1 besides its roles in DNA damage repair processes and cell cycle

checkpoint signaling. This could improve the understanding of the observed phenotypes in cell lines mutated in *NBS1* and of the clinical symptoms of NBS patients and give a new molecular explanation for the impaired proliferation capacity and increased radio-sensitivity of NBS cells.

1.2 Goal of the study

As mentioned before, deregulation of the IGF-1 system or *NBS1*-dependent processes result in an impaired cell proliferation. Therefore, the goal of this study is to elucidate the functional roles of *NBS1* in the regulation of cell cycle progression, cell proliferation, and cell survival.

For this purpose, the RNA interference technology was exploited to specifically repress *NBS1* levels in the telomerase-immortalized human retinal pigment epithelial (hTERT-RPE) cell line. The impact of *NBS1* on cell cycle regulation was determined by analyzing the cell cycle distribution, the expression pattern of cell cycle-specific cyclins, and the cellular proliferation rate of *NBS1* siRNA-transfected cells.

The functional relevance of *NBS1* in mitogenic signaling, especially mitogen-stimulated cell cycle transition at G1 to S phase was evaluated. In this study, the influence of *NBS1* on IGF-1-stimulated signaling was analyzed. This is the major pathway which is responsible for initiating cell proliferation by controlling the cell cycle during G1 to S phase, and signal transduction via this pathway was studied in *NBS1*-transfected cells. Furthermore, the influence of *NBS1* on the expression levels of IGF1R, *SOS1*, and *SOS2*, the key molecules for initiating the IGF-1 response were determined.

In order to link the data observed from the studies above to the enhanced radio-sensitivity of NBS cells, which is the most prominent phenotype of these cells, the impact of IGF-1-induced signaling on the cell survival after irradiation was analyzed in *NBS1*-transfected cells.

Summarizing, acquired data in this study established a link between the IGF-1-mediated proliferation and the impaired proliferation of NBS cells. Additionally, experiments were designed to give an explanation for the severe radio-sensitivity caused by *NBS1* deficiencies by a further mechanism besides impaired DSB-induced signaling or enhanced radiation-induced apoptosis.

1.3 Review of literature

1.3.1 Nijmegen breakage syndrome

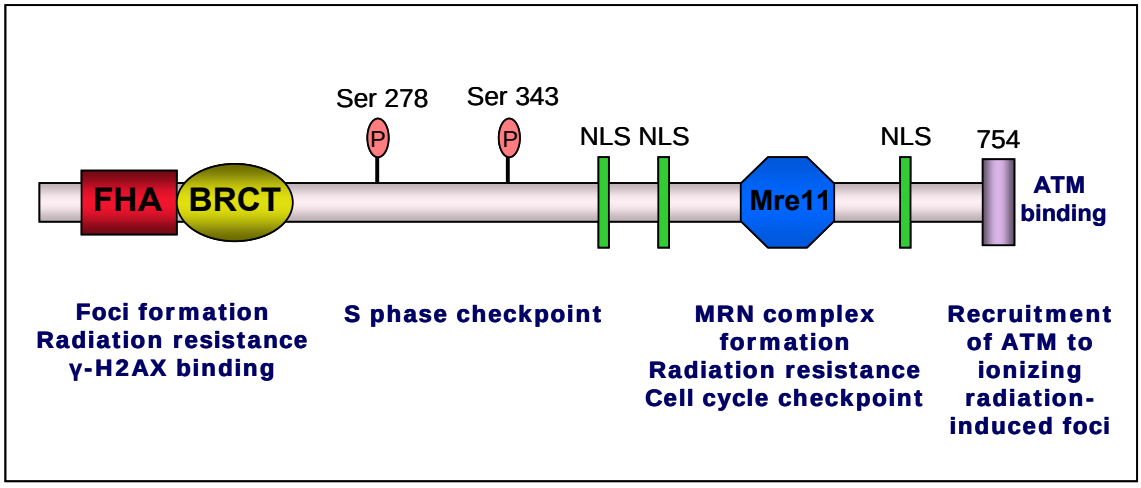
1.3.1.1 Nijmegen breakage syndrome 1 gene and its product

The Nijmegen breakage syndrome 1 protein (Nbs1), also called nibrin and p95, is a 754 amino acid protein with a predicted molecular weight of 95 kD. Nbs1 was identified in 1998 as the product of the *NBS1* gene which is located on the long arm of chromosome 8 (8q21) [1,2,4]. The entire gene consists of 16 exons and spans over 51 kb of DNA. Biallelic (homozygous or compound heterozygous) mutations in the *NBS1* gene may cause the Nijmegen Breakage Syndrome (NBS) [5].

More than 90% of all NBS patients are homozygous for a 5 bp truncating mutation, 657del5 [4]. The NBS cells harbouring this mutation contain a predicted 26 kD amino-terminal truncated protein, $Nbs1^{p26}$, and a 70 kD protein, $Nbs1^{p70}$, lacking the native N-terminal [17]. The $Nbs1^{p26}$ results from a frame shift at the codon encoding Nbs1 amino acid 218 and premature termination of translation at codon 219. The $Nbs1^{p70}$ is produced by internal translation initiation within the *NBS1* mRNA using an alternative open reading frame generated by the 657del5 frame shift [4,17]. $Nbs1^{p26}$ is actually observed in cells from patients and heterozygotes with the 657del5 mutation, whereas $Nbs1^{p70}$ is of much lower abundance and so far only observed in EBV-transformed lymphoblastoid cells [17]. The *NBS1* 657del5 mutation is a hypomorphic defect because the truncated protein products seem to be partially functional which diminishes the severity of the NBS phenotype [17].

1.3.1.2 The structure and function of Nbs1

Nbs1 contains three functional regions: the N-terminus (1-196 a.a.), a central region (278-343 a.a.), and the C-terminus (682-746 a.a.). The N-terminal region has two functional domains, a forkhead-associated (FHA) domain (24-108 a.a.) and a breast cancer carboxy-terminal (BRCT) domain (108-196 a.a.) [1,2,4,18] (see Fig. 1). FHA and BRCT domains are widely conserved in eukaryotic nuclear proteins that are related to cell cycle check points or DNA repair [19]. The FHA domain is believed to be a phospho-specific protein-protein interaction motif that recognizes phosphorylation of the target protein [20]. The FHA/BRCT domain directly binds to phosphorylated histone H2AX which occurs near sites of DNA strand breakage and recruits the Mre11/Rad50/Nbs1 (MRN) complex to the vicinity of sites of DSBs [21]. Cells derived from NBS patients that express Nbs1 truncated proteins with a mutation in either of these domains manifest a defect in ionizing radiation-induced MRN foci formation and show hypersensitivity to ionizing radiation, suggesting a role of both domains for cell survival after exposure to ionizing radiation [22,23].



modified from Tauchi 2002 [24] and Lee 2006 [25]

Figure 1. Scheme of the functional domains of Nbs1. The forkhead-associated (FHA) and Brca1 COOH-terminus (BRCT) domains in the NH_2-terminal region bind to γ-H2AX and are required for ionizing radiation (IR)-induced foci formation and radiation resistance. Phosphorylation of Ser278 and Ser343 by ataxia telangiectasia mutated protein (ATM) is essential for activation of the S phase checkpoint. The nuclear localization signal (NLS) is used to target the protein to the nucleus. The Mre11-binding domain is responsible for binding to Mre11 during formation of the Mre11/Rad50/Nbs1 (MRN) complex. The ATM-binding domain at the COOH-terminus binds to ATM and mediates recruitment of ATM to ionizing radiation-induced foci.

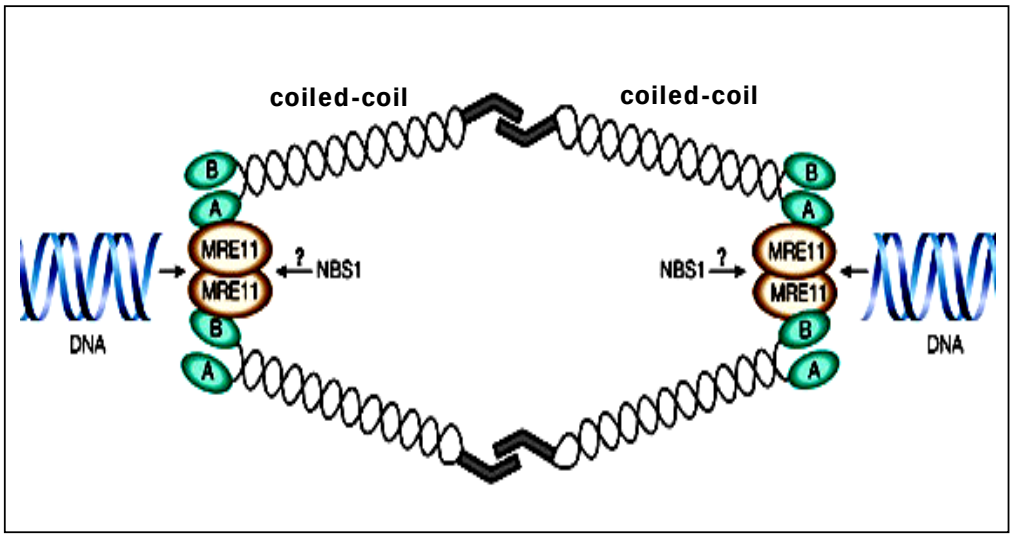
Several SQ motifs, consensus sequences of phosphorylation by ATM or ATR (an ATM and Rad3-related) kinase, are found in the central region of Nbs1. In particular, the serine residues at 278 and 343 are phosphorylated by ATM in response to radiation and these phosphorylations confer functions in signal transduction for damage response to Nbs1 [26-29]. Cells expressing Nbs1 proteins with mutations at these phosphorylation sites exhibit defective S phase checkpoint control, suggesting that Nbs1 phosphorylation by ATM is required at least for activation of the S phase checkpoint in response to ionizing radiation [30,31]. A defect in the S phase checkpoint was early recognized in NBS cells as radio-resistant DNA synthesis (RDS) which is identified as a failure to halt DNA synthesis after DNA has been damaged by ionizing radiation [19].

The C-terminal region of Nbs1 contains three potential nuclear localization signals (NLSs) at amino acid residues 461–467, 590–594, and 751–754 which are used to target the protein to the nucleus, and it has binding domains for Mre11 and ATM. The Mre11-binding domain has been localized to amino acids 682–693. Deletion of this region of the Nbs1 protein results in cellular phenotypes including defects in MRN foci formation, radiation hypersensitivity and the impairment of checkpoint control [23,32]. The extreme C-terminal region of Nbs1 at amino acids residues 734–754 mediates the interaction of Nbs1 with ATM and the recruitment of ATM to sites of DNA damage, thereby promoting ATM-dependent signaling [33].

Nbs1 is the regulatory subunit of the MRN complex which plays important roles in the processes of telomere maintenance, recognition and repair of DSBs, and transduction of damage-response signals to the cell cycle check point apparatus [34]. The primary function of the MRN complex is to sense DSBs and then to amplify the initial signal and convey it to downstream effectors, such as ATM, p53, SMC1 and BRCA1, that regulate cell cycle checkpoints and DNA repair [5,19,34-36].

The MRN complex appears as a bipolar structure with a global head and two long tails [37] (see Fig. 2). The head consists of two Rad50 ABC ATPase domains, along with the Mre11 dimer [38,39]. The precise location of Nbs1 has not been revealed, however mutagenesis and biophysical data suggest that it binds to the $Mre11_2$:$Rad50_2$ head, possibly forming a hexameric $Mre11_2$/$Rad50_2$/$Nbs1_2$ complex [40]. Rad50 contains walker A and B motives that are required for nucleotide binding and are separated by two coiled-coil regions that are

required for intramolecular interactions [37,41]. Mre11 has a single- and double-stranded (ds) DNA endonuclease, as well as a 3′–5′ dsDNA exonuclease function [42]. Mre11/Rad50 has a structural role in bridging DNA ends through the coiled-coil regions of Rad50 where Mre11 can process these ends by its nuclease activity [43]. Up to date, there is no evidence for any enzymatic activity of Nbs1, but rather, it serves as a molecular chaperone, guiding the MRN complex to the nucleus and targeting the complex to the sites of DSBs following irradiation [32,44].



modified from van den Bosch [45]

Figure 2. Scheme of the structural model of the MRN complex architecture. Mre11 (brown) binds to the coiled-coil domains of Rad50, adjacent to the ABC domains (green) of Rad50. The Rad50 antiparallel coiled-coil domains protrude from the ABC domains. The apex of the coiled-coil domains contains the zinc hook, implicated in metal-mediated intramolecular or intermolecular joining of MRN complex via the Rad50 coiled-coils. The precise location of Nbs1 has not been revealed.

1.3.1.3 Growth retardation and developmental defect in NBS

Growth retardation and developmental defects are the main clinical manifestations of NBS. Children with NBS, in spite of being born at term, are characterized by a significantly lower birth weight and head circumference in comparison with sex-matched controls, as well as lower birth length and chest circumference [46]. Microcephaly, specifically head circumference below the third percentile, is the most striking symptom of NBS. Most patients are born with microcephaly, but in individuals who were born with a head

circumference within the reference range, progressive and severe microcephaly develops during a few months of life [47]. The craniofacial characteristics of NBS patients are observed as a sloping forehead and receding mandible, a prominent mid-face with a relatively long nose, and relatively large ears [5,48]. Short structure is apparent after approximately 2-years of age, and it is a result of trunk shortening [5]. The poor development of secondary sex characteristics as the results of lack of genital organs and breasts are usually observed in NBS female patients who reach pubertal age [49]. Several studies in mice models strongly support the clinical symptoms of NBS patients. Mice with targeted hypomorphic mutations of *NBS1* show growth retardation and impaired cellular proliferation. Lymphoid and germ cell development are especially disturbed [50]. *NBS1* null mice lead to early embryonic lethality in utero which is associated with poorly developed embryonic and extraembryonic tissues [51]. The clinical symptoms in NBS patients and mouse models of NBS suggest essential functions of Nbs1 in cell proliferation and normal growth and development of mammals.

1.3.2 The insulin-like growth factors system (review in ref. [6-10])

The insulin-like growth factor system (also known as IGF axis) is a cell signaling system including two ligands, the insulin-like growth factor 1 and the insulin-like growth factor 2 (IGF-1 and IGF-2), two cell surface insulin-like growth factor receptors (IGF1R and IGF2R), at least six different insulin-like growth factor binding proteins (IGFBP1-6) [10], and multiple IGFBP proteases [52].

1.3.2.1 The insulin-like growth factors

The insulin-like growth factors (IGFs) are polypeptides with high sequence similarity to insulin. IGFs are part of a complex system that is involved in the regulation of a diverse array of biological functions, both normal and pathological in vertebrate species. There are two main IGFs, IGF-1 and IGF-2, that have been isolated and characterized [53]. IGF-1 and IGF-2 are 70% identical to one another, and 50% to pro-insulin. Both IGFs have characteristics of a circulating hormone and a tissue growth factor.

IGF-1 is a single chain polypeptide of 70 amino acids, with a molecular weight of 7,649 D [53]. The gene encoding IGF-1 is located on chromosome 12q22-q23 [54]. IGF-1 is a growth factor that circulates at high levels in the blood stream. The majority of circulating IGF-1 is produced in the liver. However, many other tissues including bone, adipose tissue, kidney, and muscle also produce IGF-1 and are sensitive to its action, especially during postnatal development [9]. The regulation of IGF-1 production in hepatic tissue is mainly mediated by growth hormones and insulin. In turn, IGF-1 feeds back to suppress growth hormones and insulin release. In addition to growth hormones, developmental factors as well as the nutrition status can modify IGF-1 production [9,55]. The action of IGF-1 is mediated by binding to specific receptors present on most cell types. IGF-1 is one of the most potent natural stimulators of cell growth (increase in the size of the cells), cell proliferation (increase in the number of cells) and a potent inhibitor of programmed cell death. In mammals, IGF-1 is required for both pre- and postnatal growth, however relatively low expression during the embryonic period indicates that it is more important for post-natal growth and development [56,57].

IGF-2, like IGF-1, is a single polypeptide, with a molecular weight of about 7.4 kD [58]. The gene for IGF-2 is located on chromosome 11p15.5 [54]. The synthesis of IGF-2 is relatively independent of growth hormones. Its expression is much higher during fetal development than during post-natal life. IGF-2 plays a fundamental role in embryonic and fetal development, whereas its role in post-natal period is less important since it is substituted by IGF-1 [59].

Most circulating IGF-1 and IGF-2 molecules form a complex to a family of IGF binding proteins (IGFBPs). IGF binding protein 3 (IGFBP3) is the predominant IGFBP in serum and almost 70% of the bound IGF is carried as a trimeric complex with IGFBP3 and the acid-labile subunit (ALS) which modulates the availability of free IGFs [8,10]. IGFBPs coordinate and regulate the biological activities of IGFs by acting as transport proteins in plasma, controlling the efflux of IGFs from the vascular space, and regulating IGFs metabolic clearance. Furthermore, IGFBPs can directly modulate the interaction of IGFs with their receptors and thereby indirectly control their biological actions [10].

1.3.2.2 The biological actions of IGFs

In general, the effects of IGFs *in vitro* are either acute anabolic effects on protein and carbohydrate metabolism, or long term effects on cell proliferation and differentiation [10]. The most widely studied effect of IGFs on cell cycle progression *in vitro* is the stimulation of DNA synthesis and cell proliferation, by causing cells to traverse the successive phases of the cell cycle. IGF-1 had been shown to function as an inducing factor for cell cycle progression, and this function has been studied extensively in BALB/c-3T3 cells [60-62]. The critical function of IGF-1 for the stimulation of cell proliferation is demonstrated intensively in a variety of cell types. IGF-1 has been shown being a potent mitogenic factor stimulating cell proliferation in fibroblasts, chondrocytes, smooth muscle cells, and epithelial cells, e.g. [10].

In vivo, the absolute requirement of IGFs for normal development is demonstrated by the severe growth retardation and developmental defects in various organs, and sterility in *IGF-1* knock-out mice [56]. In humans, deletions in the *IGF-1* gene result in severe pre- and postnatal growth and developmental defects and in mental retardation [11]. Moreover, several model systems have provided evidence that proliferation and metastasis of cancer cells are increased by IGFs receptor activation, either in relation to higher levels of circulating IGFs [63] or overexpression of the IGF receptors [64]. IGFs also control tissue homeostasis throughout life by providing essential signals for the regulation of cell growth, cell proliferation, cell cycle progression, cell differentiation, and cell survival [10].

1.3.2.3 The insulin-like growth factor receptors

The biological effects of IGF-1 and IGF-2 are mediated by their cell surface receptors. IGFs bind to three receptors with differing affinities. The type 1 IGF receptor binds both IGF-1 and IGF-2 with high affinity. Virtually all of the biological activities of the IGFs result from binding to the type 1 IGF receptor. The type 2 IGF receptor binds IGF-2 with high affinity and IGF-1 with low affinity. This receptor does not transduce a signal because it lacks a tyrosine kinase domain but acts as a negative regulator of IGF activity by sequestration, endocytosis and degradation of IGF-2 [65]. The insulin receptor binds IGF-1 with an affinity approximately 100-fold lower than insulin [10].

The IGF-1 receptor (IGF1R) is a hetero-tetrameric receptor tyrosine kinase which carries out most of the biological activities of IGFs [66]. The gene encoding IGF1R is a single copy gene located on chromosome 15q26 and encodes a single polypeptide of 1,367 amino acids [54]. The IGF1R is synthesized as a single-chain pre-proreceptor which is subsequently terminally glycosylated and proteolytically cleaved to yield the α-subunit (130-135 kD) and β-subunit (90-97 kD) of the mature receptor [67]. These assemble into a disulphide-linked hetero-tetramer comprised of two extracellular α-subunits containing the extracellular cysteine-rich domain necessary for ligand recognition and binding, and two transmembrane β-subunits transmitting the ligand-induced signal. The IGF1R β-subunit consists of three domains: a juxtamembrane domain with motifs required for recruiting the major signaling adapter proteins; a tyrosine kinase domain which is essential for the catalytic activity of the receptor; and the carboxy-terminal domain which has several important residues for IGFR signaling [67,68] (see Fig. 3).

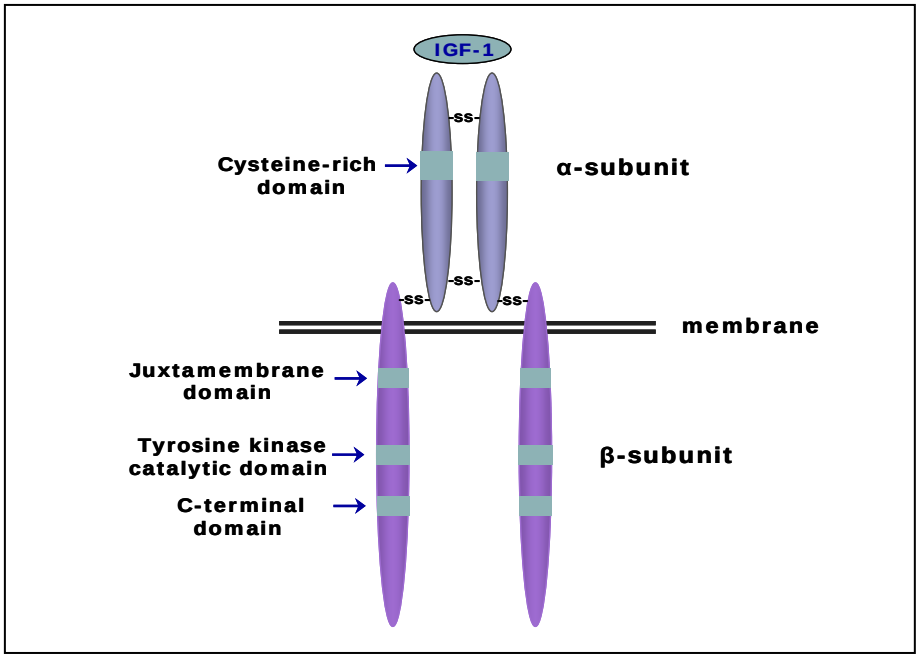


Figure 3. Scheme of the structural model fo the IGF1R. The disulphide (-ss-)- linked hetero-tetramer of the IGF1R molecule comprises of two α–subunits (light blue) and two β–subunits (light violet). Intracellular activities of the IGF1R signaling cascade are initiated via the interaction between the IGF-1 molecule (blue) with the ligand binding site of the α–subunits of IGF1R.

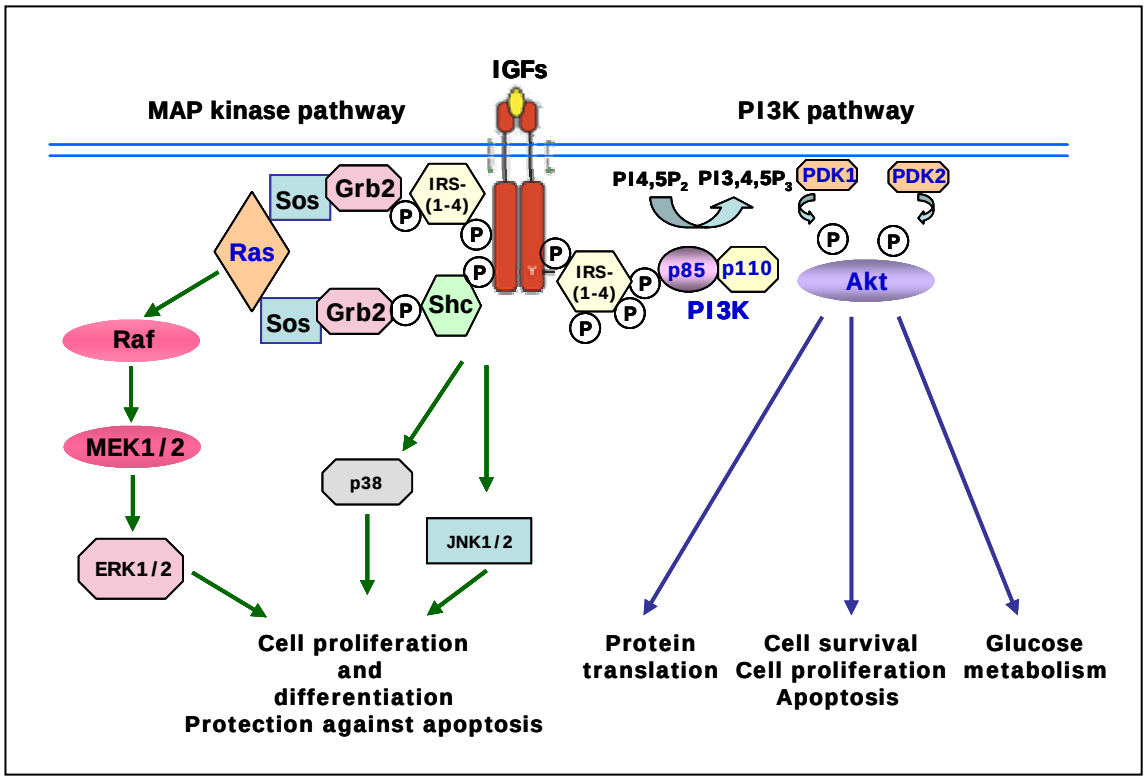
The *IGF1R* gene is constitutively expressed in most tissues and cell types, consistent with the role of IGF-1 as a progression factor through the cell cycle. Usually, high levels of expression are seen at embryonic stages which are characterized by a large proportion of actively proliferating cells. The level of *IGF1R* mRNA decreases in post-natal proliferating cells and reaches the lowest level in post-mitotic fully differentiated adult cells [10]. The importance of the IGF1R in normal mammalian development is observed in *IGF1R* null mice which are 45% of the size of wild type animals at birth, and die shortly after birth due to severe organ hypoplasia [69]. Mouse embryonic fibroblasts cultured from *IGF1R* null mice (R-cells) grow more slowly than wild type cells, and they are unable to proliferate under anchorage-independent conditions [70]. The IGF1R can induce differentiation in adipocytes, myoblasts, osteoblasts, neurons, and haematopoietic cells [71]. Activation of the IGF1R protects cells from killing induced by a variety of agents, including osmotic stress, hypoxia, radiation, and anti-cancer drugs [72,73].

1.3.3 The IGFs/IGF1R signal transduction pathways

Binding of IGFs to the extracellular part of IGF1R initiates the cytoplasmic signaling cascades that include conformational change of the receptor which enables IGF1R to bind ATP and become autophosphorylated at tyrosine residues within the β-subunits. This result in activation of the intrinsic tyrosine kinase activity of the IGF1R and subsequently tyrosine phosphorylation of several substrates which lead to the activation of intracellular signaling pathways, allowing induction of growth, proliferation, transformation, differentiation, and survival (see Fig. 4).

The proximal substrates for IGF1R include the insulin receptor substrate 1-4 (IRS1-4) [74-77] and the Src-homology collagen protein (Shc) [78]. Once activated, Shc and IRSs bind to a complex of the growth factor receptor bound-2 (Grb2) and Son of sevenless (Sos). This complex allows the activition of Ras and its downstream cascade, the Ras/Raf/mitogen activated protein kinase and extra-cellular-signal-regulated kinase kinase (MEK)/extra-cellular-signal-regulated kinase (ERK) pathway. The Ras/Raf/MEK/ERK cascade is involved in cell proliferation, differentiation, and protection against apoptosis.

Activated IRS proteins also bind to the p85 regulatory subunit of the phosphatidylinositol 3-kinase (PI3K). The activation of PI3K leads to an increase in the phosphatidyl inositol 3,4,5 triphosphate (PIP3) level that induces the recruitment of Akt (protein kinase B). This allows the constitutively activated 3'-phosphoinositide-dependent kinases (PDK)-1 and PDK-2 to phosphorylate and activate Akt. Many downstream targets of Akt have been shown to prevent apoptosis and to stimulate cellular proliferation or glucose transport [6-10,79]. The mammalian Forkhead members of the class O (FOXO) transcription factors are well-characterized substrates of Akt. Phosphorylation of FOXOs by Akt results in the retention of FOXOs in the cytoplasm [80]. In the absence of PI3K/Akt signaling, FOXOs translocate to the nucleus and activate transcription of their target genes, including those that encode proteins that induce cell cycle arrest (e.g., p130 and p27) and apoptosis (e.g., Fas ligand, Trail, and Bim) [81].



modified from Dupont, 2003 [82]

Figure 4. Scheme of the IGF1R activation and downstream signalings. Following ligand (IGFs) binding to IGF1R, tyrosine kinase activity of IGF1R is activated, and this stimulates signaling through intracellular networks that regulate cell proliferation and cell survival. Key downstream networks include the Ras/Raf/MEK/ERK and the PI3K/Akt pathways. Two other mitogen-activated protein (MAP) kinases, p38 and JNK, are also activated in response to IGF-1. The MAP kinases are involved in cell survival, differentiation, proliferation, and protection against apoptosis. The PI3K/Akt pathway is involved in protein translation, cell survival, proliferation, apoptosis, and glucose metabolism (see more details in the text).

1.3.3.1 The Ras/Raf/MEK/ERK signaling (review in ref. [83-86])

One of the most important pathways activated by IGFs is the Ras/Raf/MEK/ERK cascade [7]. The Ras/Raf/MEK/ERK cascade is a signal transduction pathway that transmits signals from extra-cellular stimuli to regulate cellular processes such as proliferation, differentiation, cell cycle progression, and prevention of apoptosis [85].

Following binding of ligands to their specific cell surface receptors, activation of the coupling complexes Shc/Grb2/Sos or IRSs/Grb2/Sos occur and induce the activation of Ras, a small GTP-binding protein. The inactive GDP-bound conformation of Ras exchanges into the active GTP-bound conformation [84]. The GTP-bound active Ras can then recruit Raf, a serine/threonine kinase, to the cell membrane for subsequent activation by phosphorylation [87].

Raf is normally activated by a complex series of events including: recruitment to the plasma membrane mediated by an interaction with Ras [88], dimerization of Raf proteins [89], phosphorylation/dephosphorylation at different domains [90], dissociation from the Raf kinase inhibitory protein (RKIP) [91], and association with scaffold complexes [92]. Raf activity is further modulated by chaperone proteins including 14-3-3 [93] and heat shock protein 90 (Hsp90) [94].

Activated c-Raf phosphorylates and activates MEK1/2 [95], which in turn activate the effector proteins ERK1 and ERK2 by phosphorylation of the Thr and Tyr residues within their activation loop [96]. ERK1/2 as well as their direct target substrate, p90 ribosomal s6 kinase ($p90^{RSK}$), enter into the nucleus upon activation, where they phosphorylate and activate transcription factors including c-myc, AP-1, TCF/Elk-1, c-Ets-1, and c-Ets-2 leading to the expression of the immediate early gene *FOS* and others [97-99] (see Fig. 5).

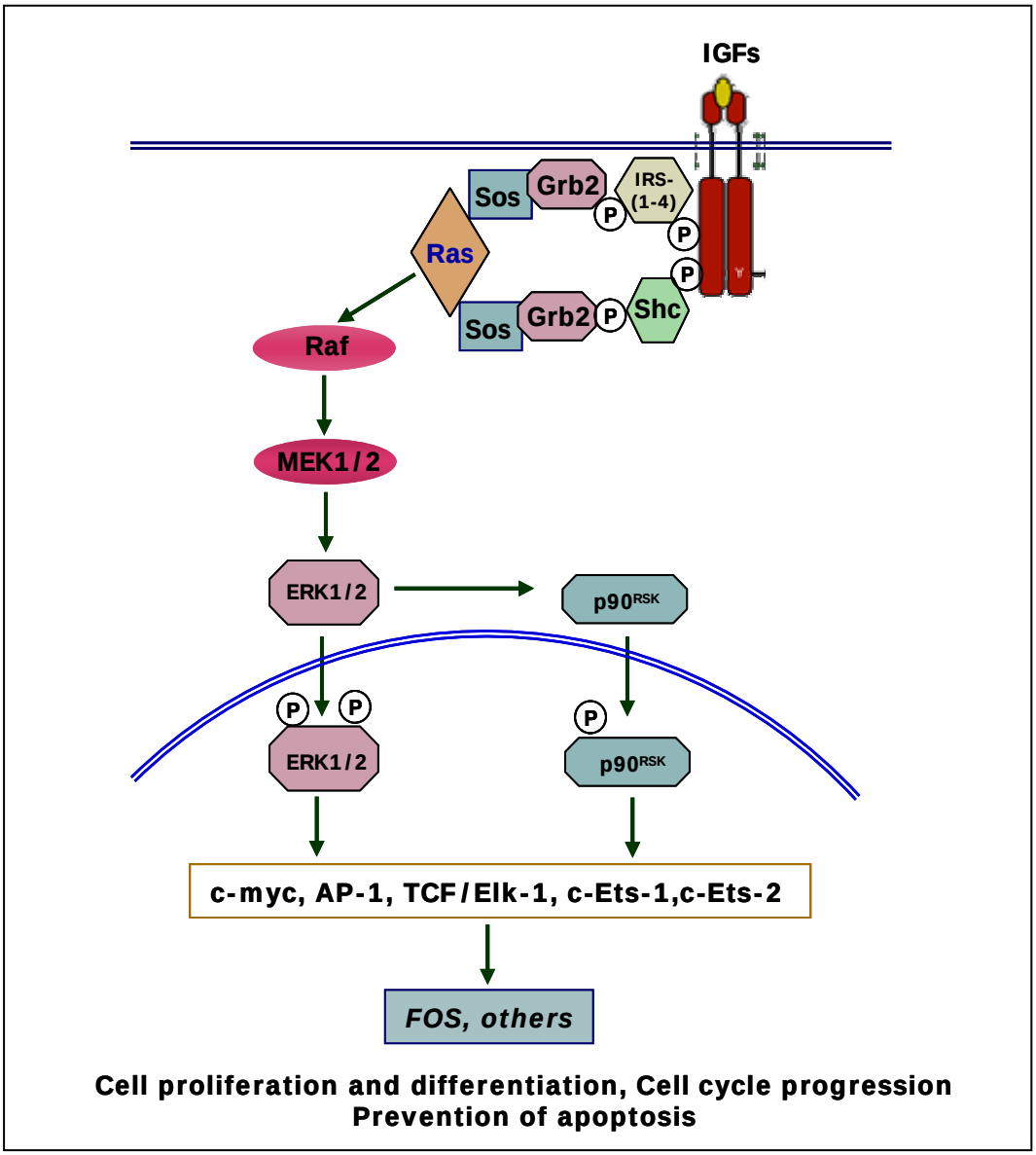


Figure 5. Scheme of the Ras/Raf/MEK/ERK cascade. Growth factor stimulation of the Ras/Raf/MEK/ERK pathway leads to sequential activation of Ras and Raf, which in turn activates MEK1 and MEK2. MEK1/2 are dual-specificity kinase that are essential to the propagation of growth factor signaling and are known to amplify signals to ERK1/2 that in turn can phosphorylate and activate a range of proteins including several transcription factors leading to the expression of *FOS* and others (see more details in the text).

1.3.3.2 c-Fos, a downstream target of the Ras/Raf/MEK/ERK cascade

c-Fos is a member of the Fos family of transcription factors including c-Fos, FosB, Fra-1, and Fra-2. c-Fos is the product of *FOS,* a proto-oncogene belonging to the immediate early genes (IEGs) that are rapidly activated in response to various cellular stimuli [100,101]. The proper regulation of *FOS* expression is important in many cellular processes including proliferation, differentiation, and oncogenic transformation [102]. *FOS* is located on the long arm of chromosome 14 (14q21-31); it contains four exons, spans approximately 4 kb, and encodes a 380 amino acid protein of 55 to 62 kD [103,104].

c-Fos has a bZIP region consisting of a basic DNA-binding domain, a leucine zipper domain, and a transactivation domain at the C-terminus [105]. The promoter of *FOS* is organized into the serum response element (SRE), the c-Sis inducible element (SIE), the TCF (ternary complex factor), the calcium response element (CRE), and the AP-1/CRE [101,106]. The SRE is involved in the induction of c-Fos in response to growth factors, phorbol esters, and ionizing radiation [101]. Serum and growth factors potently induce *FOS* expression by activating ERK. Activated ERK translocates to the nucleus to phosphorylate TCFs thereby provoke the transcriptional activity of TCFs that bind to *FOS* promoters.

Fos proteins together with Jun family members, c-Jun, JunB, and Jun D, can form the complexes that bind to AP-1 (activating protein-1) regulatory elements in the promoters and enhancer regions of numerous mammalian genes [107]. The AP-1 binding site occurs with a high frequency in the genome, and it is likely that Fos-Jun family proteins regulate all genes that contain AP-1 recognition sequences [108].

1.3.3.3 Cyclin D1, the target of the IGF-1 signaling cascade

Cyclin D1, a 295 amino acids protein with a molecular weight of about 34 kD, is the product of the *CCND1* (also known as *PRAD1*) gene located on the long arm of chromosome 11 (11q13) [109-112]. This gene spans about 15 kb and has five exons. Induction of *CCND1* is induced by growth factors including IGF-1, IGF-2, and epithelial growth factor, and hormones including androgen, retinoic acid, and peroxisome proliferator-activated receptor γ ligand [113]. Growth factor stimulation typically activates *CCND1* mRNA production through coordinated activation of several families of transcription factors, including activator protein-1 (AP-1) [114-116], nuclear factor-κB (NF-κB) [117], Sp1 [118], cyclic AMP response element-binding protein (CREB) [118], ternary complex factor (TCF) [119]. Once produced, the *CCND1* transcript is processed by post-transcriptional mechanisms including mRNA stability, sub-cellular localization, and targeted protein degradation [120-122] (see Fig. 6). Following mitogen stimulation, the cyclin D1 protein appears rapidly in early G1 phase before cyclin E and declines rapidly when the growth factors are withdrawn [123].

IGF-1 is one of the best-known growth factors that activate the *CCND1* expression. Activation of Ras/Raf/MEK/ERK, the major pathway downstream of IGF-1 signaling, induces transcription of the *CCND1* gene [124,125]. The PI3K/Akt and/or mTOR, other major pathways downstream of IGF-1, also activate the *CCND1* transcription and mRNA translation [125]. Moreover, activation of PI3K/Akt also induces a stabilization of cyclin D1 protein by inactivating glycogen synthase kinase 3 beta (GSK-3β) [125]. Abundance of cyclin D1 is regulated by its cellular localization and protein degradation [126]. During G1 phase, cyclin D1 localizes to the nucleus and re-localizes to the cytoplasm during S phase [123]. The phosphorylation of cyclin D1 at threonine 286 residue by GSK-3β in the nucleus results in the relocalization of cyclin D1 to the cytoplasm during S phase where it is degraded by the proteaosome [127-129]. The level of cyclin D1 increases again during G2 phase and sustains through M and G1 phases [130].

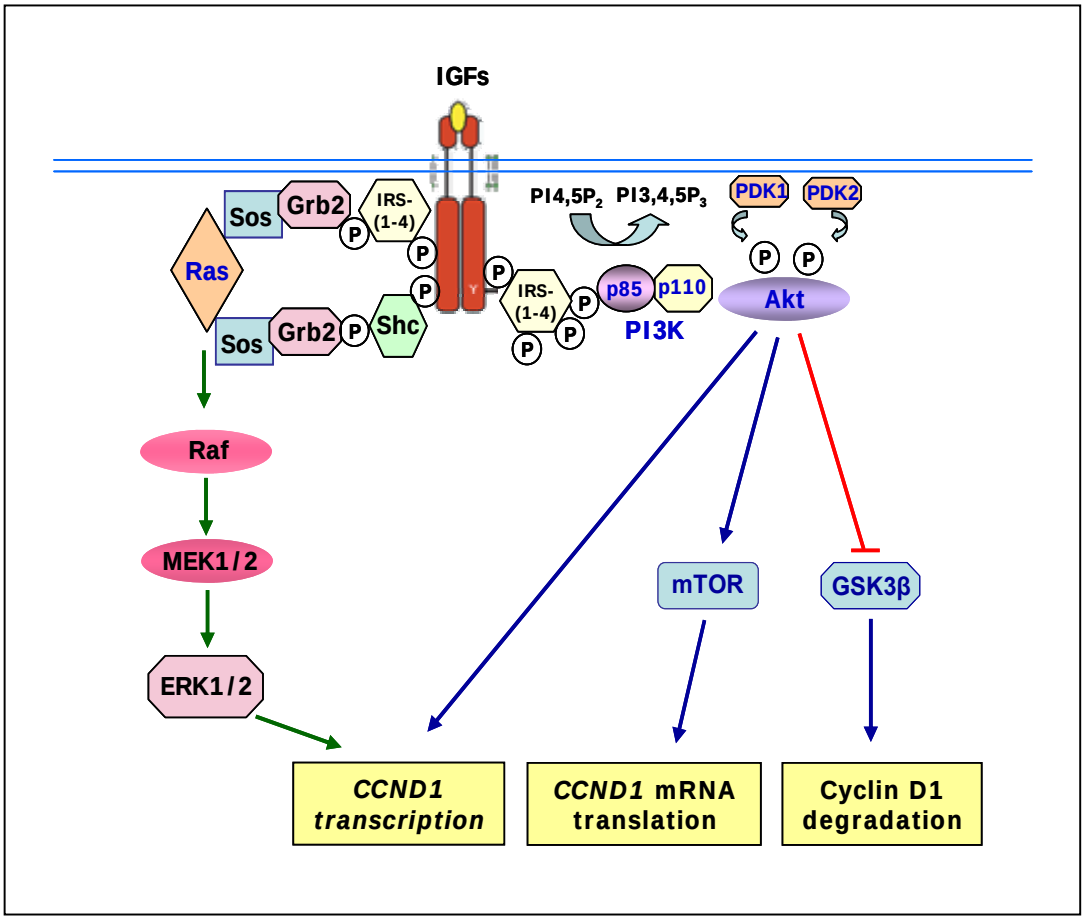


Figure 6. Scheme of the regulation of the *CCND1* gene and cyclin D1 protein by the IGF-1 signaling cascade. The major pathways downstream of the IGF-1 signaling cascade which regulate *CCND1* transcription, translation, and cyclin D1 protein stabilization are illustrated (see more details in the text).

1.3.4 The regulation of cell cycle progression through G1 phase

1.3.4.1 The cell cycle

The cell cycle is ubiquitous, complex process for the growth and proliferation of the cells and organismal development [131,132]. The cell cycle can be subdivided into interphase and mitosis phase (M). There are three stages in interphase, Gap1 phase (G1), Synthesis phase (S), and Gap2 phase (G2). Mitosis phase is divided into the four stages prophase, metaphase, anaphase, and telophase [131,133]. During mitosis, the chromosomes are separated and the cell divides into two daughter cells. After division, the cells are back in G1 phase and the cell cycle is completed. The duration of the cell cycle varies between different cell types. In most mammalian cells, it lasts between 10 and 30 hours. Cells in G1 phase do not always continue through the cycle. Instead, they can exit from the cell cycle and enter a resting stage (G0) [134,135].

1.3.4.2 **The regulation of the cell cycle** (review in ref. [123,132,136-138])

For all living eukaryotic organisms it is essential that the different phases of the cell cycle are precisely coordinated. The phases must follow in correct order, and one phase must be completed before the next phase can begin. The cell cycle is regulated in eukaryotic cells by the periodic activation of different cell cycle phase-specific proteins known as cyclin-dependent kinases (Cdks) and cyclins. Cdks are serine/threonine protein kinases that are activated at specific points in the cell cycle. The Cdks are important for the cell cycle because their inactivation state prevents mitosis. Cdks are regulated by several factors such as phosphorylation at threonine and tyrosine residues and binding to cyclins. Cyclins target Cdks to the nucleus where they can phosphorylate a variety of substrates, thereby catalyzing the process of cell division.

The expression levels of cyclins during cell division are periodic (see Fig. 7). In general, before a cell can enter a new phase of cell cycle, the cyclin of the previous phase is degraded, and the cyclin of the next phase is synthesized. Following mitogen stimulation of quiescent cells, genes encoding D-type cyclins are activated at the beginning of G1 phase

and followed by expression of E-type cyclins promoting G1/S phase transition [120,139]. Cyclin A accumulates during S phase and peaks at G2 [140]. Its activation pushes the progression of the cell through S and G2 phases. The synthesis of Cyclin B is initially induced and accumulates at late S phase but peaks at G2/M phase triggering the G2/M transition [141].

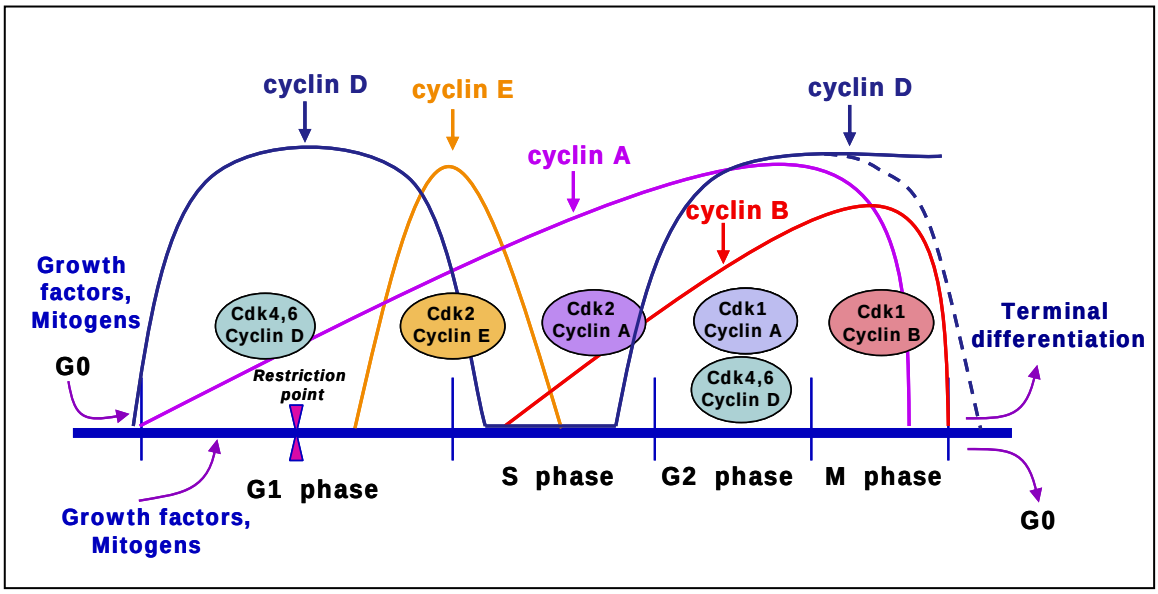


Figure 7. Scheme of the periodic expressions of cyclins and cyclin-Cdk complexes during cell cycle progression. The different stages of the cell cycle are quiescence (G0), G1 phase, the DNA synthesis phase (S), G2 phase, and mitosis phase (M).The major mammalian cyclin-Cdk complexes active in specific phases of cell cycle are illustrated. A blue broken line represents the declination of cyclin D level when the cell terminates from the cell cycle or when growth factors are withdrawn (see more details in the text).

1.3.4.3 The regulation of G1 to S phase transition

Progression through G1 phase in mammalian cells requires growth factor induced signal transduction [142]. Growth factors exert most of their action on cells at the G1/S restriction point [143]. After the restriction point in G1 is passed, the cell cycle progresses largely independently of growth factors [144]. The transition that occurs at the restriction point in G1 commits the cells to the proliferative cycle. Passage through the restriction point and transition to S phase is initiated by D-type cyclins (D1, D2, D3), the first cyclins produced in the cell cycle [145-147]. Following its expression, cyclin D initiates cell cycle progression towards S phase by its binding to either cyclin dependent kinase 4 (CDK4) or cyclin dependent kinase 6 (CDK6), dependent on cell type, and activates the kinase moiety [148].

Once activated, the cyclin D-CDK4/6 complex promotes cell cycle progresstion by inactivating the retinoblastoma tumor suppressor protein (Rb) [149-151]. In its hypophosphorylated state, Rb silences specific genes through its ability to form transcriptional repressor complexes with E2F transcription factors on the promoters of genes that are active in the S phase of the cell cycle [152]. The cyclin D-CDK4/6 complex mediates phosphorylation of Rb (pRb). pRb dissociates from E2F, thus releasing its transcriptional repressor function. In the absence of Rb, E2F (along with its binding partner DP-1) mediates the trans-activation of E2F target genes including *CCNE* (encodes cyclin E), *CCNA* (encodes cyclin A), *POL* (encodes DNA polymerase), *TK* (encodes thymidine kinase), etc. genes that facilitate the G1/S transition and S phase progression [152,153].

One of the most important targets activated by cyclin D1 is the *CCNE* gene. Cyclin D1 induces the expression of *CCNE* by two distinct mechanisms, either through E2F activation or through the sequestration of the Cip/Kip cell cycle inhibitors such as p21 or p27 [154]. Cyclin E binds to CDK2, forming the cyclin E-CDK2 complex, which pushes the cell from G1 to S phase (see Fig. 8).

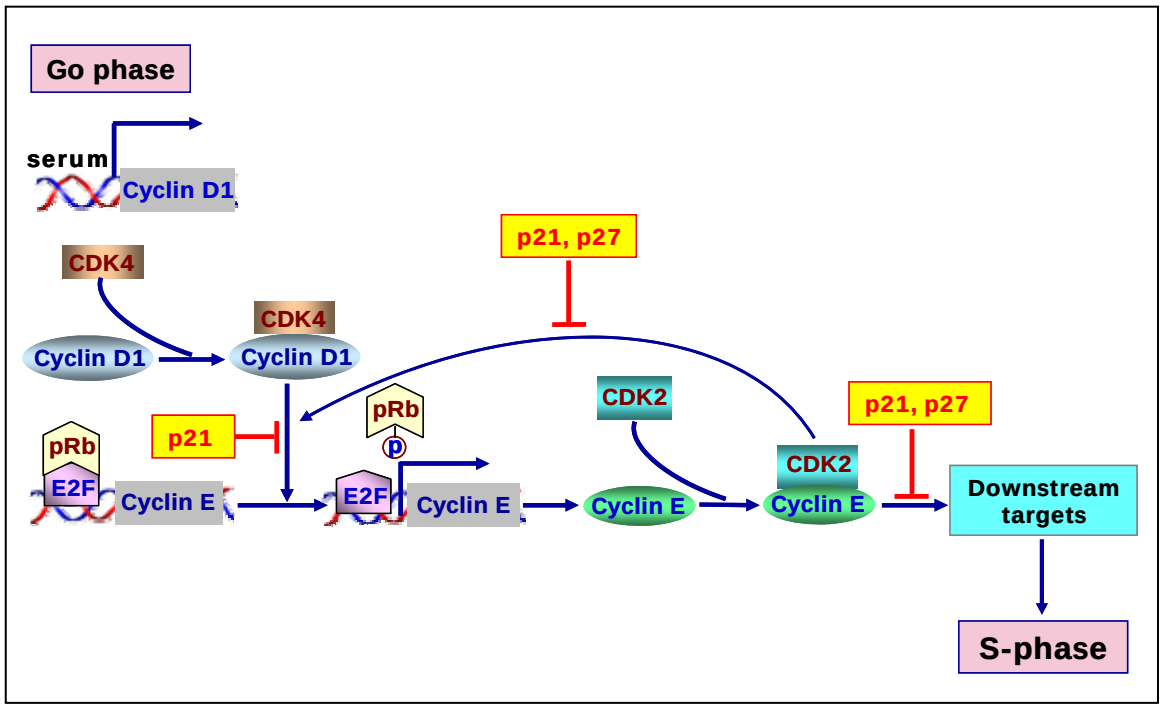


Figure 8. Scheme of the regulation of G1 to S phase transition by cyclin D1. At the beginning of G1 phase, growth factors or mitogens induce the expression of genes encoding D-type cyclins. The induced cyclin D associates to Cdk4/6 which phosphorylate and inactivate the retinoblastoma protein (Rb). The inactivated Rb fails to suppress the E2F transcription factor which controls the expression of genes required for the G1/S transition and S phase progression including the cyclin E gene.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 List of antibodies used in this study

2.1.1.1 Antibodies for immunofluorescence staining and PI3K activity assay

antibody, catalog number, source, working dilution [antibody (µl):blocking buffer (µl)]	**Supplier**
Alexa Fluor 488, A11029, goat anti-rabbit IgG, 1:200	Invitrogen, Karslruhe, Germany
Cy3^{TM}, 200-162-037, goat anti-mouse IgG, 1:500	Jackson ImmunoResearch Lab, Inc., West Grove, USA
Erk1/2, 9102, rabbit, 1:100	Cell Signaling Technology/ New England Biolab, Frankfurt a. Main, Germany
Nbs1, 1D7, mouse, 1:250	BioMol, Hamburg, Germany
p85 subunit of PI3 kinase, 06-197, rabbit (2 µl/1.5 mg of protein)	Upstate/Millipore GmbH, Schwalbach, Germany
phosphotyrosine , 05-777, mouse, (2 µl/1.5 mg of protein)	Upstate/Millipore GmbH, Schwalbach, Germany

2.1.1.2 Antibodies for Western blot

antibody, catalog number, source, working dilution [antibody (µl):blocking buffer (ml)]	**Supplier**
β-Actin, I-19 sc-1616, goat, 1: 4	Santa Cruz Biotechnology Inc., USA
Akt1, 2967, rabbit, 1:1	Cell Signaling Technology/ New England Biolab, Frankfurt a. Main, Germany

antibody, catalog number, source, working dilution [antibody (µl):blocking buffer (ml)]	**Supplier**
phospho-S473-Akt, 9271, rabbit, 1:1	Cell Signaling Technology/ New England Biolab, Frankfurt a. Main, Germany
Cyclin A, 611268, mouse, 1:2	BD Biosciences, Germany
Cyclin D1, CC12, mouse, 7:1	Calbiochem-Novabiochem, Bad Soden, Germany
Cyclin E, ab3927, mouse, 1:2	Genetex, Abcam, Germany
Erk1/2, 9102, rabbit, 1:1	Cell Signaling Technology/ New England Biolab, Frankfurt a. Main, Germany
phospho-Thr202/Tyr204-Erk1/2, 9101, rabbit, 1:1	Cell SignalingTechnology, Frankfurt a. Main, Germany
c-Fos, sc-52, rabbit, 1:1	Santa Cruz Biotechnology Inc., USA
FOXO1, sc-11350, rabbit, 1:1	Santa Cruz Biotechnology Inc., USA
Mre11, ab3621-50, rabbit, 1:1	Genetex, Abcam, Germany
Nbs1, 1D7, Mouse, 1:1	BioMol, Hamburg, Germany
Phospho-Nbs1 (Ser343), 05-663, mouse, 2:1	Upstate/Millipore GmbH, Schwalbach, Germany
Rad50, GT70228, Mouse, 1:2	Genetex, Abcam, Germany
phospho-s338-c-Raf, 56A6-9427, rabbit, 1:1	Cell Signaling Technology/ New England Biolab, Frankfurt a. Main, Germany
Sos1, sc-256, rabbit, 2:1	Santa Cruz Biotechnology Inc., USA

antibody, catalog number, source, working dilution [antibody (µl):blocking buffer (ml)]	**Supplier**
Sos2, sc-258, rabbit, 2:1	Santa Cruz Biotechnology Inc., USA
Donkey anti-goat IgG-HRP, sc-2033, 0.3:20	Santa Cruz Biotechnology Inc., USA
Goat anti-mouse IgG-HRP, sc-2005, 0.3:20	Santa Cruz Biotechnology Inc., USA
Goat anti-rabbit IgG-HRP, sc-2004, 0.3:20	Santa Cruz Biotechnology Inc., USA
IGF-1Rα, N-20 sc-712, rabbit, 5:1	Santa Cruz Biotechnology Inc., USA

2.1.2 List of buffers, medium, and solutions used in this study

2.1.2.1 Buffers and solutions for immunofluorescence staining.

1% Bovine Serum Alblumin (BSA)

Dissolve 1 g BSA in 100 ml PBS and stir until it dissolves.

4% Paraformaldehyde (PFA)

Dissolve 4 g PFA on day of use in 100 ml PBS by heating at 60°C and stirring until it dissolves. Cool before use.

0.2% Triton X-100

Prepare stock of 20% Triton X-100 in PBS; rotate tube overnight to dissolve. Dilute to 0.2% with PBS

2.1.2.2 Buffers and solutions for PI3K activity assay.

Lysis buffer

20 mM Tris/HCl, pH 7.5
137 mM NaCl
1 mM $CaCl_2$
1 mM $MgCl_2$
0.1 mM Na_3VO_4 (add before use)
0.3% Triton X-100

Reaction buffer

20 mM Tris/HCl, pH 7.5
100 mM NaCl
0.5 mM EGTA

2.1.2.3 Buffers and solutions for Western blot analysis

Blocking buffer for antibody detection

5% non fat dry milk powder in TBS-T solution

Laemmli gel loading buffer (4X)

100 mM Tris-HCl pH 6.8

4% SDS

0.2% Bromophenol blue

20% Glycerol

200 mM DTT

5 x Laemmli Running buffer

15.1 g Tris-Base (25 mM)

94 g Glycine (250 mM)

50 ml 10% SDS

Adjust the volume to 1 l with water.

NOV stock solution (100 mM)

18.3 mg/ml water

PMSF stock solution (100 mM)

17.4 mg PMSF/ml isopropanol

RIPA-Buffer–Cell lysis buffer

150 mM NaCl

10 mM Tris-HCl, pH 7.2

0.1% SDS

1% Triton X-100

1% Deoxycholate

5 mM EDTA

Before using add 1:100 of 100 mM PMSF and 100 mM of NOV

PBS (Phosphate buffered saline)

9.6 g PBS dissolved in 1 l water and sterilized by autoclaving

SDS-PAGE (separating gel, 10%)

375 mM Tris-HCl, pH 8.8

10% Acrylamid/Bisacrylamid (37.5/1) (Protogel^{TM})

0.1% SDS

0.1% APS

0.01% TEMED

SDS-PAGE (stacking gel, 5%)

125 mM Tris-solution pH 6.8

5% Acrylamid / Bisacrylamid (37.5/1) (Protogel^{TM})

0.1% SDS

0.1% APS

0.01% TEMED

Stripping Puffer

2% SDS (20 g/l)

62.5 mM TrisHCl (9.9 g/l)

100 mM ß-Mercaptoethanol (7 ml/l)

10 x TBS-T (pH 7.6)

20 mM Tris-Base (2.4 g/l)

137 mM NaCl (8 g/l)

0.1% Tween 20 (1 ml/l)

Towbin Puffer (blotting buffer)

3 g Tris-Base

14.4 g Glycine

200 ml Methanol, adjust the volume to 1 liter with distilled water.

2.1.2.4 Medium for cell culture

For RPE cell lines

DMEM-F12 (1:1) (PAA)

10% FCS (PAA)

17.3 ml of 7.5% sodiumbicarbonate

40 U/ml penicillin, 40 µg/ml streptomycin

2.1.2.5 Solutions for cell cycle analysis

Solution I (prepare 500 ml stock)

NaCl	584	mg/l
Na-citrate	1,000	mg/l
RNase A from bovine pancreas	10	mg/l
Nonidet P-40	0.3	mg/l

Solution II (prepare 500 ml stock)

Citric acid	15	g/l
Sucrose	0.25	mM

Add 50 ug/ml propiodium iodide to each solution before use.

2.1.2.6 Solutions for cell treatment

Aphidicholin

Dissolve aphidicholin in DMSO to a concentration of 2 mg/ml.

Aliquot and store at -20°C.

IGF-1 stock solution

Dissolve lyophilized IGF-1 in sterile water to a concentration of 0.1 mg/ml.

Aliquot and store at -20°C.

U0126 stock solution

Dissolve U0126 in DMSO to a concentration of 10 nM. Aliquot and store at -20°C.

Wortmannin stock solution

Dissolve wortmannin in DMSO to a concentration of 50 mM and store at 4°C.

2.1.3 List of chemicals and materials used in this study

Chemical or material	Supplier
Absolute Ethanol	Merck KG aA, Darmstadt, Germany
Agarose gel	Biozym, Hess. Oldendorf, Germany
Ampicillin	Serva, Heidelberg, Germany
APS	Merck KG aA, Darmstadt, Germany
Aphidicolin	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
[γ-^{32}P]ATP	Hartmann-Analytik, Braunschweig, Germany
Blotting Grade Blocker Non-Fat Dry Milk	Biorad, Munich, Germany
Boric acid	Merck KG aA, Darmstadt, Germany
Bradford reagent	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
Bromophenol blue	Molecular Diagnostics, Mannheim
BSA	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
Citric acid	Merck KG aA, Darmstadt, Germany
Chloroform	Merck KG aA, Darmstadt, Germany
Colorimetric cell proliferation kit I (MTT)	Roche Molecular Biochemicals, Mannheim, Germany
Deoxycholate	Merck KG aA, Darmstadt

Chemical or material	Supplier
DEPC	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
DMEM	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
DMSO	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
DTT	Serva, Heidelberg, Germany
EDTA	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
Ethidium bromide	Serva, Heidelberg, Germany
FCS	PAA Laboratories, Austria
LightCycler Faststart Reaction Mix SYBR Green I	Roche Molecular Biochemicals, Mannheim, Germany
Magnesium chloride	Merck KG aA, Darmstadt, Germany
2-Mercaptoethanol	Merck KG aA, Darmstadt, Germany
Methanol	Merck KG aA, Darmstadt, Germany
Neomycin	Calbiochem-Novabiochem, Bad Soden, Germany
Nonidet P-40	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
Nuclear and cytoplasmic extraction kit (NE-PER)	Perbio Science Deutschland GmbH Bonn, Germany
Paraformaldehyd	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
Penicillin/Streptomycin	Gibco BRL, Karlsruhe, Germany
Phosphate Buffered Saline	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
PMSF	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
Polyfect Transfection Reagent	QIAGEN GmbH, Hilden, Germany

Chemical and material	Supplier
Ponceau S	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
Propidiumiodide	Sigma, USA
Protogel™	Natioal Diagnostics., Atlanta, USA
Roti-buffer	Roth GmbH, Karlsruhe, Germany
SDS	Serva, Heidelberg, Germany
SiLentFect Lipid	Biorad, Munich, Germany
siRNA (*NBS1* and scramble)	Ambion (Europe) Ltd, U.K.
siRNA (*NBS1* stealth and scramble stealth)	Invitrogen, Karlsruhe, Germany
Sodium citrate	Merck KG aA, Darmstadt, Germany
Sodium bicarbonate	Merck KG aA, Darmstadt, Germany
Sodium chloride	Merck KG aA, Darmstadt, Germany
Sodiumorthovanadate	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany
Sucrose	Merck KG aA, Darmstadt, Germany
SuperScript Reverse Transkriptase	Invitrogen, Karlsruhe, Germany
TEMED	Pharmacia Biotech GmbH, Freiburg, Germany
Tris	Merck KG aA, Darmstadt, Germany
Triton X-100	Merck KG aA, Darmstadt
Trypsin	GibcoBRL, Karlsruhe, Germany
TRIzol	Invitrogen, Karslruhe, Germany
Tween-20	Merck KG aA, Darmstadt, Germany
U0126	Cell Signaling Technology/ New England Biolab, Frankfurt a. Main, Germany
Vectashield-mounting medium	Vector Laboratories, Burlinggame, USA
Wortmannin	SIGMA-Aldrich Chemie GmbH, Deisenhofen, Germany

2.1.4 List of instruments used in this study

Instrument	Supplier
Agarose gel electrophoresis-Apparatus	(Wide-Mini-) Sub Cell GT, Biorad, Munich, Germany
Blotting-Apparatus	Trans-Blot SD Semi-Dry Transfer Cell, Biorad, Munich, Germany
Blotting-paper	WhATMann 3 mm, Schleicher & Schuell GmbH, Dassel, Germany
Cell culture flask	• Greiner Labotechnik Bio-One GmbH, Frickenhausen, Germany
Cell culture dish	• Nunc, Dänemark, Germany
Centrifuge	• Eppendorf centrifuge 5415c, Eppendorf, Hamburg, Germany • Multifuge 3SR, Heraeus, Hanau, Germany • Sigma 2K15 and Sigma 3K15, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany
CO_2 - Incubator	Sanyo, Japan
Cryo tube	Kisker-Biotech, Mühlhausen, Germany
Fluorescence-activated cell sorting and software	BD LSRII (Scanner), FACS-Scan, FACStar Plus (Sorter), BD FACSDiva software, Becton Dickinson, USA
Glass ware	• Braun, Melsungen, Germany • Schott, Mainz, Germany
Incubator	• Heraeus, Hanau, Germany • Memmert, Schwabach, germany • Sanyo, Japan

Instrument	Supplier
Laminar flow clean bench	Larminar Flow, BDK, Sonnenbühl-Genkingen, Germany
Light Cycler	Roche Molecular Diagnostics, Mannheim, Germany
Magnetic stirrer	Ikamag RCT, IKA-Labortechnick, Staufen i.Br., Germany
Microscope	• Axiovert 25CF, Zeiss, Jena, Germany • LSM 510, Zeiss, Jena, Germany • Olympus LH50A, Olympus Optical Co., Hamburg, Germany
Nitrocellulose Membrane	Protran 0,2 mm, Schleicher &Schuell
PAGE-Apparatus	Hoefer Mighty SmallTM SE245, Dual Gel Caster, Amersham Pharmacia Biotech, Freiburg, Germany
Pipette	Eppendorf, Hamburg, Germany
pH-Meter	InoLab pH Level 1, UK
Power supply unit	• Biorad, munich, Germany • Consort electrophoresis power supply, Fröbel Labortechnik GmbH, Lindau, Germany • Electrophoresis Power Supply-EPS600, Amersham Pharmacia Biotech, Freiburg, Germany
Film	X-Omat AR Film, Kodak, Stuttgart, Germany
Film processing machine	Optimax Typ TR, MS Labogeräte, Wiesloch, Germany
Reaction tube	0.5 ml, 1.5 ml, 2.0 ml Eppendorf Hamburg, Germany

Instrument	Supplier
Reaction tube	• 15 ml, 20 ml Falcon USA and Greiner Labotechnik Bio-One GmbH, Frickenhausen, Germany • FACS-tube, Falcon, USA • PCR-tube, Biozym Diagnostik, hess-Oldendorf, Germany
Refrigerator	• Liebherr GmbH, Lienz, Germany • Privileg, Quelle, Fürth, Germany • Sanyo, Japan
Scanner	Umax, powerLook 1000, Willich
Photometer	BioFotometer, Eppendorf, Hamburg, Germany
UV-Transilluminator	Vilber lourmat, AGS, Heidelberg, Germany
Water Bath	Frigomix U1, Braun, Melsungen, Germany

2.1.5 List of oligonucleotide primers for real time PCR

Target gene, catalog number	Supplier
B2M, QT00088935	QIAGEN GmbH, Hilden, Germany
CCND1, QT00495285	QIAGEN GmbH, Hilden, Germany
FOS, QT00007070	QIAGEN GmbH, Hilden, Germany
NBS1	Metabion international AG, Germany
SOS1, QT00030814	QIAGEN GmbH, Hilden, Germany
SOS2, QT00096936)	QIAGEN GmbH, Hilden, Germany

2.2 Methods

2.2.1 Cell culture

The telomerase-positive, immortalized human retinal pigment epithelial cell line, (hTERT-RPE) was cultured in Dulbecco's Modified Eagle Medium (DMEM-F12) containing 2.5 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 0.25% sodium bicarbonate, 40 units/ml penicillin G, and 40 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.2.2 Transient cell transfection with short interfering RNAs

The day before transfection, approximately 8×10^4 cells were seeded into a 60 mm culture plate containing 5 ml DMEM/F12 medium and cultivated overnight such that they are 70% confluent in the following day. 15-60 min prior to transfection, the medium was aspirated from the plates and 2.5 ml fresh medium were added to each plate. For each well to be transfected, transfection solutions were prepared by adding 3 μl of siLentfect Lipid to 250 μl of serum free medium. After that, *NBS1* siRNA (si*NBS1*; 5′-AUGAUGUGGCCAUAGAAGATT-3′, 5′-UCUUCUAUGGCCACAUCAUCC -3'), or scramble siRNA, or NBS1 stealth siRNA (unknown sequence), or scramble stealth siRNA were added to 250 μl of serum free medium as a final concentration of 30 nM after adding to the culture dishes. The diluted siRNAs were added to the diluted siLentFect Lipid and then mixed by pipetting. The mixed solutions were incubated for 20 min at room temperature. 500 μl of the RNA-siLentFect Lipid complexes were directly added to each culture dish containing 2.5 ml serum-containing medium. The culture plates were swirled to ensure uniform distribution of the transfection complexes and then incubated at cell culture condition. The transfection mixture was removed 8 h after transfection, and cells were washed with PBS before being incubated with fresh medium until the expression of target gene was fully repressed.

2.2.3 Cell treatments

For cell treatment, the medium was aspirated from the dishes and replaced with 5 ml of fresh medium containing the following reagents. For aphidicolin treatment, cells were treated for 24 h with 10 μg/ml of aphidicolin diluted in DMSO. Subsequently, cells were released from blockade by washing twice with PBS before adding 5 ml fresh medium. For IGF-1, U0126/IGF-1 and wortmannin/IGF-1 treatment, cells were cultured in serum-free medium for 48 h, pretreated with 10 μM U0126 or 100 nM wortmannin for 1 h and then stimulated with 100 ng/ml IGF-1. The treated cells were collected at different time points for cell cycle analysis, quantitative real time PCR analysis, and Western blot analysis.

2.2.4 Cell cycle analysis by flow cytometry

DNA staining of isolated nuclei for cell cycle analysis was performed using a modified method according to Nüsse et al. (24). At each indicated time point, the treated cells were detached with trypsin, collected by centrifugation at 300 ×g for 5 min. The cell pellet was gently resuspended in 500 μl of a solution I and vortexed for a short time interval. The cell suspensions were incubated for 30 min at room temperature, followed by the addition of 500 μl of a solution II. The cell suspensions were mixed and stored at 4°C before flow cytometric measurement. Cell cycle distributions were analyzed on a fluorescence-activated cell sorting (FACS) with two signal detectors, the photodiode and the photomultipier tubes, equipped with a laser operating at 20 mW, generating a light of 488 nm wavelength. PI emission was measured at wavelengths between 600 to 650 nm.

2.2.5 Cell proliferation and viability assays

To determine the cell proliferation rate, RPE cells were transfected with *NBS1* siRNA or scramble siRNA. At 1 to 4 days after siRNA transfection, cells were harvested by trypsinization, and counted with a hemocytometer and a particle counter. The colorimetric cell proliferation kit I (MTT) was used for the measurement of cell proliferation after stimulation with growth factor IGF-1. 24 h after transfection with siRNA, cells were seeded into 24-well plates and incubated for 4 h. After starvation for 24 h, cells were pre-treated with vehicle alone or 10 μM U0126 and subsequently stimulated with 300 μl medium

containing 10% FCS and 100 nM IGF-1 per well. 48 h after stimulation, 30 µl MTT labeling reagent was added, and cells were incubated for another 4 h. Following the addition of 300 µl solubilization solution, the samples were incubated for an additional 12 h and absorbance was determined against a background control (medium only) at 595 nm. Cell proliferation was expressed as n-fold increase of absorbance in treated cells relative to that of untreated control cells.

2.2.6 Colony forming assay and cell irradiation

20 h after siRNA transfection, the cells were prepared into a single-cell suspension by trypsinization, and the cell concentration was counted. An appropriate number of cells were seeded into 6-well plates and then cultivated for 12 h to allow the cells to attach to the plate. The number of cells per plate initially seeded varies with the dose, so that the number of colonies surviving is in a range that can be counted conveniently. The cells pre-treated with or without 100 nM IGF-1 were irradiated using a Cesium-137 source with a single dose of 0, 3, 6, or 9 Gy at a dose rate of 1.76 Gy/min at room temperature. After that, the medium was replaced by fresh medium and the cells were allowed to grow for 7-10 days until the surviving cells produced macroscopic visible colonies that can be counted easily. The cells were fixed with 100% ethanol for 10 min and then stained with crystal-violet for 10 min. Colonies containing more than 50 cells were counted and survival fractions were calculated from the ratio of colonies produced to the cells plated, with a correction necessary for plating efficiency (PE) (see equation below).

$$\text{Plating efficiency (PE)} = \frac{\text{Number of colonies counted}}{\text{Number of cells seeded}} \times 100\%$$

Plating efficiency (PE) is the percentage of cells seeded that grow into colonies without irradiation.

$$\text{Survival fraction (SF)} = \frac{\text{Number of colonies counted}}{\text{Number of cells seeded}} \times \text{PE} / 100\%$$

Survival fraction (SF) is the fraction of cells surviving at a giving dose of radiation.

2.2.7 PI3K activity assay

PI3K activity was determined as described with slight modifications [155]. RPE cells were transfected with scramble siRNA or *NBS1* siRNA. 48 h after transfection, cells were treated with or without 100 nM IGF-1 for 10 min. After that the medium was removed and cell extracts were prepared from approximately $60x10^6$ exponentially growing cells. Cell extracts were prepared by adding lysis buffer directly to the cells. Immunoprecipitation was performed by using antibodies against p85α (as a positive control) or against P-Tyr with an equal amount of total protein (1.5 mg). Immunocomplexes were immobilized on protein-G coated agarose beads and washed twice in lysis buffer, once in lysis buffer containing 0.5 M LiCl and twice in reaction buffer. Subsequently, the beads were resuspended in 50 µl of reaction buffer and 5 µg phosphatidylinositol was added from a sonified stock solution. Then, 5 µl of a solution of 200 mM $MgCl_2$ and 200 µM ATP containing 20 µCi of [γ-^{32}P] ATP were added to the reaction mixture. The tubes were incubated at room temperature for 45 min before the reaction was stopped by adding 150 µl of stop solution ($CHCl_3$/ CH_3OH/ HCl conc., 100:200:2) and 120 µl of $CHCl_3$. The organic phase was separated by centrifugation and washed once with CH3OH/HCl, 1N (1:1). The samples were concentrated by over night drying. The dried reaction product was re-dissolved in 10 µl

CH_3Cl/ MeOH (1:1) and separated by thin layer chromatography on a silica gel plate coated with 1 % K-oxalate, 2 mM EDTA in H_2O/ CH_3OH (3:2). The plates were developed in $CHCl_3$/ CH_3OH/ H_2O/ NH_4OH (45:35:8.5:1.5) and phosphorylated lipids were visualized by autoradiography.

2.2.8 Real time PCR analysis

2.2.8.1 RNA preparation

At each indicated time point, medium was removed and the treated cells were lyzed directly in a culture plate using TRIzol reagent. Total RNA was prepared according to the manufacturer's instruction. The RNA pellet was dissolved in RNAse-free water and the RNA concentration was determined by measuring the optical density (O.D.). The dissolved RNA was used for first-strand cDNA synthesis or stored at -80°C. The O.D. at wavelength of 260 nm and a light pass of 1 cm correspond to approximately 40 μg/ml of RNA. The equation for calculating the RNA concentration is as following;

$$\text{RNA concentration (ug/ml)} = OD_{260} \times 40 \text{ dilution factor}$$

2.2.8.2 First-strand cDNA synthesis

After genomic DNA elimination, the RNA samples were reverse-transcribed using the QuantiTect reverse transcription kit, according to the manufacturer's instruction. The synthesized first-strand cDNA is ready for real time PCR analysis.

2.2.8.3 Quantitative Real time PCR

Quantitative real time PCR was performed using the LightCycler FastStart Reaction Mix SYBR Green I. The primer pair for *NBS1* amplification was 5'- CAG ACC TTA ATT CCT GAC TGT C -3' as the forward primer and 5'- TTT ACA GTG GGT GCA TCT TGT G -3' as the reverse primer. The primers for *CCND1*, *FOS*, *SOS1*, *SOS2*, and *B2M* amplification were purchased from Qiagen (unknown sequence). The relative expressions of the genes of interest were calculated by the 'delta-delta Ct' method [153]. The relative expression data were normalized by using *B2M* as a reference gene.

2.2.9 Western blot analysis

2.2.9.1 Whole cell lysate preparation

At each indicated time point, the cells were detached with trypsin, collected by centrifugation at 300 ×g for 5 min. Whole cell lysates were prepared by suspending the cells in lysis buffer and incubating the suspension for 10 min on ice. Cell lysates were collected after centrifugation at 16,000 ×g for 10 min. Protein concentration was determined using the Bradford reagent. Cells lysates were mixed with Laemmli's buffer and then boiled at 95°C for 5 min.

2.2.9.2 Nuclear and cytoplasmic extraction

At each indicated time point, the cells were detached with trypsin, collected by centrifugation at 300 ×g for 5 min. Nuclear and cytoplasmic extracts were prepared using Pierce Nuclear and Cytoplasmic Extraction Reagent Kit (NE-PER) according to the manufacturer's instruction. Cells extracts were mixed with Laemmli's buffer and then boiled at 95°C for 5 min.

2.2.9.3 SDS polyacrylamide gel elctrophoresis and immunoblotting

30 µg of protein from each sample were separated by electrophoresis in 8% to 12% SDS-poly acrylamide gels. Proteins were then transferred to a blotting membrane and the membrane was incubated in blocking buffer containing 8% skim milk for 1 to 3 h at room temperature. The membrane was washed 3 times with TBS-T and followed by incubation in Roti-block solution containing primary antibody overnight at 4°C. After washing 3 times with TBS-T, the membrane was incubated with horseradish peroxidase-labeled secondary antibody diluted in blocking buffer for 1 h. The membrane was washed 3 times with TBS-T and then developed using chemiluminescence detection on X-ray films.

2.2.10 Immunostaining and confocal microscopy

To study ERK1/2 and Nbs1 subcellular localization by immunostaining, approximately 3×10^5 cells were seeded into 4-well chamber slides and transfected with siRNA. After 24 h, cells were cultured in medium containing 0.2% FCS for 48 h, and medium was then replaced by fresh medium containing 10% FCS and 100 ng/ml of IGF-1. After treatment, the cells were fixed for 15 min in 4% paraformaldehyde and permeabilized with 0.2% Triton-X-100 in PBS for 5 min. After blocking with 1% BSA for 60 min, the cells were probed with anti-Nbs1 and anti-Erk1/2 antibodies over night at 4°C, followed by incubation for 1 h at room temperature in the dark with fluorescence-labeled secondary antibodies Cy-3, goat-anti-mouse IgG for Nbs1 and Alexa Fluor 488, goat anti-rabbit IgG for Erk1/2. The nuclei were stained with Hoechst 33342 and cover slips were mounted on slides with Vectashield.

3. RESULTS

3.1 Suppression of endogenous Nbs1 levels in RPE cells by short interfering RNA

To determine the functional role of *NBS1* in cell cycle progression and cellular proliferation, small interfering RNAs (siRNAs) were used to specifically repress *NBS1* levels in RPE cells. Two oligonucleotide siRNA duplexes were used to target the human *NBS1* mRNA while the non-targeting scramble siRNA (mock) duplexes were used as a negative control. The first *NBS1* siRNA duplex (si*NBS1*) was used in all subsequent experiments while the second *NBS1* Stealth siRNA duplex (si, 2) was used in some key experiments to rule out the off-target effect of siRNAs (result 3.8 and Fig. 33-37). The transfection reactions were performed by using siLentfect reagent. The *NBS1* siRNA and scramble siRNA were used in a final concentration of 20 nM. After transient transfection, the expression level of endogenous *NBS1* mRNA was determined by quantitative real-time PCR. The result shown in Fig. 9 showed that the level of endogenous *NBS1* mRNA in *NBS1* siRNA-transfected cells was effectively repressed to less than 10% of the level in scramble siRNA-transfected cells as assessed at 24 to 90 h after transfection.

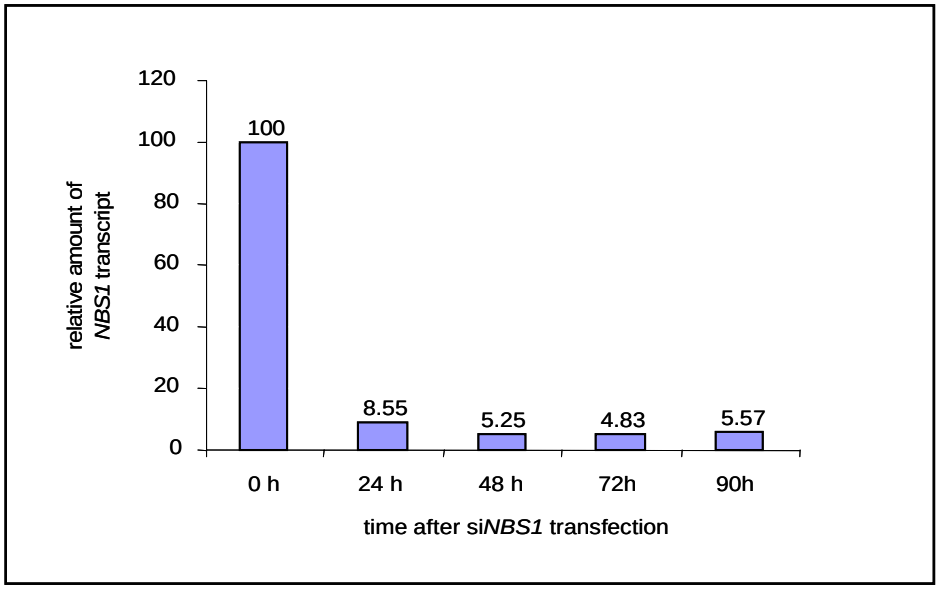


Figure 9. *NBS1* siRNA (si*NBS1*)-mediated down-regulation of endogenous *NBS1* mRNA. At 24, 48, 72, 90 h after transfection, the RPE cells were harvested for mRNA extraction. The extracted mRNAs were reverse-transcribed and cDNA products from the reaction were used as a template for quantification of the *NBS1* mRNA level in si*NBS1*-transfected cells relative to scramble siRNA-transfected cells by quantitative real time PCR. *B2M* was used for normalization of the cDNA input in the reaction. This result is representative for two independent experiments.

In addition, the protein level of Nbs1 was also determined. The whole cell lysates extracted from scramble and *NBS1* siRNA-transfected cells were examined by Western blot analysis. A representative result is shown in Fig. 10A. In *NBS1* siRNA-transfected cells, Nbs1 was almost completely absent at 48-90 h after transfection as detected by Western blot analysis. It has been reported that Mre11 and Rad50 are required for the stability of proteins component of the MRN complex. Deficiency of either Mre11 or Rad50 reduced the levels of the other two proteins of the MRN complex [156,157]. Therefore, the levels of Mre11 and Rad50 were analyzed in *NBS1* siRNA-transfected cells compared to scramble siRNA-transfected cells (Fig. 10B). The results showed that the absence of Nbs1 did not affect the expression or the stability of its complex partners Mre11 and Rad50.

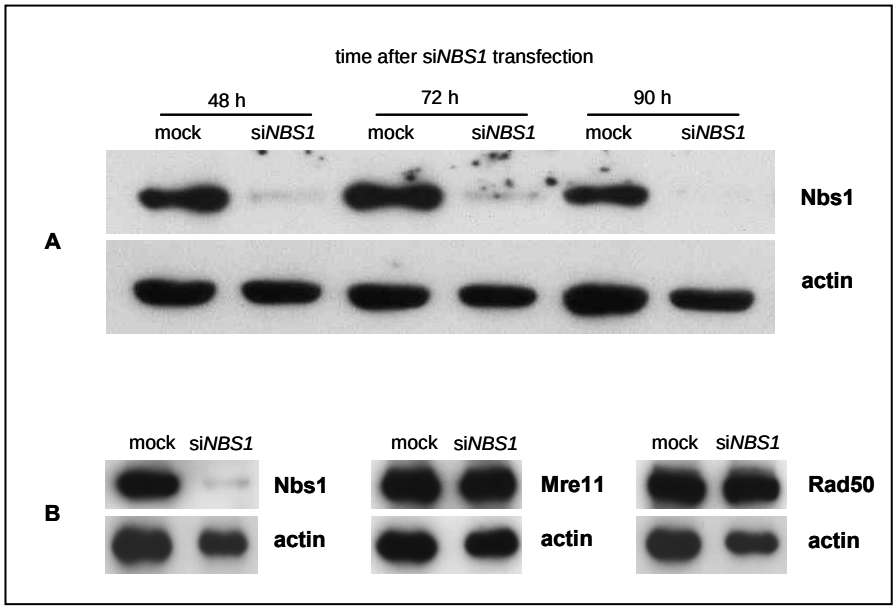


Figure 10. The protein levels of Nbs1, Mre11, and Rad50 from the lysates of scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (*siNBS1*). At the indicated time points after transfection, the RPE cells were harvested for whole cell lysate preparation. The whole cell lysates were analyzed by Western blot analysis. Antibodies against Nbs1, Mre11, or Rad50 identified Nbs1 as a 95 kD band, Mre11 as a 85 kD band, or Rad50 as a 153 kD band. Detection of actin was used as a loading control. (A) The levels of Nbs1 were determined at 48, 72, 90 h after transfection. (B) The levels of Nbs1 (left panel), Mre11 (middle panel), and Rad50 (right panel) were determined at 72 h after transfection. Each result is representative for two independent experiments.

3.2 The impact of Nbs1 on the regulation of cell cycle progression and proliferation of RPE cells

3.2.1 The impact of Nbs1 on the regulation of cell cycle progression

Flow cytometry allows to measure the DNA content of an individual cell, and reveals cell distribution over DNA content, which indicates the position of cells in the cell cycle. Therefore, flow cytometry was used to determine the cell cycle distribution in this study. DNA content distribution of a typical exponentially growing cell population is composed of two peaks of G1/G0 and G2/M phase cells and a valley of S phase cells. G2/M phase cells have twice the amount of DNA of G1/G0 phase cells, and S phase cells possess varying amounts of DNA between G1 and G2 cells. To demonstrate that *NBS1* is essential for cell cycle progression, *NBS1* siRNA or scramble siRNA-transfected cells were synchronized in G1/S phase by aphidicholin treatment. The cells were treated with 10 μg/ml aphidicolin for 40 h and they were then released from the arrest by incubation in complete medium. The cells entering into S and G2/M phase were harvested at the indicated time points and the cell cycle distribution was monitored by flow cytometry.

Representative results are shown in Fig. 11. After release from G1 arrest, 45% of the scramble siRNA-transfected cells progressed into S phase within 6 h and up to 58% of the cells reached G2/M phase within 28 h (Fig. 11, right panel). Delayed cell cycle progression was observed in *NBS1* siRNA-transfected cells. When compared to scramble siRNA-transfected cells, suppression of endogenous *NBS1* in RPE cells caused a 3.8 fold reduction in S phase cells and a significantly delayed progression to G2/M phase (2.5 fold reduction) as observed at 6 and 20 h after release from G1 arrest, respectively (Fig. 11, left panel).

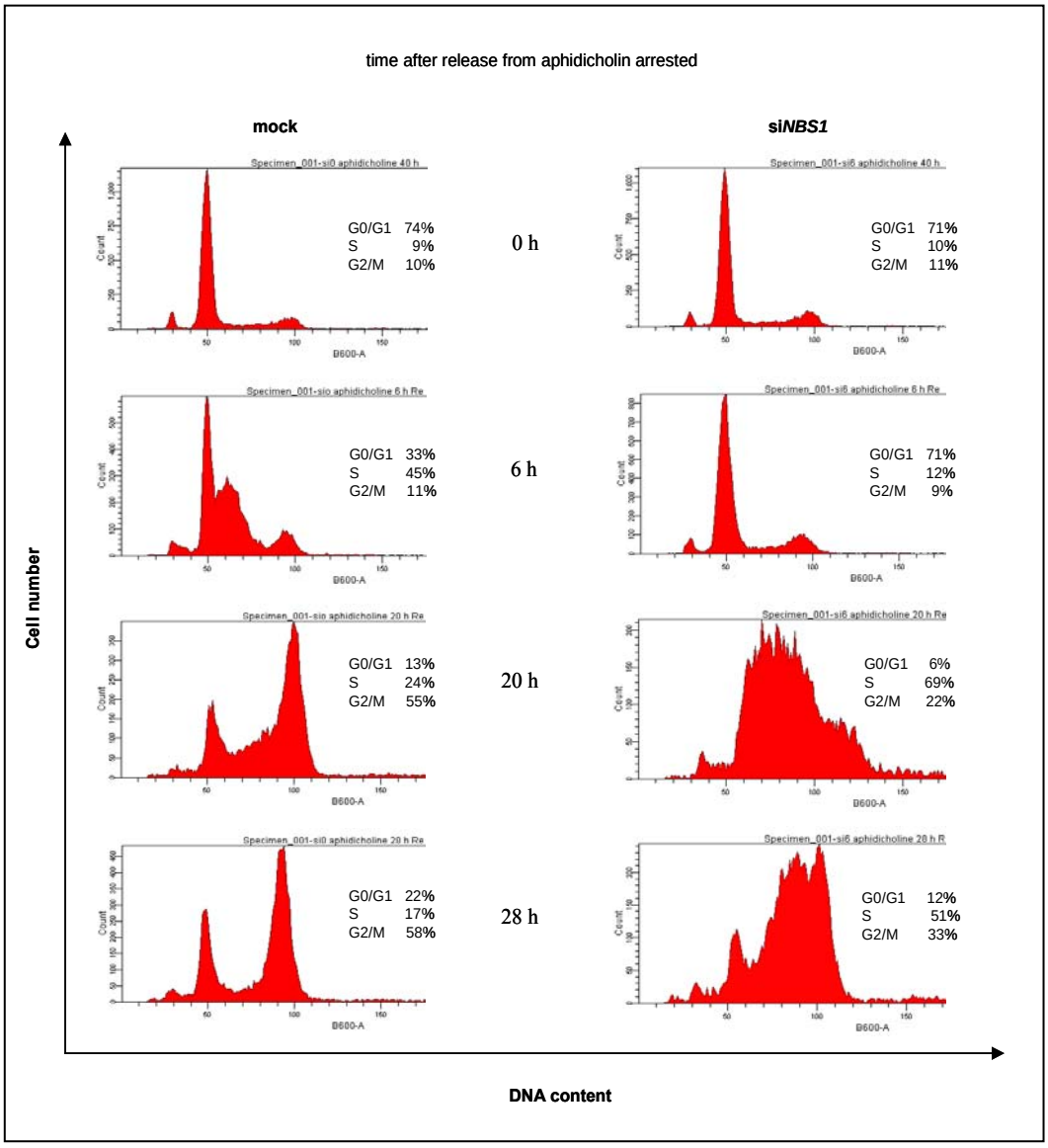


Figure 11. Cell cycle analysis of scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (*siNBS1*). After transfection, RPE cells were cultivated for 40 h to allow si*NBS1*-mediated depletion of *NBS1* expression. The cells were synchronized at G1/S phase by treatment with 10 µg/ml aphidicolin for 24 h. Subsequently, the treated cells were released from the blockade and collected at the indicated time points for cell cycle analysis by flow cytometry. This result is representative for two independent experiments.

Cyclins are key regulators of the cell cycle progression. Periodical oscillation of cyclins is central to cell cycle regulation. Progression from G1 is initiated by cyclin D and followed by the expressions of cyclin E and cyclin A. To examine, whether delayed cell cycle progression in *NBS1* siRNA-transfected cells is related to the disturbances of periodical oscillations of cyclins, the protein levels of cyclin E and cyclin A were determined. Whole cell lysates, collected at 6, 20, and 28 h after release from G1 arrest, were determined by Western blot analysis. The results revealed the highest level of cyclin E in scramble siRNA-transfected G1-arrested cells (time zero after release), which correlates with the largest population of cells in G1 phase. At the later time points, the level of cyclin E was significantly reduced due to the decline of cell numbers in G1. In contrast, the level of cyclin E in G1 arrested cells was much lower in *NBS1* siRNA-transfected as compared to scramble siRNA-transfected cells. Moreover, within the time of observation no reduction of the cyclin E level was observed in scramble siRNA-transfected cells after release from G1 arrest (Fig. 12A). An increase in the amount of cyclin A was clearly observed at 20 and 28 h after release from G1 arrest in scramble siRNA-transfected cells (Fig. 12B). However, the level of cyclin A increased at a slower rate in *NBS1* siRNA-transfected cells compared to scramble siRNA-transfected cells and reached the same level at 28 h after release. This result correlated with the delayed cell cycle progression of *NBS1* siRNA-transfected cells that was observed from the cell cycle analysis.

The disturbance in the periodic oscillation of cyclin E and delayed expression of cyclin A in *NBS1* siRNA-transfected cells suggested that Nbs1 is involved in the regulation of cylin E and cyclin A expression.

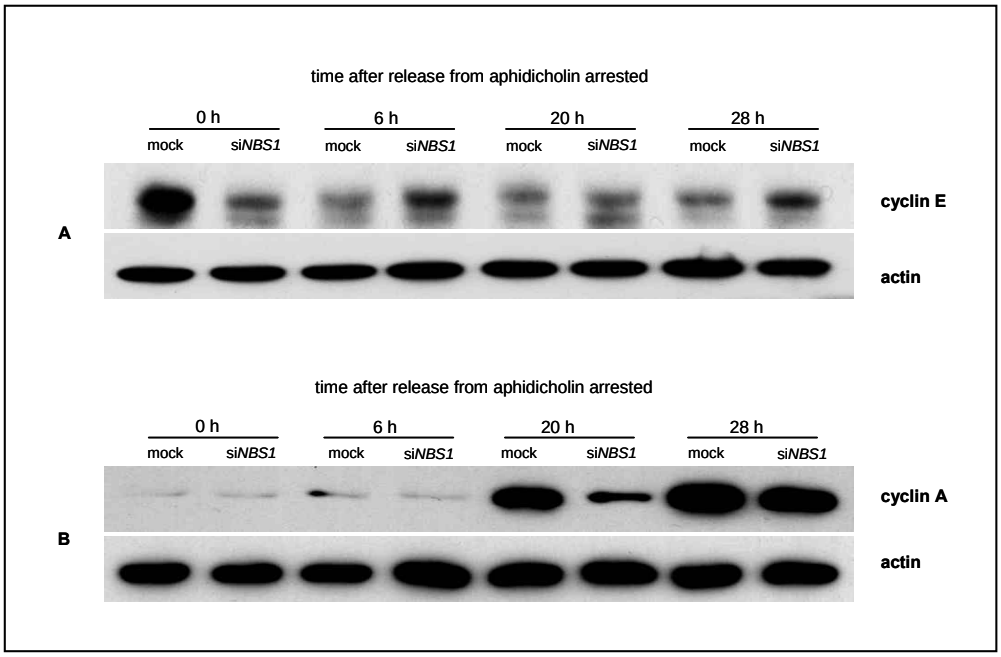


Figure 12. The protein levels of cyclin E and cyclin A from lysates of scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*) after release from G1/S arrest. After transfection, RPE cells were cultivated for 40 h to allow si*NBS1*-mediated depletion of *NBS1* expression. The cells were synchronized at G1/S phase by treatment with 10 µg/ml aphidicolin for 24 h. Afterward the treated cells were released from the blockade and then collected at the indicated time points. The whole cell lysates were analyzed by Western blot analysis. Antibodies against cyclin E or cyclin A identified cyclin E as a 50 kD band (A), or cyclin A as a 60 kD band (B). Detection of actin was used as a loading control. Each result is representative for two independent experiments.

3.2.2 The impact of Nbs1 on the proliferation capacity of RPE cells

To investigate the consequences of *NBS1* silencing on cell proliferation, the proliferation capacity of *NBS1* siRNA-transfected cells was compared to scramble siRNA-transfected cells. 24 h after transfection with siRNAs, 10,000 cells were seeded into 30 mm dishes and cultivated for 96 h in medium containing 10% FCS. At the indicated time points, cells were trypsinized and the cell numbers were determined by using a particle counter and a microscope counting chamber. The results showed that the cellular proliferation rate of *NBS1* siRNA-transfected cells was significantly reduced by 60% (determined at day 4), as compared with scramble siRNA-transfected cells (Fig. 13).

The IGF-1 growth factor is a potent mitogen for cell proliferation in various cellular systems [158]. Thus, the impact of Nbs1 on proliferation of RPE cells after IGF-1 treatment was also determined. One day after *NBS1* siRNA transfection, the cells were seeded into 6 well plates and cultured in medium containing 10% FCS in the presence or absence of IGF-1 (100 ng/ml) for 1 to 4 days. The result showed a clearly enhanced cellular proliferation rate following IGF-1 treatment in scramble siRNA-transfected cells (~2 folds increased, as determined at day 4), while IGF-1-enhanced cellular proliferation rate was not observed in *NBS1* siRNA-transfected cells (Fig. 13). This finding indicated that *NBS1* is required for IGF-1-stimulated proliferation of RPE cells.

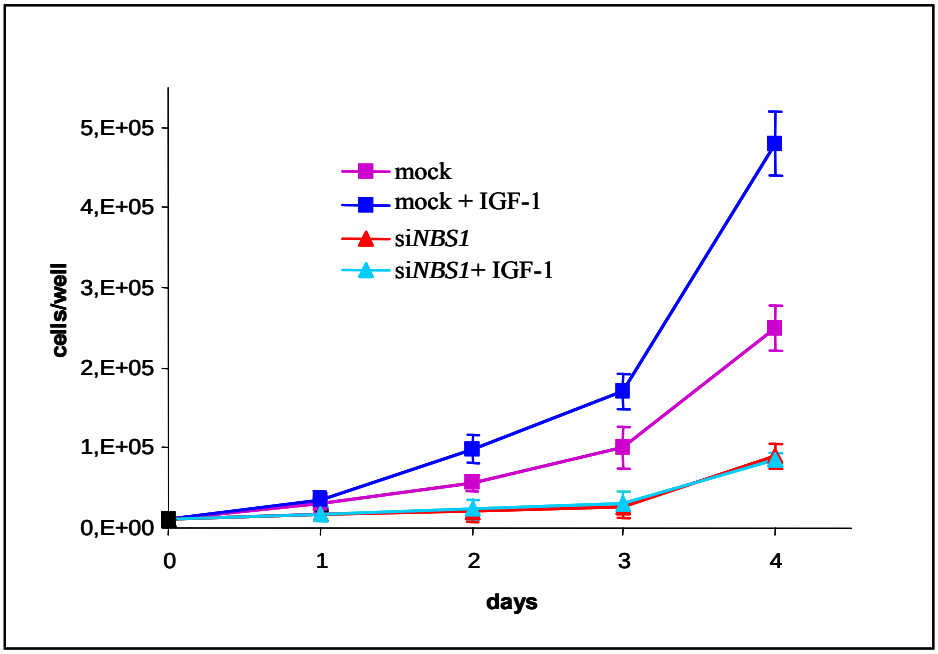


Figure 13. Cellular proliferation rate of scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*) under normal condition and in response to IGF-1 stimulation. One day after transfection, RPE cells were seeded into 6 well plates and incubated in medium containing 10% FCS either supplemented with or without IGF-1 (100 ng/ml). Medium was changed every day. At the indicated time points, the cells were trypsinized and the cell number was determined in duplicate. The cell number is presented as the mean ± SD of three independent experiments.

3.3 The impact of Nbs1 on the downstream signaling cascades of the IGF1R

3.3.1 The impact of Nbs1 on the PI3K/Akt cascade

The previous findings indicated that Nbs1 has a role in IGF-1-induced cell proliferation. For further elucidation, the impact of Nbs1 on the initiation of signaling cascades downstream of the IGF-1 receptor was investigated. First, the activity of the PI3K/Akt, one of the key pathways downstream of the IGF-1 signaling, was investigated. 24 h after siRNAs transfection, cells were serum-starved for 24 h and then treated with or without IGF-1. The treated cells were collected at different time points after IGF-1 stimulation and cell lysates were prepared for the determination of PI3K activity (as described in 2.2.7). The result from the PI3K activity assay showed that under serume deprivation condition, the activity of PI3K was almost undetectable in scramble siRNA and *NBS1* siRNA-transfected cells (Fig. 14). Upon IGF-1 stimulation, a strong increase of PI3K activity was observed in both scramble siRNA and *NBS1* siRNA-transfected cells. Remarkably, the PI3K activity was higher in scramble siRNA-transfected cells than in *NBS1* siRNA-transfected cells.

Upon IGF1R stimulation, PI3K is activated which in turn activates Akt, a key downstream target of the cascade, by phosphorylating the serine residue 437 of Akt. Therefore, Western blot analysis was performed to determine the phosphorylation level of Akt. The result showed that the basal level of phosphorylated Akt at serine 473 was lower in *NBS1* siRNA-transfected cells as compared to scramble siRNA-transfected cells while the total level of Akt was equivalent (Fig. 15A). Remarkably, in scramble siRNA-transfected cells, IGF-1 triggered a strong increase of Akt phosphorylation which reached a maximum level (~40 fold) at 30 min after stimulation (Fig. 15B) and the phosphorylation could be blocked by the PI3K inhibitor wortmannin (Fig. 15C). In contrast, IGF-1-induced phosphorylation of Akt was almost undetectable in *NBS1* siRNA-transfected cells. These results revealed that *NBS1* is involved in PI3K/Akt activation in response to IGF-1 treatment in RPE cells, which is consistent with recent findings showing an increase of PI3K activity and Akt phosphorylation upon over-expression of *NBS1* in human hypopharyngeal cancer cell lines [159].

It had been reported that activation of PI3K/Akt upon mitogenic stimulation results in the phosphorylation of FOXO1, leading to nuclear exclusion and degradation of the FOXO1 protein [160,161]. Therefore, the protein level of FOXO1 which is negatively regulated by the PI3K/Akt pathway was also determined in this study. The result showed that under serum deprivation condition, the level of FOXO1 was lower in scramble siRNA-tranfected cells than that of *NBS1* siRNA-transfected cells (Fig. 15 D). A rapid decreasing of FOXO1 level was clearly observed at 15 min and 30 min after IGF-1 treatment in scramble siRNA-tranfected cells. In contrast, the level of FOXO1 was not altered in *NBS1* siRNA-transfected cells after IGF-1 treatment. This result correlated well with a deteriorated activity of PI3K and Akt in *NBS1* siRNA-transfected cells in response to IGF-1.

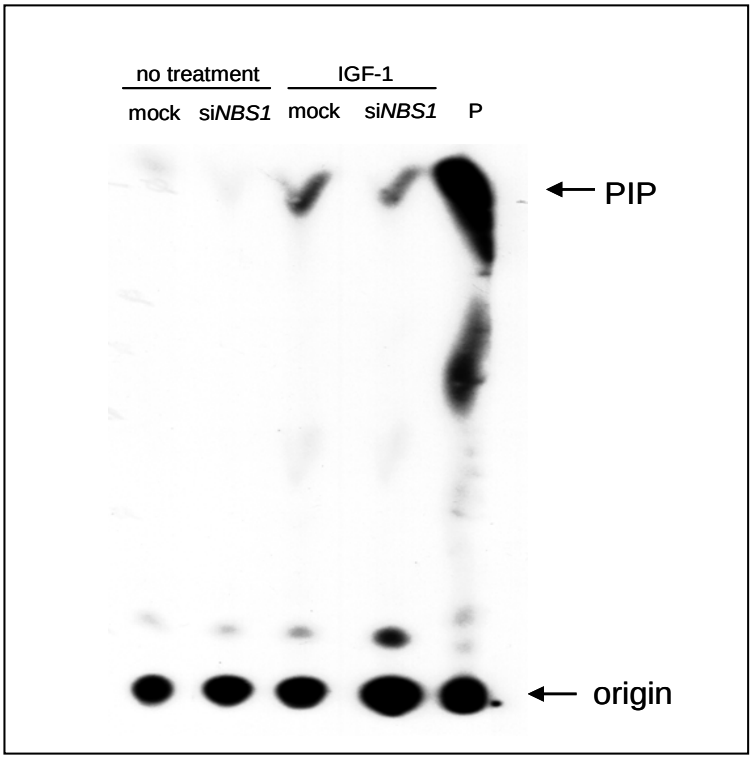


Figure 14. The impact of Nbs1 on the PI3K activity in response to IGF-1. 24 h after transfection, RPE cells were deprived of serum for 24 h. The serum deprived cells were subsequently treated with or without 100 ng/ml IGF-1 for 10 min. The treated cells were collected for cell lysate preparation. The PI3K activity was analyzed using p-Try immunoprecipitate of whole cell lysate extracted from scramble siRNA-transfected cells (mock), *NBS1* siRNA-transfected cells (si*NBS1*). An immunoprecipitate generated with an antibody against the p85 subunit of PI3K served as positive control (P). The acitivity of PI3K is reflected by an intensity of the phosphoinositide phosphate (PIP) signal. This result is representative for two independent experiments.

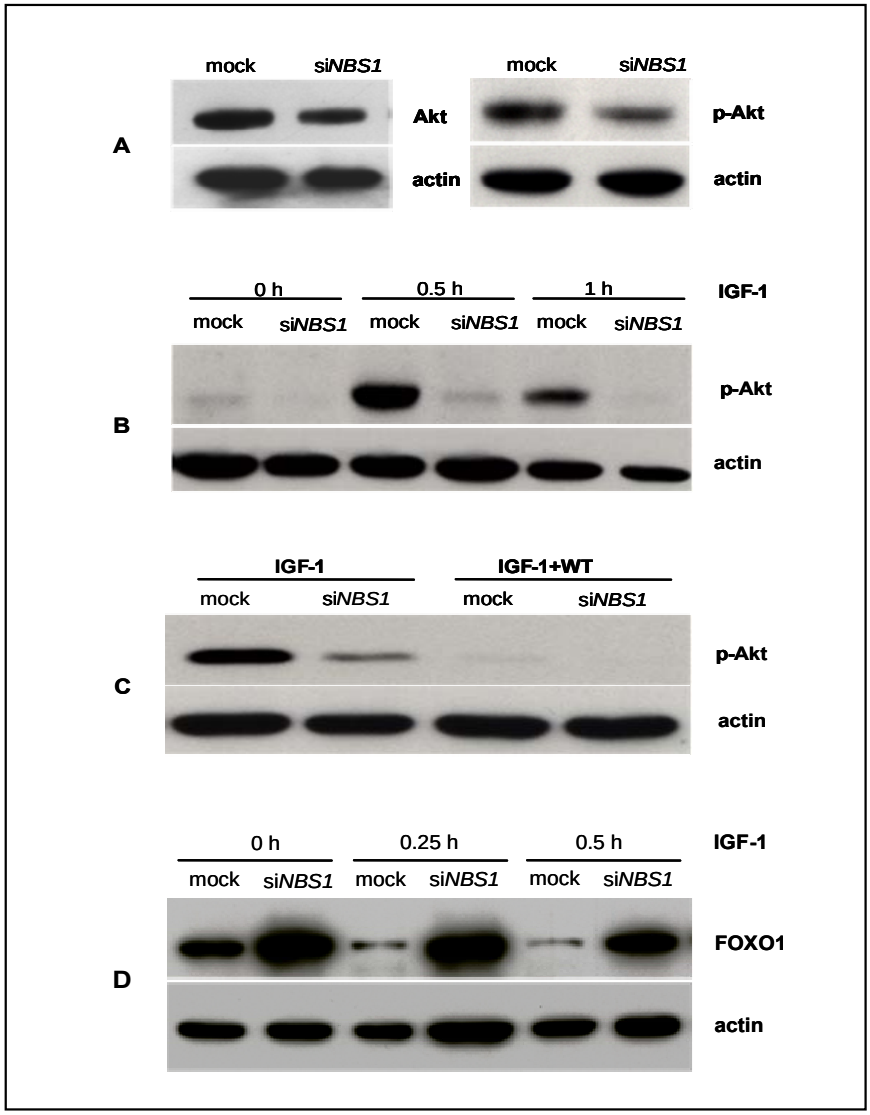


Figure 15. The impact of Nbs1 on the activation of Akt in response to IGF-1. 24 h after transfection, RPE cells were deprived of serum for 24 h, treated with 100 ng/ml IGF-1 or pretreated with 10 μM wortmannin for 1 h. The treated cells were collected at different time points for protein extraction. The levels of Akt and p-Ser437-Akt in the protein extracts from scramble siRNA-transfected cells (mock) or *NBS1* siRNA-transfected cells (si*NBS1*) were determined by Western blot analysis. Antibodies against Akt or p-Ser437-Akt identified Akt and p-Ser437-Akt as a 60 kD band. The antibody against FOXO1 identified FOXO1 as a 80 kD band. Detection of actin was used as a loading control. (A) the levels of Akt and p-Ser437-Akt under normal growth conditions, (B) the levels of p-Ser437-Akt upon IGF-1 stimulation after serum starvation, (C) the levels of p-Ser437-Akt upon IGF-1 stimulation after serum starvation with or without wortmanin (WT) pretreatment, and (D) the levels of FOXO1 upon IGF-1 stimulation after serum starvation. Each result is representative for two independent experiments.

3.3.2 The impact of Nbs1 on the activation of ERK1/2

The activation of Ras/Raf/MEK/ERK is required for the biological activity of IGF-1 regarding the regulation of cell proliferation, cell differentiation, and cell survival. Thus, I assessed whether *NBS1* might be involved in Ras/Raf/MEK/ERK-mediated signaling in response to IGF-1 stimulation. The activities of ERK1/2, the downstream effectors of the Ras/Raf/MEK/ERK cascade, were determined by measuring their phosphorylation status. IGF-1 triggered a strong increase of p-ERK1/2 levels in scramble siRNA-transfected cells which reached a maximum level (~13 fold) at 2 h after stimulation, while only slightly increased of pERK1/2 levels (~2 fold) were observed in *NBS1* siRNA-transfected cells (Fig. 16). The total ERK1/2 protein levels in scramble siRNA-transfected cells and *NBS1* siRNA-transfected cells were equivalent and not altered following IGF-1 stimulation (Fig. 16).

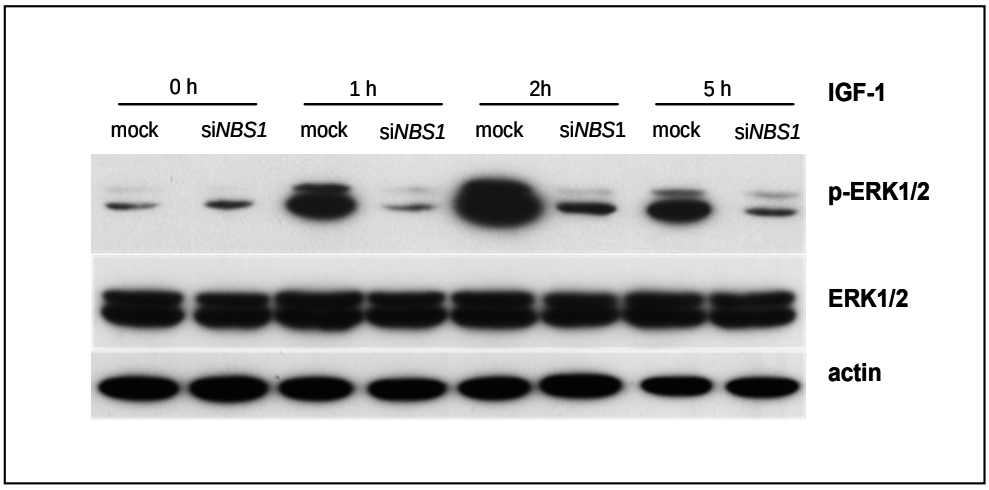


Figure 16. The impact of Nbs1 on the activation of ERK1/2 in response to IGF-1. One day after transfection, RPE cells were deprived of serum for 24 h, treated with 100 ng/ml IGF-1. The treated cells were collected at different time points for protein extraction. The levels of ERK1/2, and p-ERK in the protein extracted from scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*) were determined by Western blot analysis. Antibodies against ERK1/2 or p-ERK1/2 identified ERK1/2 or p-ERK1/2 as 42 and 44 kD bands. Detection of actin was used as a loading control. This result is representative for two independent experiments.

Several signaling kinases participate in the transmission of signals via the ERK cascade. Therefore, the MEK1/2-specific inhibitor U0126 was applied to analyze whether the increased phosphorylation levels of ERK1/2 after IGF-1 stimulation occurred via the Ras/Raf/MEK pathway. The effects of U0126 on ERK1/2 phosphorylation and cellular proliferation after IGF-1 stimulation were analyzed. The results clearly showed that ERK1/2 phosphorylations were abolished by U0126 (Fig. 17). Additionally, the phosphorylation level of p90RSK, a downstream effector of ERK1/2, was also investigated. As the consequence of the deteriorated activity of ERK1/2 in *NBS1* siRNA-transfected cells, the phosphorylation level of p90RSK at serine 380 which is phosphorylated by ERK1/2 upon growth factor stimulation was much lower in *NBS1* siRNA-transfected cells as compared to scramble siRNA-transfected cells (Fig. 18).

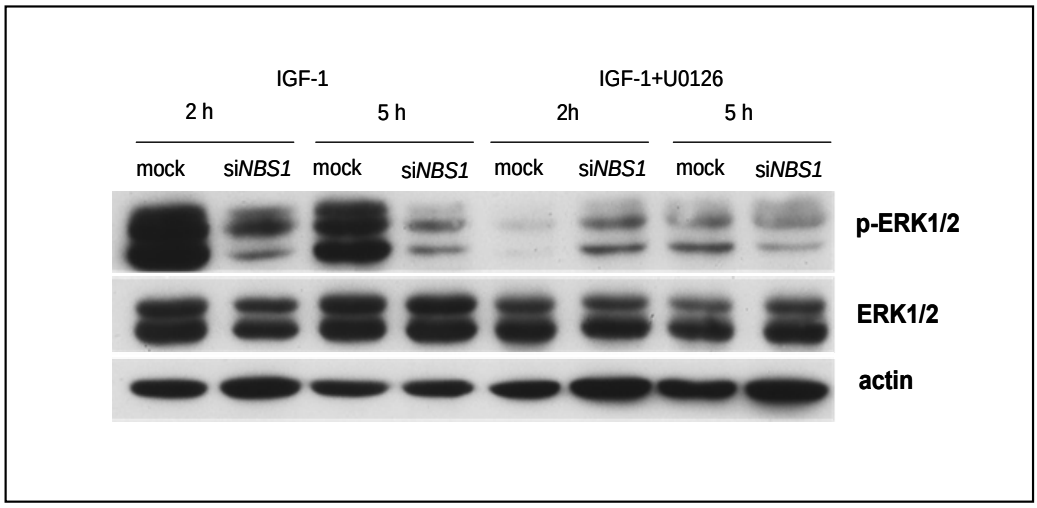


Figure 17. The effect of the MEK1/2 specific inhibitor U0126 on the IGF-1 induced phosphorylation of ERK1/2. One day after transfection, RPE cells were deprived of serum for 24 h, treated with 100 ng/ml IGF-1 or pretreated with 10 μM U0126 for 1 h. The treated cells were collected at different time points for protein extraction. The levels of ERK1/2, and p-ERK1/2 in the protein extracted from scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*) were determined by Western blot analysis. Antibodies against ERK1/2 and p-ERK1/2 identified ERK1/2 and p-ERK1/2 as 42 and 44 kD bands. Detection of actin was used as a loading control. This result is representative for two independent experiments.

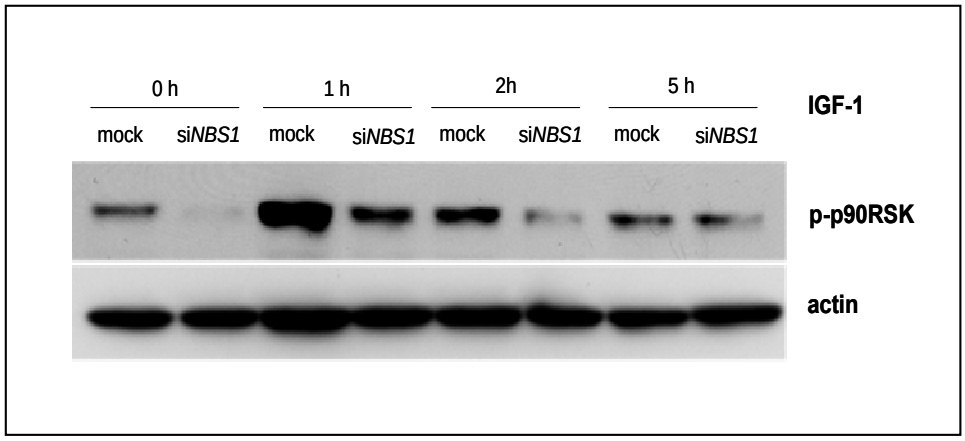


Figure 18. The impact of Nbs1 on the phosphorylation level of p90RSK upon IGF-1 stimulation. One day after transfection, RPE cells were deprived of serum for 24 h and treated with 100 ng/ml IGF-1. The treated cells were collected at different time points for protein extraction. The levels of p-p90RSK in the protein extracted from scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*) were determined by Western blot analysis. Antibodies against p-p90RSK identified p-p90RSK as a 90 kD band. Detection of actin was used as a loading control. This result is representative for two independent experiments.

Translocation of ERK1/2 from the cytoplasm into the nucleus is crucial for their biological activities [162,163]. Therefore, immunofluorescence staining was performed to monitor the subcellular distribution of ERK1/2 after IGF-1 stimulation in *NBS1* siRNA-transfected cells versus scramble siRNA-transfected cells. Without stimulation, ERK1/2 localized mainly in the cytoplasm (Fig. 19A). Following IGF-1 stimulation, nuclear translocation of ERK1/2 was clearly observed in scramble siRNA-transfected cells but not in *NBS1* siRNA-transfected cells, as determined at 1 h after IGF-1 treatment (Fig. 19B). Loss of nuclear translocation of ERK1/2 following IGF-1 stimulation in *NBS1* siRNA-transfected cells correlated well with the low levels of ERK1/2 phosphorylation which were demonstrated by Western blot analysis.

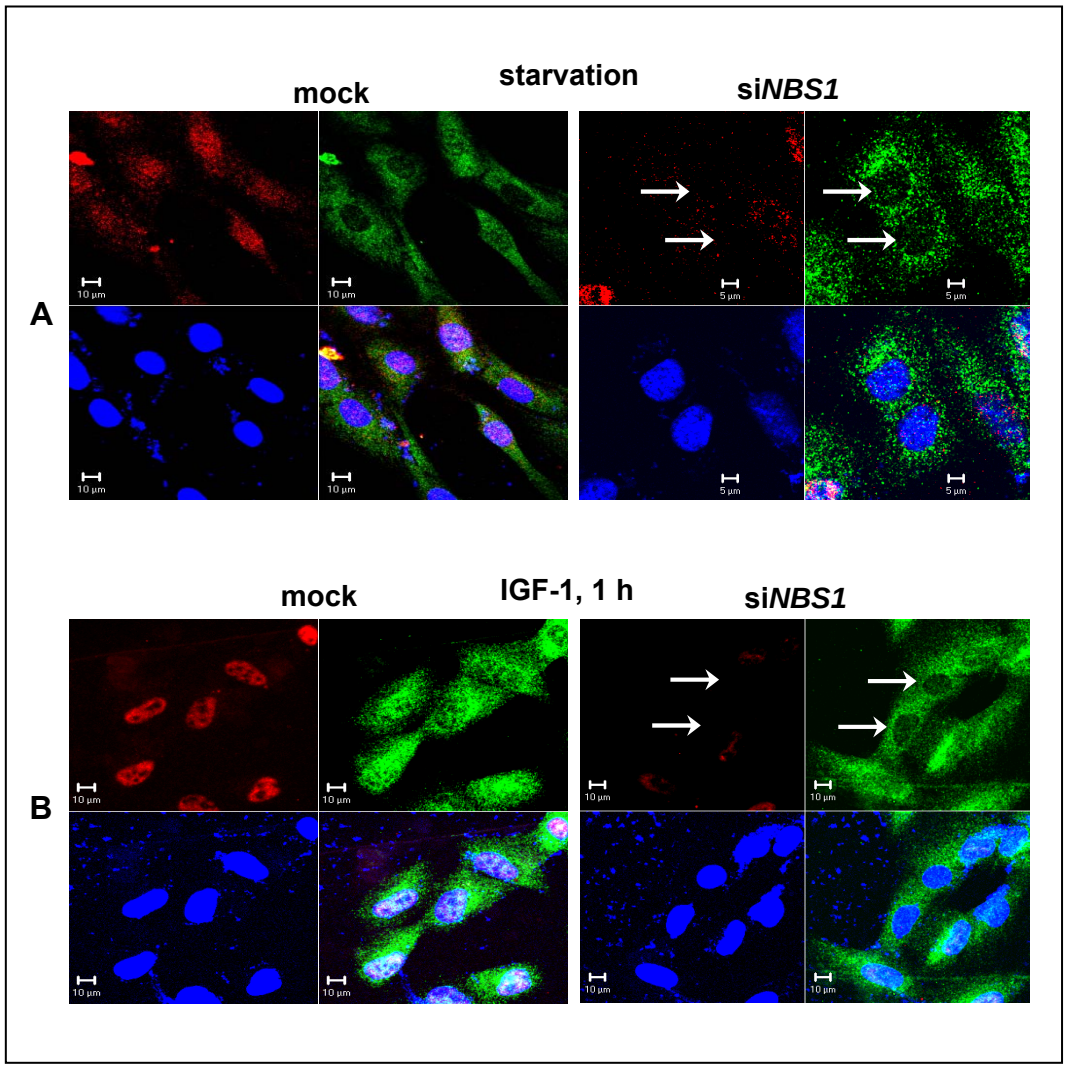


Figure 19. Subcellular distribution of ERK1/2. The ERK1/2 subcellular localization in RPE cells was determined by immunostaining; about $3x10^5$ cells were seeded into 4-wells chamber slides and transfected with scramble siRNA (mock) or *NBS1* siRNA (si*NBS1*). 24 h after transfection, the siRNA transfected cells were cultured in medium containing 0.2% FCS for 48 h. Subsequently, medium was replaced by complete fresh medium containing 100 ng/ml IGF-I. After treatment, the cells were fixed and immunostained as described in materials and methods. (A) under serum deprivation condition, (B) after IGF-I treatment for 60 minutes, (Nbs1) red, (ERK1/2) green, (nucleus) blue, and (white arrow) nucleus of a *NBS1* depleted cell. Each result is representative for two independent experiments.

3.4 The impact of Nbs1 on IGF-1-induced cell proliferation via the Ras/Raf/MEK/ERK pathway

3.4.1 The effect of the MEK1/2-specific inhibitor (U0126) on IGF-1-induced cell proliferation

Activation of the Ras/Raf/MEK/ERK pathway has been shown to be important for IGF-1-stimulated cell proliferation in various cells types [10]. To examine whether the Ras/Raf/MEK/ERK pathway is involved in IGF-1-enhanced proliferation of RPE cells, the cellular proliferation activity was determined after inhibition of the Ras/Raf/MEK/ERK cascade by U0126. 24 h after transfection with siRNAs, the cells were seeded into 24-well plates, incubated for 4 h, and starved for 24 h. Then, the cells were pre-treated with vehicle alone or 10 μM U0126 and subsequently stimulated with medium containing 10% FCS and 100 ng/ml IGF-1. Proliferation activities of the cells were measured by MTT assays at 48 h after treatment. The results are shown in Fig. 20; they indicated that the proliferation activity of scramble siRNA-transfected cells was strongly enhanced by IGF-1. In the presence of the inhibitor U0126, this induction was reduced by 50%. In *NBS1* siRNA-transfected cells, the proliferation activity enhanced by IGF-1 was 2-fold lower as compared to in scramble siRNA-transfected cells and additional treatment with the MEK inhibitor had no additional impact.

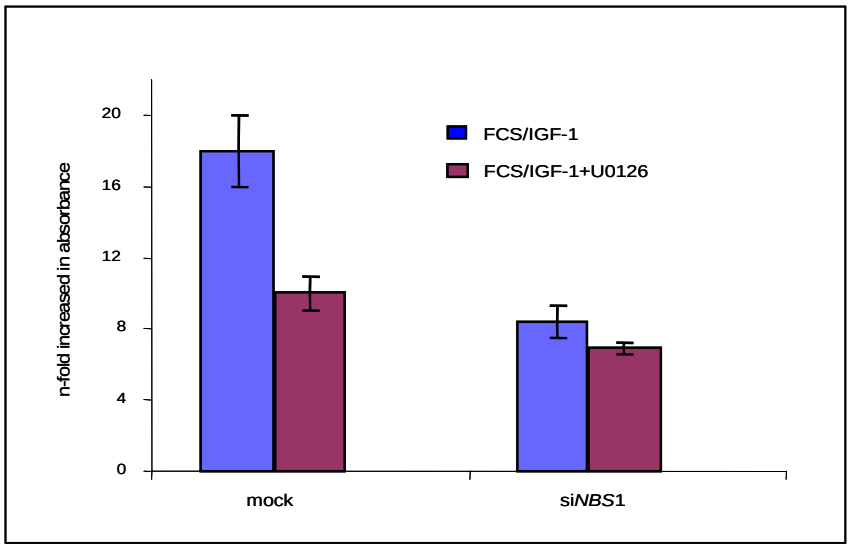


Figure 20. Effect of the MEK1/2-specific inhibitor U0126 on the proliferation activities of scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*) after stimulation with IGF-1. One day after transfection with siRNAs, RPE cells were deprived of serum for 24 h, pretreated with 10 μM U0126 for 1 h and then stimulated with 10% FCS containing 100 ng/ml IGF-1 for 48 h. or left unstimulated in medium without serum. Proliferation activity was measured by MTT assay. The result is shown as the mean ± SD of n-fold increase in absorbance relative to the unstimulated serum deprived cells from three independent experiments.

3.4.2 The impact of Nbs1 on the IGF-1-induced cyclin D1 expression

A disturbance of the IGF-1 signaling cascade was found to be the explanation for the impaired proliferation ability of *NBS1* siRNA-transfected cells. Moreover, using the MEK1/2 specific inhibitor indicated that the Ras/Raf/MEK/ERK cascade, a downstream network of IGF-1, is involved. It has been well documented, that cyclin D1 is important for cell division by initiating the cell cycle re-entry. Its expression is strongly up-regulated upon growth factor stimulation through the Ras/Raf/MEK/ERK cascade via activation of the AP-1 and Ets-1 transcription factors [123]. Thus, it can be assumed that induction of the cyclin D1 expression in response to IGF-1 is impaired in *NBS1* siRNA-transfected cells. *CCND1* expression was determined by quantitative real time PCR. One day after siRNA transfection, the cells were grown under serum deprivation conditions for 2 days to synchronize cells in early G1 phase. After that, the cells were treated with 100 ng/ml IGF-1 and then collected at several time points for RNA and protein extraction. As expected, the levels of *CCND1* mRNA were low in scramble siRNA transfected cells and *NBS1* siRNA-transfected cells under starvation conditions. Following IGF-1 stimulation, a strong induction of *CCND1* expression was clearly observed in scramble siRNA-transfected cells, whereas it was only slightly increased in *NBS1* siRNA-transfected cells (Fig. 21).

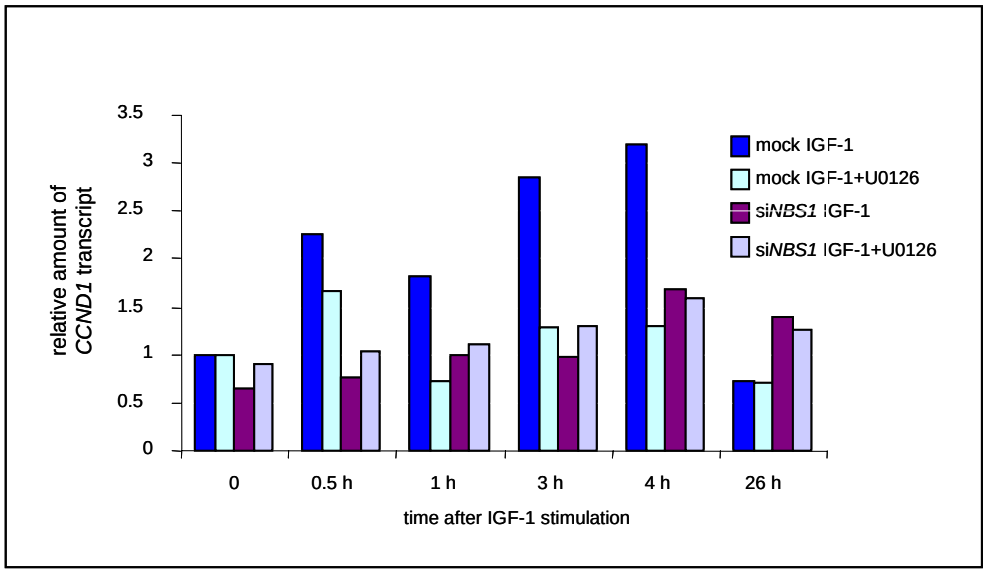


Figure 21. The expression of *CCND1* upon IGF-1 stimulation in scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*). At indicated time points upon IGF-1 treatment of RPE cells in the presence or absence of U0126, the mock and si*NBS1* transfected cells were harvested for mRNA extraction. The extracted mRNA was reverse-transcribed and cDNA products from the reaction were used as a template for quantification of *CCND1* mRNA by quantitative real time PCR. *B2M* was used for normalization of the cDNA input in the reaction. This result is representative for two independent experiments.

Comparable to the expression at mRNA level, Western blot analysis also revealed a low basal level of cyclin D1 protein in scramble siRNA-transfected cells, and it was nearly undetectable in *NBS1* siRNA-transfected cells. The level of this protein was robustly increased after IGF-1 stimulation in scramble siRNA-transfected cells. However, the level of cyclin D1 was only slightly increased in *NBS1* siRNA-transfected cells (Fig. 22A). Furthermore, in order to prove that *NBS1* is involved in IGF-1-enhanced cyclin D1 expressions in RPE cells via the Ras/Raf/MEK/MAPK cascade, cyclin D1 expression was determined after pretreatment with U0126 prior to IGF-1 stimulation. As expected, enhanced cyclin D1 expression at mRNA and protein level in scramble siRNA-transfected cells by IGF-1 was completely abolished by U0126 but this effect was not observed in si*NBS1*-transfected cells (Fig. 21 and 22B).

Taken together, these results indicate that impaired cell proliferation and delayed cell cycle progression as seen in *NBS1* siRNA-transfected cells were likely due to the involvement of *NBS1* in the Ras/Raf/MEK/ERK cascade, which at least in part induces cyclin D1 expression.

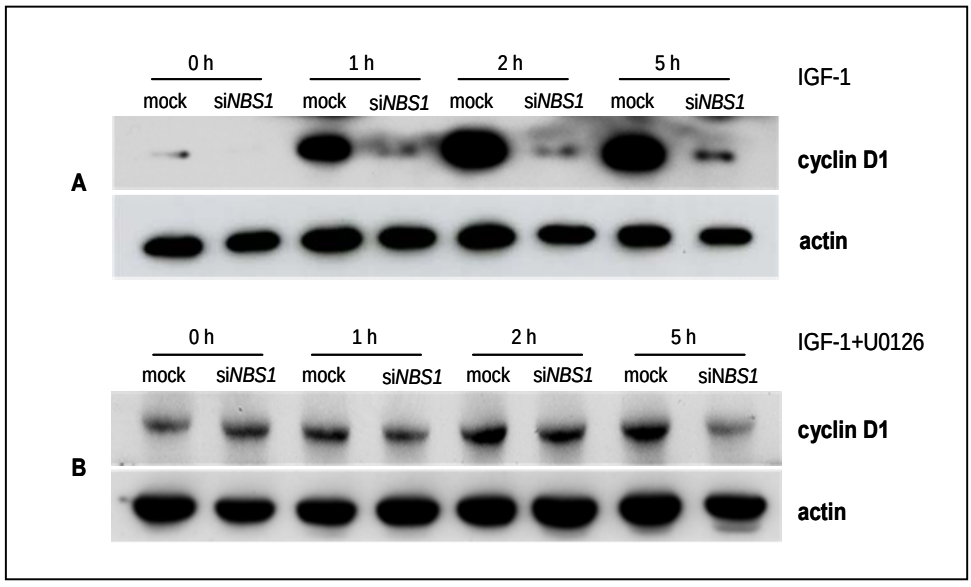


Figure 22. The level of cylin D1 upon IGF-1 stimulation in lysates of scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*). At indicated time points after IGF-1 treatment of RPE cells in presence or absence of U0126, scramble siRNA and *NBS1* siRNA-transfected cells were harvested for whole cell lysate preparation. The level of cyclin D1 was determined by Western blot analysis. Antibody against cyclin D1 identified cyclin D1 as a 34 kD band. Detection of actin was used as a loading control. (A) The level of cyclin D1 at the indicated time points after IGF-1 stimulation. (B) The level of cyclin D1 at the indicated time points after U0126 pretreatment plus IGF-1 stimulation. Each result is representative for two independent experiments.

3.4.3 The impact of Nbs1 on the IGF-1-induced c-Fos expression

c-Fos belongs to the family of AP-1 transcription factors which are important regulators of the early transcriptional processes after extracellular stimulation. In response to IGF-1, AP-1 is strongly up-regulated and activated by Ras/Raf/MEK/ERK resulting in the up-regulation of cyclin D1 expression [164]. Since induction of cyclin D1 expression upon IGF-1 stimulation is diminished in *NBS1* siRNA-transfected cells, expression of *FOS* was investigated both on the transcript and protein levels. RNA and protein were isolated from samples treated with IGF-1 alone or with IGF-1 plus U0126 pretreatment and subjected to quantitative real time PCR and Western blot analysis, respectively. The result shown in Fig. 23 for scramble siRNA-transfected cells, mRNA level of *FOS* was strongly increased to the maximum level of ~120 fold induction 30 min after IGF-1 stimulation and then declined rapidly within 1 h. U0126 pretreatment powerfully suppressed IGF-1-induced up-regulated *FOS* expression via inhibition of MEK1/2. In contrast to scramble siRNA-transfected cells, induction of *FOS* was much lower in *NBS1* siRNA-transfected cells upon IGF-1 stimulation in the presence or absence of U0126.

Results from Western blot analysis of c-Fos protein correlated with the *FOS* mRNA expression level (Fig. 24A). The level of c-Fos protein increased within 2 h upon IGF-1 stimulation in the scramble siRNA-transfected cells which was effectively abolished by pretreatment with U0126 (Fig. 24B). In contrast, only a slight increase in the level of c-Fos protein was observed in *NBS1* siRNA-transfected cells.

These findings confirmed the assumption that *NBS1* is involved in the IGF-1 signaling pathway promoting cell cycle progression and cell proliferation, which is conferring its influences on the regulation of cyclin D1 expression via c-Fos through its role in Ras/Raf/MEK/ERK signaling.

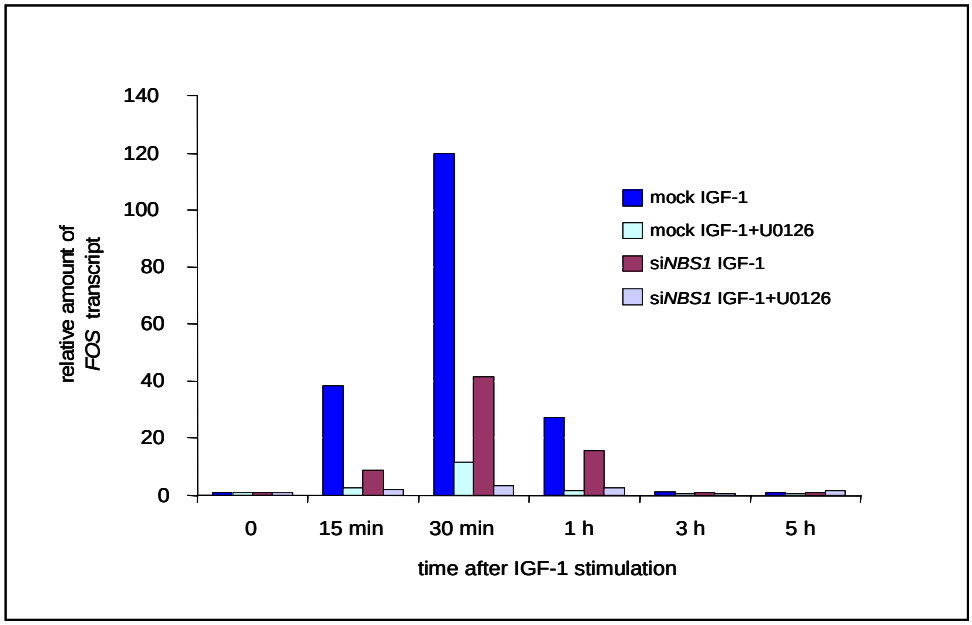


Figure 23. The expression of *FOS* upon IGF-1 stimulation in scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*). At indicated time points upon IGF-1 treatment of RPE cells in the presence or absence of U0126, the mock and si*NBS1* transfected cells were harvested for mRNA extraction. The extracted mRNA was reverse-transcribed and cDNA products from the reaction were used as a template for quantification of *FOS* mRNA by quantitative real time PCR. *B2M* was used for normalization of the cDNA input in the reaction. This result is representative for two independent experiments.

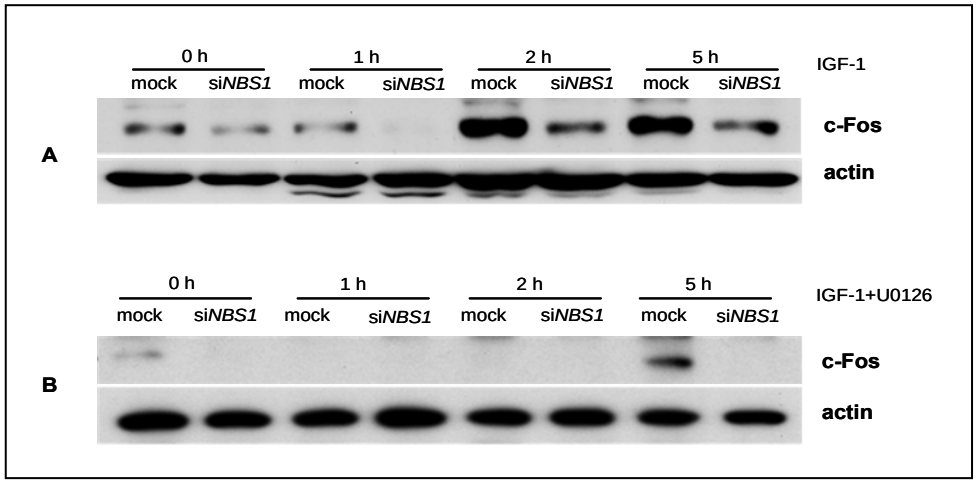


Figure 24. The level of c-Fos upon IGF-1 stimulation from lysates of scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*). At indicated time points upon IGF-1 treatment of RPE cells in the presence or absence of U0126, mock and si*NBS1*–transfected cells were harvested for whole cell lysate preparation. The protein level of c-Fos was determined by Western blot analysis. Antibody against c-Fos identified c-Fos as a 62 kD band. Detection of actin was used as a loading control. (A) The level of c-Fos at indicated time points after IGF-1 stimulation. (B) The level of c-Fos at indicated time points after U0126 pretreatment plus IGF-1 stimulation. Each result is representative for two independent experiments. The appearance of c-Fos at 5 h after U0126 pretretament plus IGF-1 stimulation may result from reduction of the concentration or activity of U0126 in the medium.

3.5 The molecular mechanism of Nbs1 on the IGF-1 signaling cascade

3.5.1 The influence of Nbs1 on the activation of c-Raf

The results above indicated an impact of *NBS1* on IGF-1-induced proliferation via the initiation of the signaling cascade upstream of ERK1/2, hence an active status of c-Raf was evaluated by Western blot analysis. IGF-1 triggered a strong increase of serine-338 phosphorylation of c-Raf in scramble siRNA-transfected cells, which rapidly declined within 2 h after stimulation (Fig. 25). Notably, the phosphorylation level of c-Raf was much lower and hardly any IGF-1-induced activation could be detected in *NBS1* siRNA-transfected cells. These results suggested an early role for *NBS1* upstream of c-Raf in IGF-1-stimulated activation of the Ras/Raf/MEK/ERK signaling pathway.

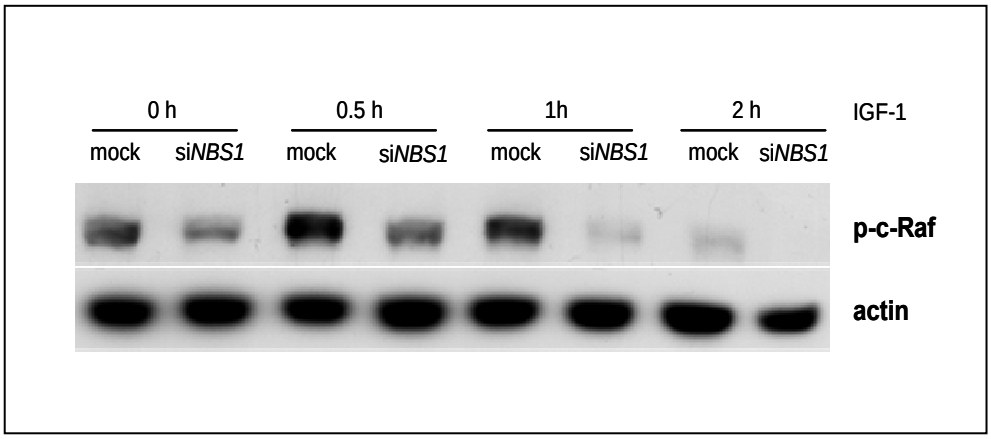


Figure 25. *NBS1* is an upstream regulator of c-Raf. One day after transfection with scramble siRNA (mock) or *NBS1* siRNA (si*NBS1*), RPE cells were deprived of serum for 24 h and treated with 100 ng/ml IGF-1. The treated cells were collected at different time points for protein extraction. The level of p-c-Raf was determined by Western blot analysis. The antibody against p-c-Raf identified p-c-Raf as a 74 kD band. Detection of actin was used as a loading control. This result is representative for two independent experiments.

3.5.2 The influence of Nbs1 on the expression of *SOS1*, *SOS2* and IGF1R

Obviously, *NBS1* influences the Ras/Raf/MEK/ERK cascade at a very early step upon receptor stimulation by IGF-1 treatment. Activation of the early factor Ras requires the action of guanine nucleotide exchange factors (like Sos1 and Sos2) to convert Ras from the inactive GDP-bound to the active GTP-bound form [165]. Therefore, I analyzed *SOS1* and *SOS2* expressions after *NBS1* siRNA transfection by quantitative real time PCR. The results shown in Fig. 26 demonstrated that the amount of *SOS1* and *SOS2* transcripts of *NBS1* siRNA-transfected cells were decreased 4-5 fold as compared to scramble siRNA-transfected cells. Reduced amounts of Sos1 and Sos2 were also detected on the protein level in *NBS1* siRNA-transfected cells (Fig. 27). These reduced Sos1 and Sos2 levels could be a reason for the diminished activity of the Ras/Raf/MEK/ERK signaling pathway in *NBS1* siRNA-transfected cells.

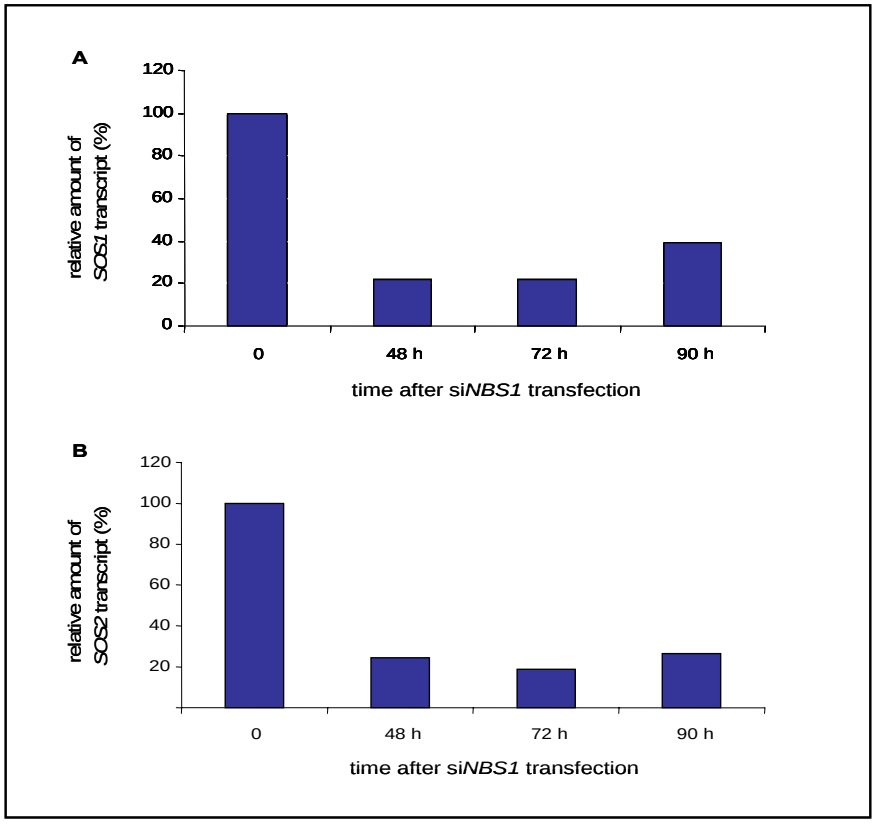


Figure 26. Basal expression of *SOS1* and *SOS2* transcripts in scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*). Relative amounts of *SOS1* (A) and *SOS2* (B) mRNAs were quantified in extracts of RPE *NBS*1 siRNA-transfected cells and in that of scramble siRNA-transfected cells by quantitative RT-PCR. Samples were prepared at the indicated time points after transfection. Each result is representative for two independent experiments.

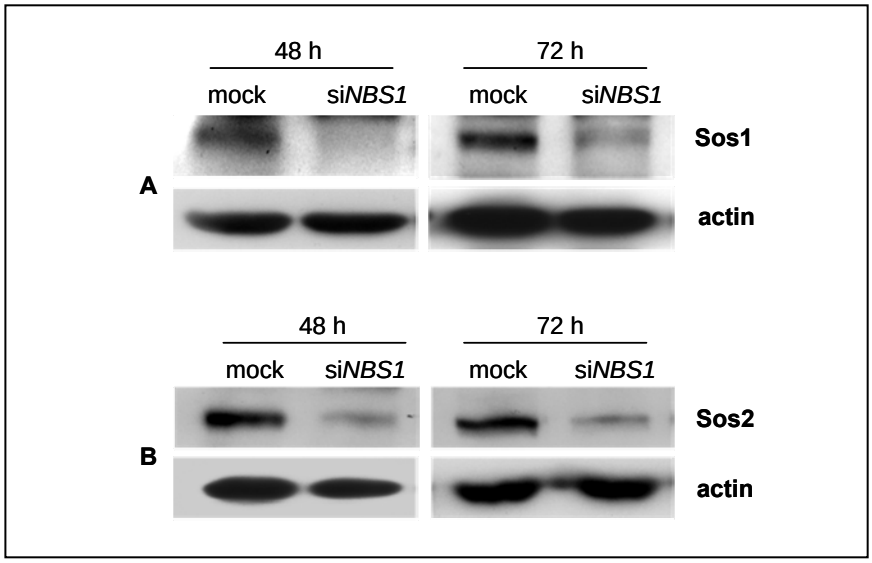


Figure 27. The basal levels of Sos1 and Sos2 from the lysates of scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*). The RPE cells were transfected with *NBS1* siRNA or scramble siRNAmock and collected at 48 h and 72 h after transfection for protein extraction. The levels of Sos1 (A) and Sos2 (B) were determined by Western blot analysis. Antibodies against Sos1 or Sos2 identified Sos1 or Sos2 as a 182 kD band. Detection of actin was used as a loading control. Each result is representative for two independent experiments.

Activation of IGF1R is the proximal event of the Ras/Raf/MEK/ERK cascade. It was reported that *IGF1R* expression levels are suppressed in A-T cells and that the transcription activity of the *IGF1R* gene is dependent on ATM activity [166]. Since Nbs1 functions up-stream and down-stream of ATM, it could be supposed that suppression of endogenous expression of *NBS1* may also suppress the expression of IGF1R. Western blot analysis revealed that basal level of the IGF-1 receptor protein was significantly decreased in *NBS1* siRNA-transfected cells as compared to that in scramble siRNA-transfected cells (Fig. 28).

Taken together, suppression of the basal expression levels of *SOS1*, SOS2, and IGF1R provided a strong explanation for the diminished activity of Ras/Raf/MEK/ERK signaling upon IGF-1 stimulation in *NBS1* siRNA-transfected cells.

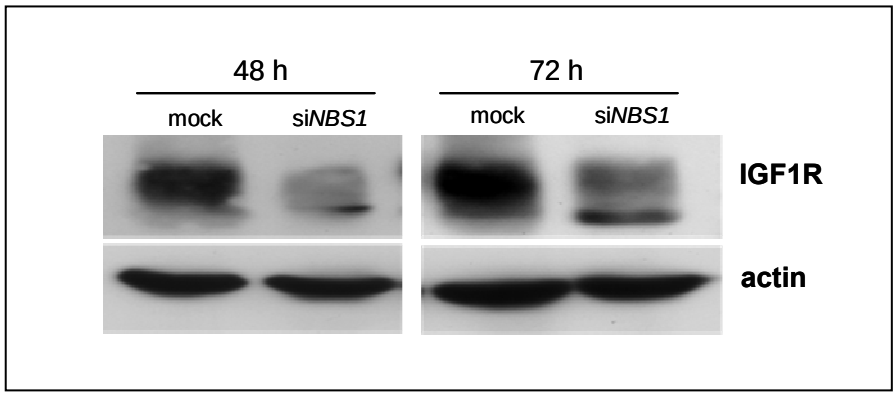


Figure 28. The basal levels of IGF1R (α-subunit) from the lysates of scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*). The RPE cells were transfected with *NBS1* siRNA or scramble siRNA and collected at 48 h and 72 h after transfection for protein extraction. The level of IGF1R (α-subunit) was determined by Western blot analysis. The antibody against IGF1R (α-subunit) identified IGF1R (α-subunit) as a 130 kD band. Detection of actin was used as a loading control. This result is representative for two independent experiments.

3.6 The influence of IGF-1 on the phosphorylation of Nbs1

There are good evidences that Nbs1 is involved in the IGF-1 signaling pathway by influencing the expressions of *SOS1*, SOS2, and IGF1R. However, it is challenging to investigate the other possible roles of Nbs1, especially its function as an additional component of this signaling cascade. Phosphorylation of Nbs1 at serine residue 343 by ATM in response to radiation is required for its functions in signal transduction for DNA damage response. Since IGF-1 stimulation has been found to enhance kinase activity of ATM [167], it is interesting to investigate whether IGF-1 can also induce phosphorylation of Nbs1. Whole cell extracts from RPE cells upon IGF-1 stimulation were analyzed by Western blotting using a Nbs1 phospho-serine 343 antibody. The results shown in Fig. 29A showed that the phosphorylation levels of Nbs1, under normal serum supplementation and serum deprivation, were very low and hardly detectable. However, phosphorylated Nbs1 was remarkably increased at the earliest time point, 15 min after exposure of the cells to IGF-1. The accumulation of phosphorylated Nbs1 was sustained for several hours, the longest time points of observation was 5 h after IGF-1 treatment. The previous results demonstrated that *NBS1* depletion caused diminished activity of the Ras/Raf/MEK/ERK cascade upon IGF-1 stimulation. The MEK specific inhibitor U0126 had no effect on the level of phosphorylated Nbs1 (Fig. 29B). This finding clearly indicated that phosphorylation of Nbs1 was not transmitted via the MEK/ERK pathway.

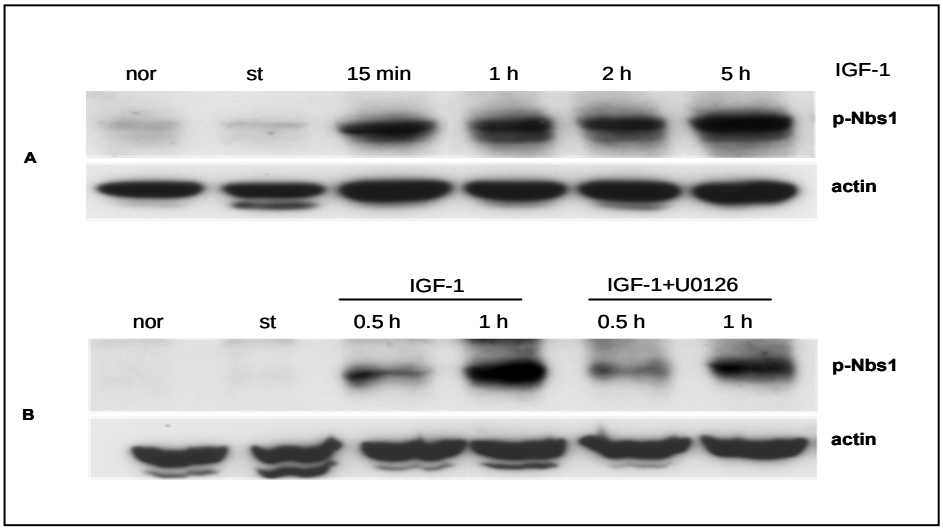


Figure 29. The influence of IGF-1 on the phosphorylation of Nbs1. RPE cells were deprived of serum for 24 h, treated alone with 100 ng/ml IGF-1 (A) or treated with 100 ng/ml IGF-1 plus 10 μM U0126 pretreatment (B). The treated cells were collected at different time points for protein extraction. The levels of p-Ser343-Nbs1 were determined by Western blot analysis. The antibody against p-Ser343-Nbs1 identified p-Ser343-Nbs1 as a 95 kD band. Detection of actin was used as a loading control. (nor) is referred to normal serum supplemented and (st) is referred to serum deprived. Each result is representative for two independent experiments.

The finding that Nbs1 was phosphorylated rapidly upon IGF-1 stimulation raised the question for the possible mechanism of this event. Under normal circumstances, Nbs1 has been found to localize only in the nucleus, and how it is activated by receptor-mediated signaling is unknown. One possible explanation for the phosphorylation of Nbs1 upon IGF-1 stimulation is the translocation of Nbs1 from the nucleus to cytoplasm. Therefore, immuno-fluorescence staining was performed for monitoring the subcellular distribution of Nbs1 in RPE cells. Nbs1 was found to localize only in the nucleus (Fig. 30A) and IGF-1 had no effect on the cellular distribution of Nbs1 (Fig. 30B). In addition, Western blot analysis of the nuclear and cytoplasmic fractions of RPE cells upon IGF-1 stimulation revealed the similar results. The Nbs1 protein was only detected in the nuclear fraction (Fig. 31, left panel) while it was not detectable in the cytoplasmic fraction during the time of observation (Fig. 31, right panel).

These results demonstrated for the first time that IGF-1 induces phosphorylation of Nbs1, however the exact mechanism is unknown.

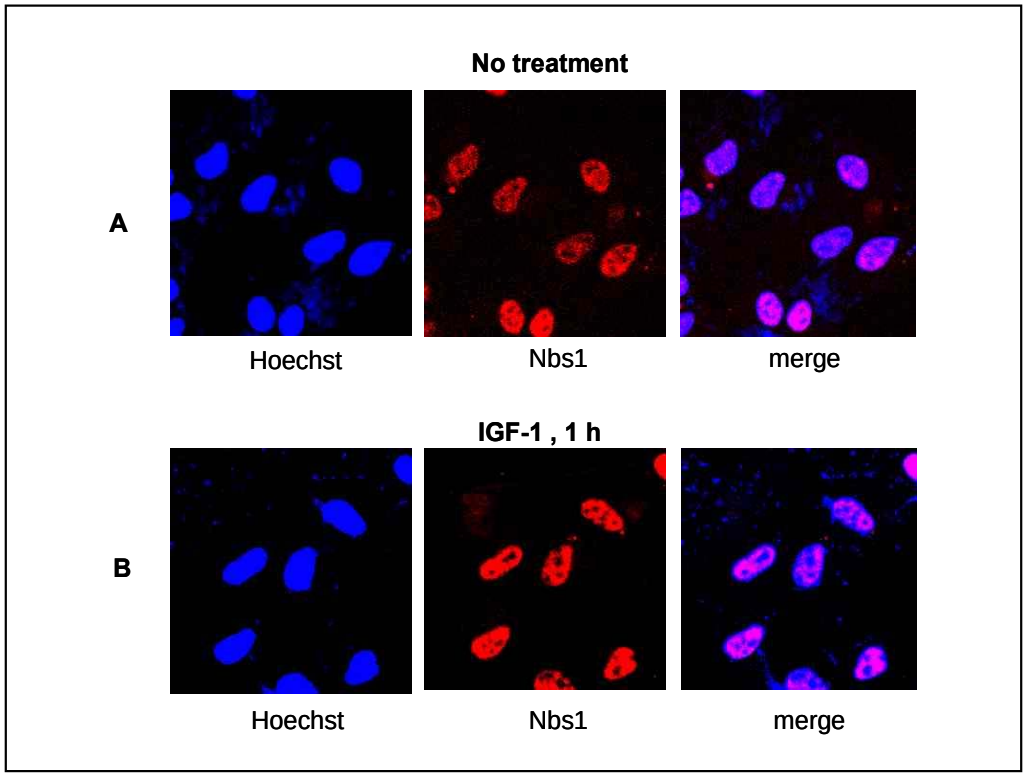


Figure 30. Subcellular localization of Nbs1. The subcellular localization of Nbs1 was determined by immunostaining. About $3x10^5$ RPE cells were seeded into 4-wells chamber slides and cultivated for 48 h, afterward the cells were treated with or without 100 ng/ml IGF-1 for 1 h. After treatment, the cells were fixed and immunostained as described in material and method. (A) no treatment (B) 1 h after IGF-1 treatment , blue (nuclei) , red (Nbs1). Each result is representative for two independent experiments.

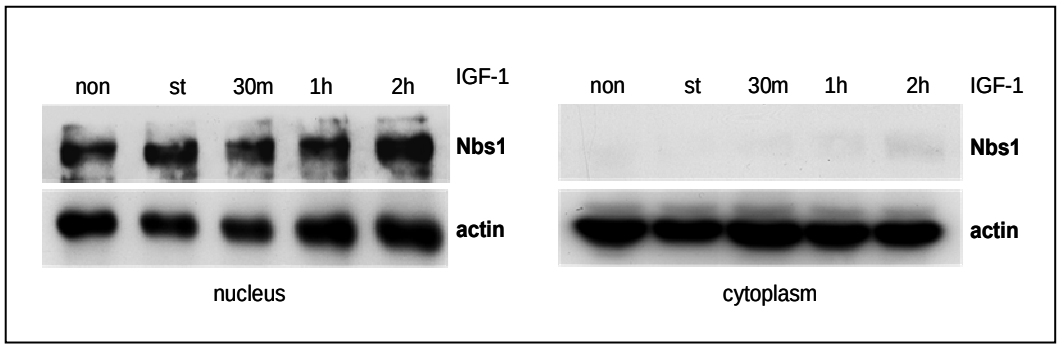


Figure 31. Subcellular localization of Nbs1. The subcellular localization of Nbs1 was determined by Western blot analysis of nuclear (right panel) and cytoplasmic (left panel) extracts of RPE cells at the indicate time points after IGF-1 treatment. The antibody against Nbs1 identified Nbs1 as a 95 kD band. Detection of actin was used as a loading control. This result is representative for two independent experiments.

3.7 IGF-1 signaling cascade mediated cellular radio-resistance is associated with the increased radio-sensitivity of *NBS1* siRNA-transfected cells

The involvement of *NBS1* in the IGF-1 signaling cascade on cell proliferation could explain the developmental defect and growth retardation phenotype in NBS patients. It also would be interesting to investigate a putative association between the IGF-1 signaling cascade-related events and increased radio-sensitivity of *NBS1* siRNA-transfected cells. Activation of IGF1R was found to possess the ability to confer clonogenic radio-resistance following ionizing radiation [168,169]. As the expression of IGF1R was strongly reduced in *NBS1* siRNA-transfected cells, it could be expected that this can contribute to enhanced radio-sensitivity in these cells. Therefore, clonogenic cell survival, as determined by colony forming assay, was used for measuring the radio-sensitivity of the cells. The scramble siRNA and *NBS1* siRNA-transfected cells were pretreated with or without 100 ng/ml IGF-1 for 1 h before gamma irradiation with the doses of 0, 3, 6, or 9 Gy. Of these, for the doses of 3 and 6 Gy, *NBS1* siRNA-transfected cells exhibited significantly enhanced radio-sensitivity as compared to scramble siRNA-transfected cells (Fig. 32A and B). For 9 Gy, the survival fractions between scramble siRNA and si*NBS1*-transfected cells were not significantly different because almost every cell was killed. Additional pretreatment with IGF-1 enhanced the survival fraction of scramble siRNA-transfected cells for the dose of 9 Gy by a factor of ~5 as compared to no treatment (Fig. 32A and B). Notably, IGF-1 treatment in *NBS1* siRNA-transfected cells did not confer any significant increase in cell survival after irradiation.

These results suggested that enhanced radio-sensitivity of *NBS1* siRNA-transfected cells could at least partially derive from the disturbance of IGF-1 signaling.

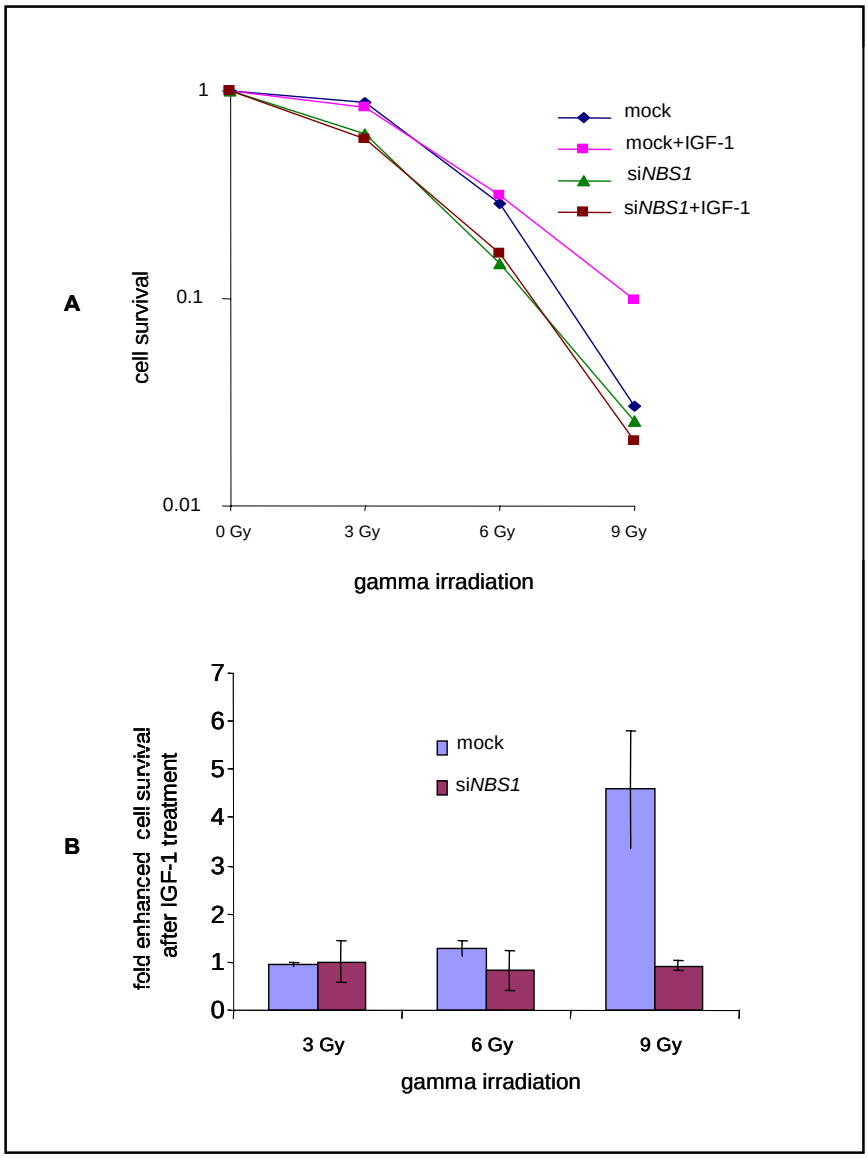


Figure 32. Cellular radio-sensitivity of scramble siRNA-transfected cells (mock) and *NBS1* siRNA-transfected cells (si*NBS1*) under normal conditions and in response to IGF-1 stimulation. One day after transfection of RPE cells with siRNA, cells were seeded into 6 well plates and incubated in medium containing 10% FCS. The cells were pre-treated with or without IGF-1 (100 ng/ml) for 1 h before gamma irradiation. Clonogenic survival of gamma-irradiated cells was determined at the tenth day after gamma irradiation. The dose survival curve shows a representative result from three independent experiments (A). The fold enhanced in cell survival after IGF-1 treatment was presented as the mean ± SD of the three independent experiments.

3.8 Validation of the key results by the second siRNA

RNAi, mediated by siRNA, is widely used to silence gene expression. However, several reports have suggested that siRNA can regulate the expression of unintended targets (off-target effect) [170,171]. To validate a confidence of the data from siRNA experiments in this study, the *NBS1* Stealth siRNA was used as a second siRNA to target *NBS1* mRNA and scramble Stealth siRNA was used as a negative control. The transfection reactions were performed by using siLentfect reagent. The *NBS1* Stealth siRNA and scramble Stealth siRNA were used in a final concentration of 20 nM. After transient transfection, the expression level of endogenous *NBS1* mRNA was determined by quantitative real-time PCR. The result showed that the level of endogenous *NBS1* mRNA in *NBS1* Stealth siRNA-transfected cells was effectively repressed to less than 10% of scramble Stealth siRNA-transfected cells as assessed at 72 to 144 h after transfection (Fig. 33).

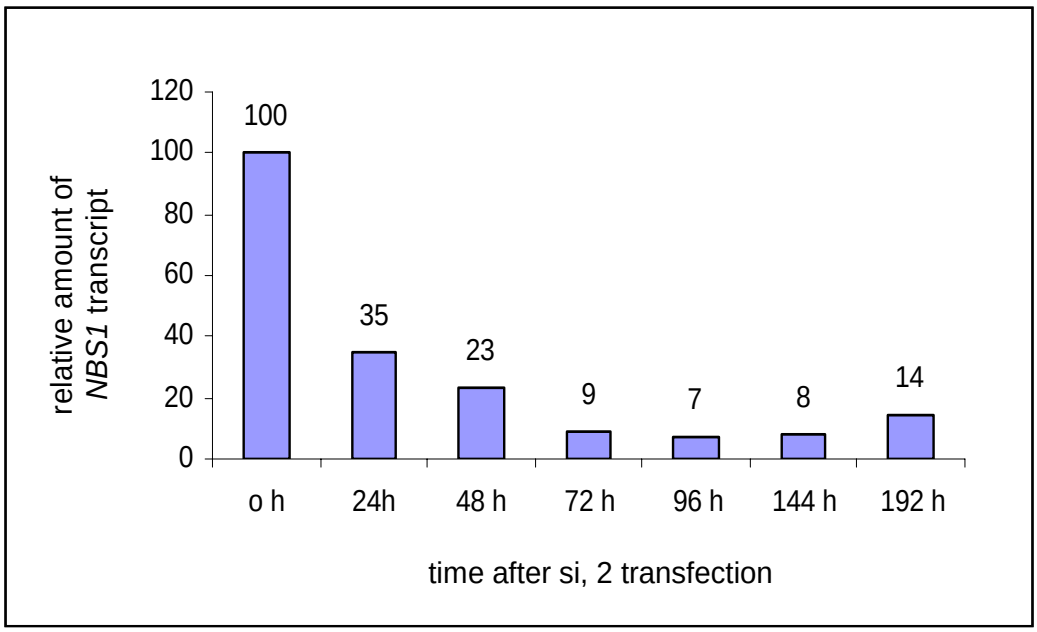


Figure 33. *NBS1* Stealth siRNA-mediated down-regulation of endogenous *NBS1* mRNA. At 24, 48, 72, 96, 144, and 192 h after transfection, the RPE cells were harvested for mRNA extraction. The extracted mRNA was reverse-transcribed and cDNA products from the reaction were used as a template for quantification of *NBS1* mRNA in *NBS1* Stealth siRNA-transfected cells (si, 2) relative to scramble Stealth siRNA-transfected cells by quantitative real time PCR. *B2M* was used for normalization of the cDNA input in the reaction. This result is representative for two independent experiments.

In addition, the protein level of Nbs1 was also determined. The whole cell lysates extracted from scramble Stealth siRNA-transfected and *NBS1* Stealth siRNA-transfected cells were examined by Western blot analysis. A representative result is shown in Fig. 34. In *NBS1* Stealth siRNA-transfected cells, the protein level of Nbs1 was almost completely absent at 96 h to 192 h after transfection as detected by Western blot analysis.

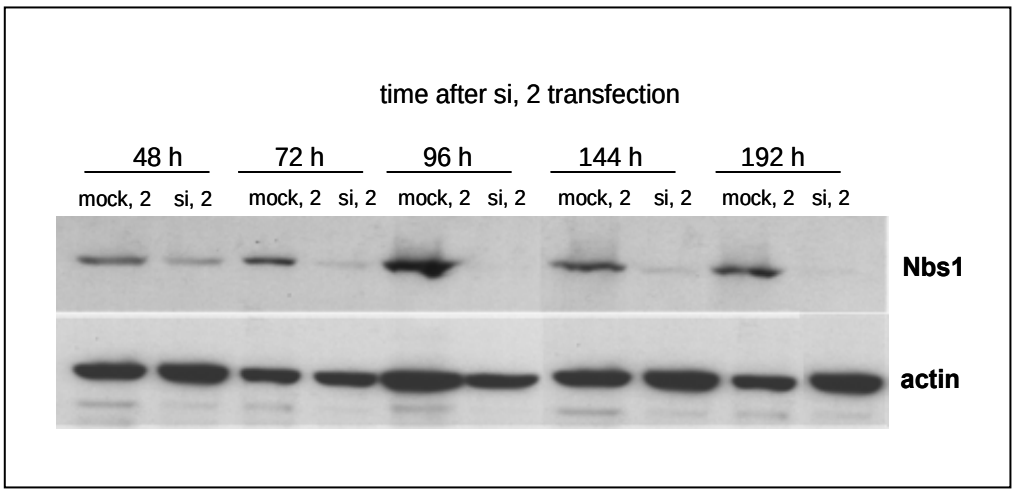


Figure 34. The protein levels of Nbs1 from the lysates of scramble Stealth siRNA (mock, 2) and *NBS1* Stealth siRNA (si, 2)-transfected cells. At indicated time points after transfection, the RPE cells were harvested for whole cell lysate preparation. The whole cell lysates were analyzed by Western blot analysis. The antibody against Nbs1, identified Nbs1 as a 95 kD band. Detection of actin was used as a loading control. (A) The levels of Nbs1 were determined at 48, 72, 96, 144, and 196 h after siRNA transfection. This result is representative for two independent experiments.

The consequence of *NBS1* silencing by *NBS1* Stealth siRNA on the proliferation capacity of RPE cells was examined firstly. 72 h after transfection, the cellular proliferation rate of the cells were determined by using the same experimental conditions as described in 3.3.2. The similar results which were observed from *NBS1* silencing by the first *NBS1* siRNA duplex were demonstrated. Without IGF-1 treatment, the cellular proliferation rate of *NBS1* Stealth siRNA-transfected cells was reduced by ~50%, as compared to scramble Stealth siRNA-transfected cells (Fig. 35). IGF-1-enhanced cellular proliferation rate was clearly observed in scramble Stealth siRNA-transfected cells but was not observed in scramble Stealth siRNA-transfected cells.

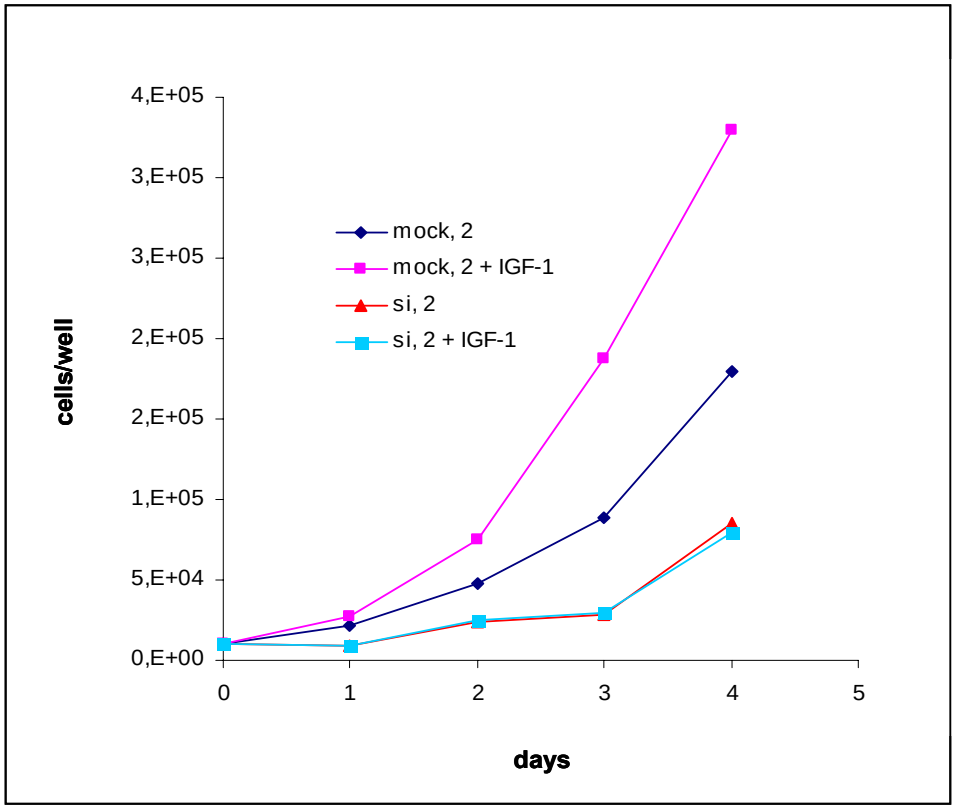


Figure 35. Cellular proliferation of scramble Stealth siRNA-transfected cells (mock, 2) and *NBS1* Stealth siRNA-transfected cells (si, 2) under normal condition and in response to IGF-1 stimulation. 72 h after transfection, RPE cells were seeded into 6 well plates and incubated in medium containing 10% FCS either supplemented with or without IGF-1 (100 ng/ml). Medium was changed every day. At the indicated time points, the cells were trypsinized and the cell number was determined in duplicate. This result is representative for two independent experiments.

Determination of the expression levels of *SOS1*, *SOS2*, and IGF1R were further repeated in order to rule out an off-target effect of the first siRNA duplex. The impact of *NBS1* on the expression levels of *SOS1*, *SOS2*, and IGF1R was also demonstrated by application of *NBS1* Stealth siRNA. Reduced amounts of *SOS1* and *SOS2* transcripts and reduced amounts of Sos1, Sos2, and IGF1R proteins were demonstrated in *NBS1* Stealth siRNA-transfected cells (Fig. 36-37).

Taken together, these results confirmed that the observed phenotypes of RPE cells after suppression of endogenous *NBS1* level by siRNAs are approved and that off-target effect of the siRNA duplexes can be ruled out.

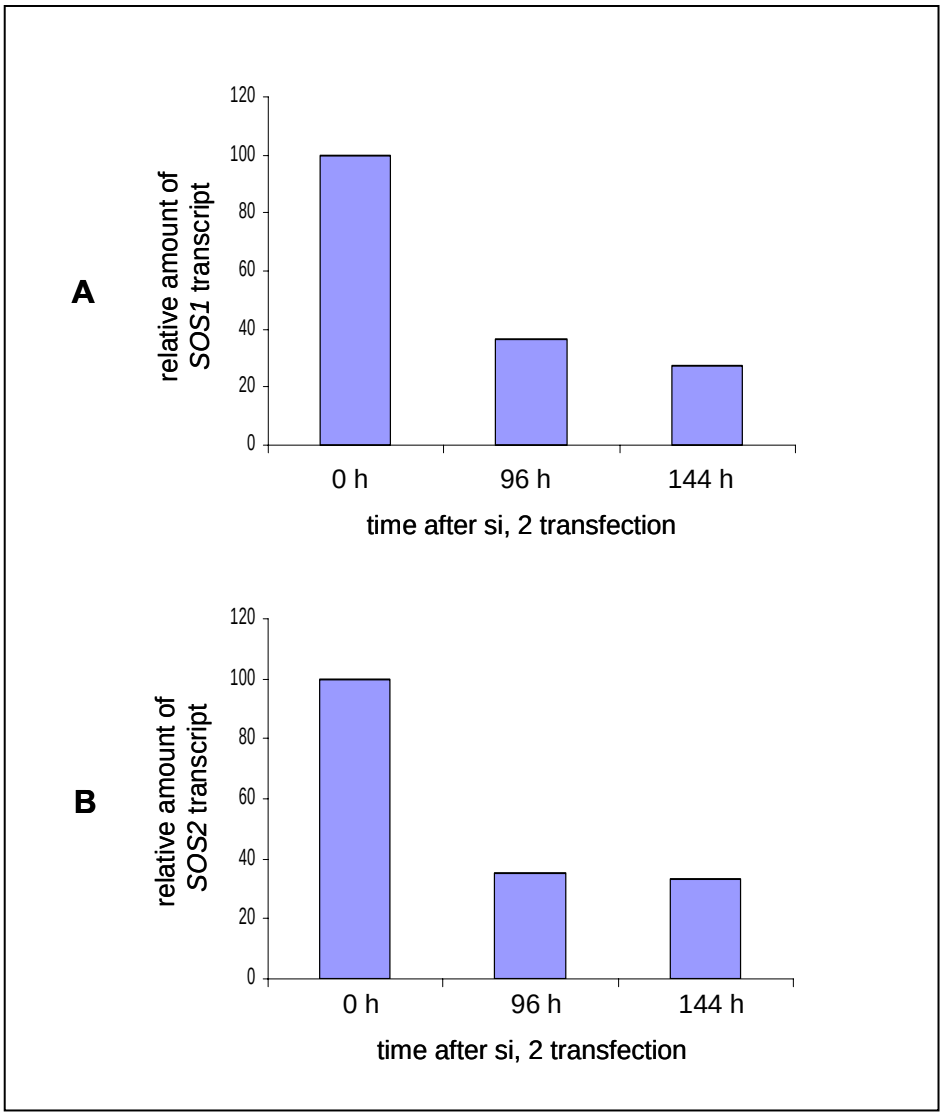


Figure 36. Basal expression of *SOS1* and *SOS2* transcripts in scramble Stealth siRNA-transfected cells and *NBS1* Stealth siRNA-transfected cells. Relative amounts of *SOS1* (A) and *SOS2* (B) transcripts were quantified in extracts of RPE *NBS1* Stealth siRNA-transfected cells (si, 2) and in that of scramble Stealth siRNA-transfected cells (mock, 2) by quantitative RT-PCR. Samples were prepared at indicated time points after transfection. Each result is representative for two independent experiments.

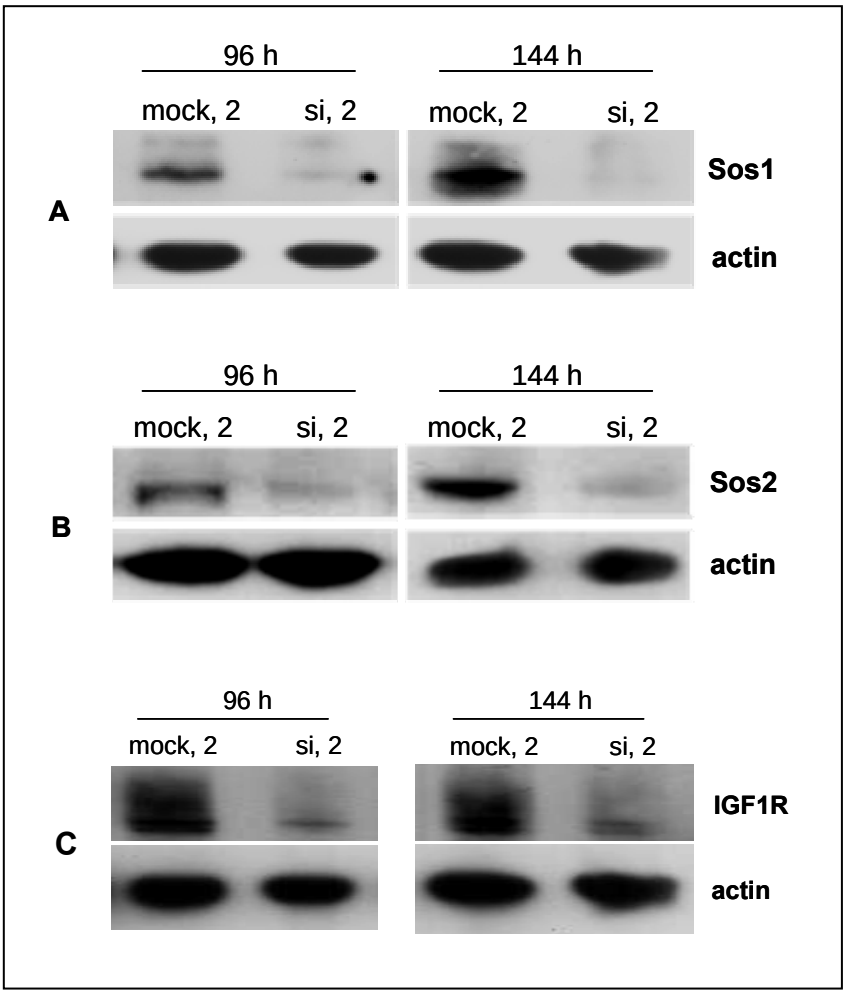


Figure 37. The basal levels of Sos1, Sos2, and IGF1R (α-subunit) from the lysates of scramble Stealth siRNA-transfected cells and *NBS1* Stealth siRNA-transfected cells. The RPE cells were transfected with scramble Stealth siRNA (mock, 2) or *NBS1* Stealth siRNA (si, 2) and collected at 96 and 144 h after transfection for protein extraction. The levels of Sos1 (A), Sos2 (B), and IGF1R (C) were determined by Western blot analysis. Antibodies against Sos1 or Sos2 identified Sos1 or Sos2 as a 182 kD band. The antibody against IGF1R (α-subunit) identified IGF1R (α-subunit) as a 130 kD band. Detection of actin was used as a loading control. Each result is representative for two independent experiments.

4. DISCUSSION AND CONCLUSION

4.1 Discussion

4.1.1 Down-regulation of *NBS1*

The experimental plan in this study was designed to analyze the function of Nbs1 without any impact of partial activities of Nbs1 truncated fragments that are expressed in NBS patient cell lines with the common 657del5 mutation. It has been proposed that the Nbs1 truncated fragments may have partial activities in growth and development of mammals [51]. The significance of the truncated products had been observed in two knock-out mice which produce no truncated Nbs1 and die early in embryonic development while the other pair of knock-down mice which do produce truncated Nbs1 fragments are viable [51,172]. Before RNAi technology was invented, functional studies of Nbs1 had been done by using patient cell lines or knock-out technology. However, the disadvantage of using patient cell lines in addition to the interference from the partial function of truncated fragments is the variation of the genetic background of individual cells. For silencing the gene of interest, the knock out technology is frequently used in animal models, but this technique is cumbersome, time consuming, and it is not applicable in humans. During the past six years, the use of RNAi technology has been growing rapidly because it is a very effective post-transcriptional gene silencing method and it is rather easy to perform. Most important is that RNAi engages an endogenous mechanism that exists in cells. For this reason, RNAi was employed to silence the expression of the *NBS1* gene in this study.

Recently, off-target effects that complicate the interpretation of data generated from siRNA-mediated knock-down experiments have been reported [170,171]. To validate the data from siRNA experiments, two siRNA duplexes were independently used in this study. The first siRNA duplex (*NBS1* siRNA) consists of two unmodified oligonucleotides while the second siRNA duplex (*NBS1* stealth siRNA) consists of a chemically modified oligonucleotide duplex. The data presented here showed that the two different oligonucleotide siRNA duplexes effectively decreased the amount of *NBS1* mRNA and Nbs1 protein. However, only the *NBS1* siRNA was used for all experiments because it provides a rapid reduction of the Nbs1 protein and its effective knock-down period is more suitable. Using *NBS1* siRNA,

the amount of *NBS1* mRNA was decreased effectively within 24 h after transfection while the Nbs1 protein was reduced by 24 h later. The disappearance of the Nbs1 protein occurs after the disappearance of *NBS1* mRNA, because more time is needed for protein degradation. The down-regulations of *NBS1* at mRNA and protein level were still effective up to 90 h, thus the further experiments were carried out within 48-90 after transfection of the cells with siRNA. To rule out a possible off-target effect, the determinations of cell proliferation capacity, expression levels of *SOS1*, *SOS2*, Sos1, Sos2, and IGF1R were repeated after suppression of endogenous *NBS1* expression by *NBS1* stealth siRNA. These are the key experiments which confirm that *NBS1* influences IGF-1 signaling promoting cell proliferation by modulating the expressions of *SOS1*, *SOS2*, and IGF1R. Using *NBS1* stealth siRNA, the amounts of *NBS1* mRNA and Nbs1 protein were effectively decreased within 72 and 96 h, respectively. Knock-down of *NBS1* at mRNA and protein level by *NBS1* stealth siRNA were still effective up to 144 h, thus the further experiments were carried out within 96-144 h after transfection.

It has been reported, that deficiency of Mre11 (in lymphoblastoid cell lines derived from ataxia-telangiectasia-like disorder patients) or Rad50 (in HCT116 and 293T cell lines after siRNA-mediated suppression of Rad50) causes a reduction of all protein components of the MRN complex [157,173]. This suggests, that either Rad50 or Mre11 or both are required for protein stability of the entire complex. The results in this report showed that in the absence of Nbs1, the levels of Mre11 and Rad50 are not reduced. Knock-down of *NBS1* does not affect the protein levels of its complex partners, thus the RNAi approach is suitable for the functional study of a single protein, Nbs1. It has been reported, that Nbs1 is required for stimulation of DNA binding activity of the MRN complex and for the nuclease activity of Mre11/Rad50 [37]. In addition, Nbs1 is essential for nuclear localization and targeting of the MRN complex to the sites of DNA double strand breaks [32,44]. It should be considered that, although Nbs1 is not required for Mre11/Rad50 stability, the presence of Nbs1 is required for the activity of the complex [37].

4.1.2 *NBS1* and cell cycle regulation

The involvement of *NBS1* in cell cycle progression of undamaged cells is clearly shown in this study. After release from G1 arrest, *NBS1* siRNA-transfected cells showed slower progression from G1 phase to the next stages of the cell cycle as compared to scramble siRNA-transfected cells. Further investigations for the underlying mechanism of the delayed cell cycle progression in theses cells was focussed on the expression patterns of cyclins. The finding, that the periodical oscillations of cyclins E and A were disturbed in *NBS1* siRNA-transfected cells provides some evidence that Nbs1 influences cell cycle progression possibly at the beginning of G1 phase or, during G1/S phase transition.

The accumulation of cyclin D1 is a key step for the cells to enter the cell cycle. Therefore, the expression pattern of cyclin D1 was determined in this study in order to clarify if *NBS1* is involved in the initiation step of cell cycle progression. Aphidicholin induces synchronization of the cells in early S phase by inhibiting the binding of 2'-deoxynucleotides-5'-triphosphates to DNA polymerases α and δ [174], thus it can not be used for the analysis of cell cycle specific events at the initiation of G1 phase. A widely used model system to investigate cell proliferation, and thus G1 phase progression, is stimulation of serum-starved cells with growth factor [132]. Therefore, serum starvation, the standard approach to arrest the cells in G0 or early G1 phase, was used in this study. IGF-1 was selected for stimulating cell proliferation because this growth hormone is very important for pre-natal and post-natal growth and development [7,10]. The data presented here showed that the expression of cyclin D1 was very low in serum-starved cells. Upon IGF-1 stimulation, the expression of cyclin D1 was rapidly increased in the scramble siRNA-transfected cells but negligible in *NBS1* siRNA-transfected cells. These results indicated the involvement of *NBS1* in the initiation event of cell cycle re-entry probably by influencing the IGF-1 signaling-induced expression of the *CCND1* gene.

IGF-1 induces cell cycle progression and cell proliferation through different signaling pathways and different targets of cell cycle components dependent on the cell types. For example, in human osteosarcoma MG63 cells, IGF-1 stimulates *CCND1* expression in an ERK1/2-dependent manner [175], but this effect is dependent on the PI3K/Akt signaling pathway in human BON neuroendocrine tumor cells [176]. In human intestinal smooth

muscle cells, effects of IGF-1 on proliferation are mediated jointly by the ERK1/2 and PI3K-dependent pathways that regulate cyclin D1 levels, and CDK4 activities. In this study, the identification of the pathway responsible for IGF-1-induced expression of cyclin D1 in RPE cells was performed by using a selective inhibitor. Application of the MEK1/2-specific inhibitor, U0126, completely abolished the induction of cyclin D1 and increased cellular proliferation induced by IGF-1. Therefore, it can be assumed that the Ras/Raf/MEK/ERK cascade is the major pathway for IGF-1-induced cell proliferation and cell cycle progression in RPE cells.

It was reported, that PI3K/Akt and/or mTOR regulate cell growth and enhance cell proliferation by affecting *CCND1* translation and cyclin D1 stabilization [177-179]. I also found that induction of Akt activity through PI3K by IGF-1 was impaired in *NBS1* siRNA-transfected cells. Since induction of *CCND1* expression was completely abolished by MEK1/2 inhibition, it can be assumed that the PI3K/Akt cascade is not essential for the regulation of *CCND1* expression in RPE cells. However, since the determination was carried out within 5 h after IGF-1 stimulation, it is possible that the Ras/Raf/MEK/ERK pathway up-regulates *CCND1* expression during an early period of time after IGF-1 stimulation and that the other pathways, especially the PI3-K/Akt pathway, are involved in the regulation of cyclin D1 at later time points.

ERK1 and ERK2 are the effector proteins of the Ras/Raf/MEK cascade. IGF-1 stimulation leads to phosphorylation of threonine and tyrosine residues within the activation loop of ERK1/2 [95]. Active ERKs phosphorylate targets in the cytoplasm and they translocate from the cytoplasm to the nucleus. Active ERK1/2 accumulate in the nucleus where they can directly phosphorylate many transcription factors involved in cell cycle regulation. The results clearly showed that *NBS1* siRNA-transfected cells failed to response to IGF-1-induced ERK1/2 activation which were demonstrated by the low levels of ERK1/2 phophorylation and no accumulation of ERK1/2 in the nucleus after IGF-1 stimulation.

The Ras/Raf/MEK/ERK cascade promotes transcription of the *CCND1* gene via the activation of its promoter at the AP-1 site. The human *CCND1* gene regulatory sequences contain two AP-1 binding sites [116]. Several AP-1 proteins including Jun and Fos bind to these sites and regulate *CCND1* transcription [114,115]. The effect of the AP-1 transcription

factor on the activation of *CCND1* depends on a complex composition and phosphorylation of Jun and Fos proteins. In general, c-Jun is a target of the c-Jun N-terminal kinases (JNKs), and c-Fos is a target of ERKs. The proper regulation of c-Fos is important in many cellular processes including proliferation and differentiation [102]. Control of cell proliferation by c-Fos is mainly mediated by its ability to regulate the expression of cell cycle regulators. For example, in response to growth factor stimulation, active ERK1/2 induce the phosphorylation of ternary complex factors especially Elk1 to induce transcription of the *FOS* gene [180]. c-Fos enters the nucleus immediately after translation. Accumulation and stabilization of c-Fos in the nucleus requires the phosphorylation at the C-terminal site by ERK1/2 and their down stream targets, ribosomal s6 kinases (RSKs), for full stabilization of the c-Fos protein [181]. The accumulation of active c-Fos in the nucleus induces the transcription of many genes including Fos family member Fra1 a transcriptional activator of *CCND1* promoter, leading to the induction of the *CCND1* gene [164].

The data reported here showed that *FOS* transcription peaks within minutes after growth factor stimulation in scramble siRNA-transfected cells. Remarkably, a big difference in the induction of the *FOS* expression between scramble siRNA-transfected cells and *NBS1* siRNA-transfected cells was clearly observed after IGF-1 stimulation. Specifically, it was confirmed by MEK inhibition that the Ras/Raf/MEK/ERK pathway regulates *FOS* expression. Taken together, these findings underline the role of Nbs1 in mitogenic signaling and cell proliferation. Obviously, Nbs1 is involved in the initiation event of cell cycle re-entry through the Ras/Raf/MEK/ERK signaling cascade which regulates the expression of the *CCND1* gene via Fos family members. The data presented here propose a model for the function of Nbs1 upstream or at the level MEK1/2 (Fig. 38).

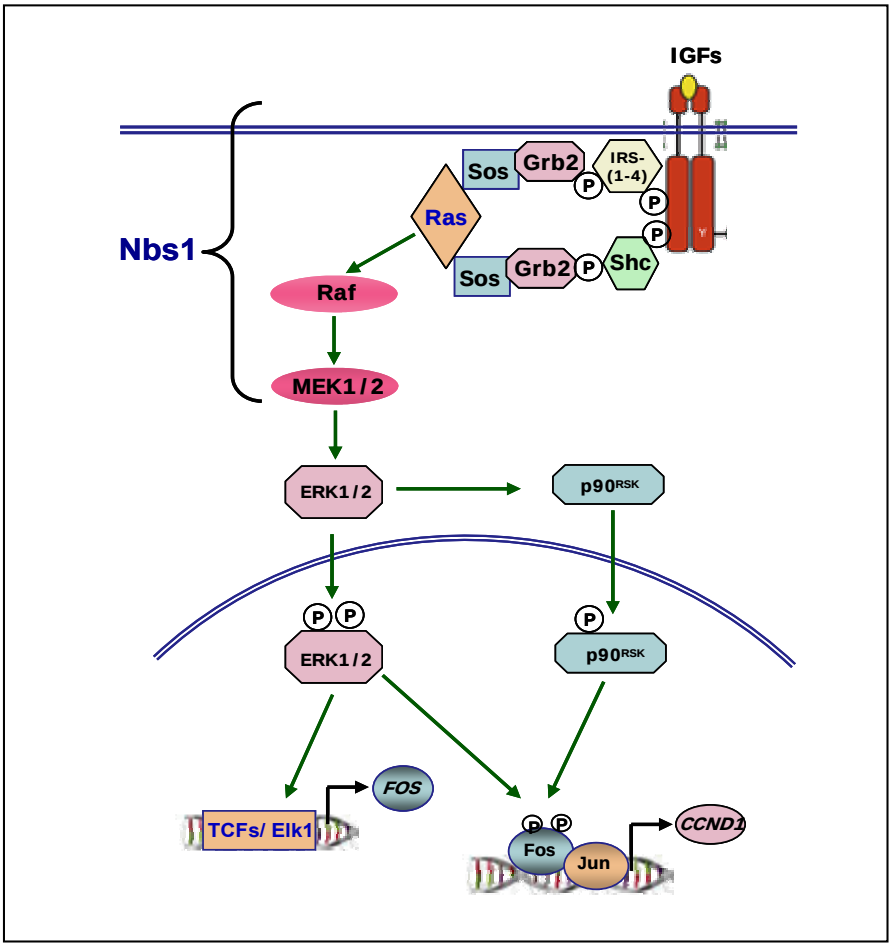


Figure 38. Scheme of a model for a putative regulation of the IGF-1 signaling cascade by Nbs1 (see more detail in the text).

4.1.3 The influence of *NBS1* on the expression of *SOS1*, *SOS2* and IGF1R

Data from this study suggested that *NBS1* is mandatory at an early time point of IGF1R activation, since phosphorylation of c-Raf was already reduced in *NBS1* siRNA-transfected cells after IGF-1 treatment. IGF-1-induced activation of the Ras/Raf/MEK/ERK cascade is mediated by the IGF1R-phosphorylated IRS-1 and Shc proteins, via interactions with Grb2 and Sos [182] (Fig. 38). In response to signals induced by activated IGF1R, the guanine nucleotide exchange factors, Sos proteins, function as Ras activators [183]. Upon IGF1R activation, Sos is induced to form a complex with the adaptor protein Grb2. The Grb2-Sos complex interacts with activated IGF1R on the cytoplasmic side of the plasma membrane.

This allows the presentation of Sos to Ras, leading to the exchange of GDP for GTP and Ras activation [184]. Since Sos proteins are critical for Ras activation, the expressions of *SOS1* and *SOS2* were quantified in *NBS1* siRNA-transfected cells and scramble siRNA-transfected cells. A 4 to 5 fold reduction of *SOS1* and *SOS2* expressions in *NBS1*-depleted cells were demonstrated. Consistent with these results, a reduced amount of *SOS2* expression in *NBS1*-mutated cells was also shown by oligonucleotide microarray analysis of the SV40-transformed NBS fibroblast cell line [185]. Therefore, it can be suggested that IGF-1-triggered Ras/Raf/MEK/ERK signaling is impaired in *NBS1* siRNA-transfected cells due to *NBS1*-dependent expression of *SOS1* and *SOS2*.

Moreover, the data reported here also showed that *NBS1* plays an important role in IGF1R protein expression. *NBS1* down-regulated cells had low levels of IGF1R. In contrast to this, Watanabe *et al.* reported that there was no significant difference in IGF1R protein levels among normal cell lines, NBS patient cell lines, and *NBS1*-complemented NBS patient cell lines [186]. The contradiction may result from the use of different cell types and experimental strategies. The NBS cell lines which were used in the study mentioned above were SV40-transformed fibroblasts, derived from a patient harbouring a homozygous *NBS1* 657del5 mutation. These patient cell lines express two truncated Nbs1 fragments which could be active in several functions, including regulation of IGF1R expression. In the experimental system used in this study, full-length Nbs1 expression was repressed. Thus, the detected phenotypes represent the actual activity of Nbs1 and are not hidden by functions maintained by the expression of two partial Nbs1 fragments expressed in NBS patient cell lines with the common founder mutation 657del5 [17]. It should be considered that only the protein level of IGF1R was determined in this study. For further investigation, detection of *IGF1R* at mRNA level by quantitative real time PCR is suggested.

The exact mechanism by which *NBS1* influences the expression of the IGF1R protein remains to be elucidated. Interestingly, ATM controls *IGF1R* gene expression in a DNA damage response pathway via a mechanism involving the zinc-finger transcription factors Sp1 and WT1 [187]. In response to DNA damage, *NBS1* was shown to act downstream and upstream of ATM [166]. Therefore, it might be possible that the impact of *NBS1* on IGF1R expression resultes from its influence via ATM-related mechanisms. Two models can be proposed for the regulation for of *IGF1R* expression by Nbs1 (Fig.39).

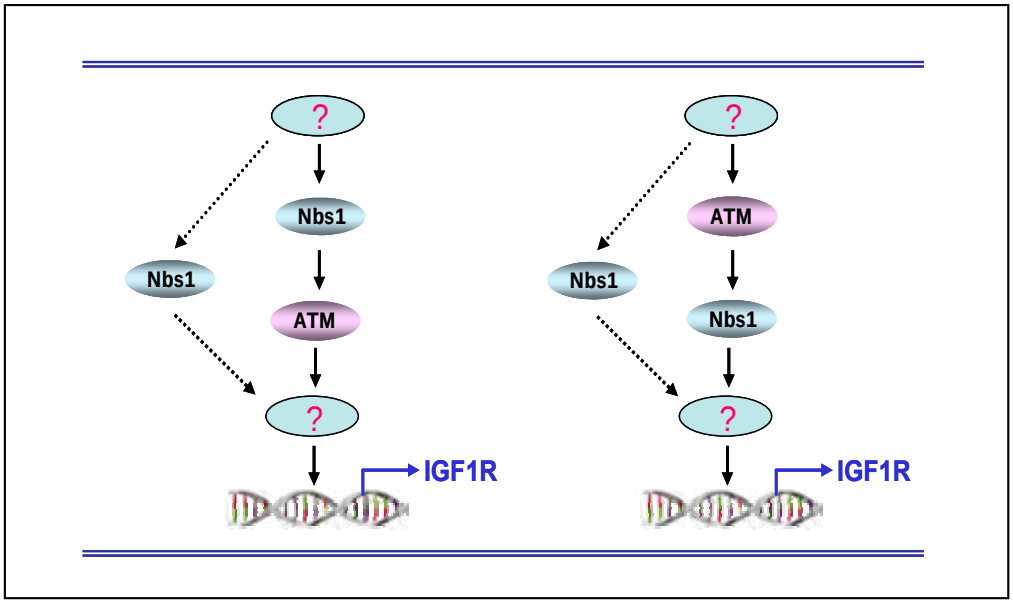


Figure 39. Scheme of a putative role of Nbs1 in the regulation of *IGF1R* expression. Arrows with broken line represent the function of Nbs1 independent of ATM. The left model represents the function of Nbs1 upstream of ATM. The right model represents the function of Nbs1 downstream of ATM. Blue circles with a question mark represent unknown molecules.

This report clearly demonstrated that *NBS1* is involved in the IGF-1 signaling pathway by influencing the expression of *SOS1*, *SOS2*, and their proteins, and IGF1R. However, the finding that phosphorylation of Nbs1 can be induced by IGF-1 suggests an additional mechanism that may responsible for the diminished activity of downstream signaling cascades of IGF-1 in *NBS1* down-regulated cells. It has been shown that IGF1R signaling can modulate ATM kinase activity in response to ionizing radiation [167]. Moreover, Suzuki *et al*. also found that phosphorylation of ATM at both threonine and tyrosine residues is stimulated by IGF-1 [188]. These reports, together with the findings reported here, are likely to be an important link between IGF-1 and Nbs1. Serine 343 of Nbs1 is phosphorylated by ATM in response to ionizing radiation suggesting that Nbs1 functions downstream of ATM [26,27]. Therefore, it is possible that IGF-1-induced phosphorylation of Nbs1 is also mediated by ATM. However, there is also evidence that Nbs1 functions upstream of ATM [166]. Thus, it is also possible that IGF-1 signaling modulates activity of Nbs1 and then activated Nbs1 in turn activates ATM kinase activity leading again indistinguishable models (fig 40).

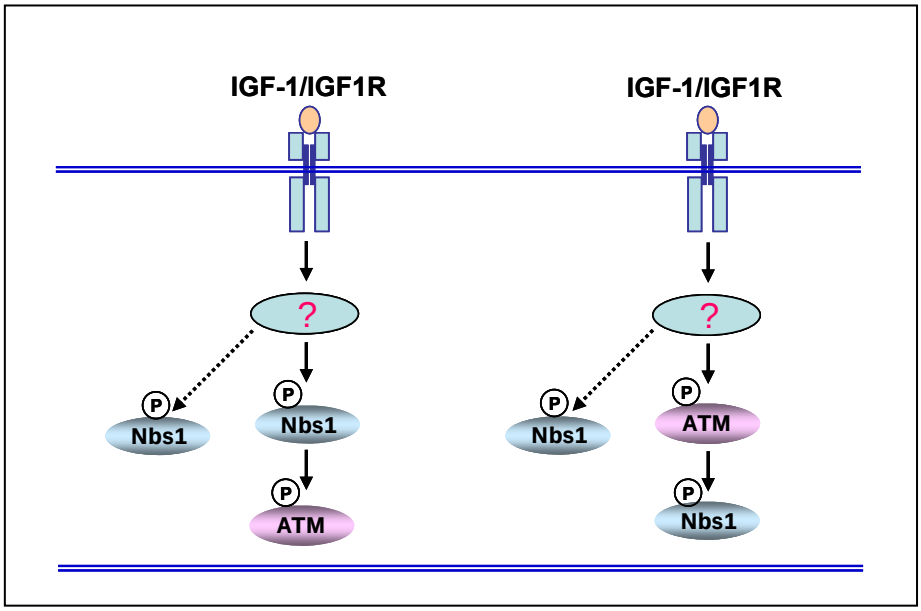


Figure 40. Scheme of the putative models for the phosphorylation of Nbs1 in response to IGF-1. Arrows with a broken line represent the phosphorylation of Nbs1 independent of ATM. The left model represents the phosphorylation of Nbs1 up-stream of ATM phosphorylation. The right model represents the phosphorylation of Nbs1 down stream of ATM phosphorylation. Blue circles with a question mark represent unknown molecules.

The molecular mechanism of *NBS1* in IGF-1 signaling remains to be elucidated. The data reported here showed that Nbs1 was mainly found in the nucleus, and that cytoplasmic translocation of Nbs1 after IGF-1 stimulation was not observed by immunofluorescence staining or Western blot analysis. Therefore, it is unlikely that Nbs1 has a direct interaction with the cytosol or membrane-localized components of the IGF-1 signaling. In response to DNA damage, Nbs1 functions together with Mre11 and Rad50 in the DNA repair process, which requires their translocation from the cytoplasm to the nucleus to participate in DNA damage processing as a trimeric complex [1]. However, it is not known whether Nbs1 functions independently or together with the Mre11/Rad50 complex in response to IGF-1. There is evidence that Nbs1 and Mre11/Rad50 may have an opposite role in the regulation of cell proliferation. Nbs1 over-expression increases cell proliferation [159], but over-expression of Rad50 inhibits cellular proliferation [189]. One possible explanation for their opposite role can be seen in the ability of Nbs1 to modulate the nuclear and cytoplasmic pools of Mre11 and Rad50. In the absence of Nbs1, Mre11 and Rad50 may translocate

freely into the cytoplasm where they can interact with each other and with IGF-1 signaling components and thereby possibly inhibit the activation of the IGF-1 signaling cascade. Therefore, I hypothesize that in the presence of Nbs1, Mre11 and Rad50 are retained within the nucleus, resulting in the reduction of their concentration in cytoplasm, thus diminishing their inhibitory effect on the IGF-1 signaling cascade.

4.1.4 The disturbance of the IGF-1 signaling cascade causes increased radio-sensitivity of *NBS1* siRNA-transfected cells

In addition of being important for stimulation of cell proliferation, the IGFs system has been characterized as a cell survival factor in certain cells [16]. IGF-1 is a highly efficient anti-apoptotic agent, not only because it has strong anti-apoptotic activities, but also because it protects cells from a variety of apoptotic stimuli, including osmotic stress [73], hypoxia [73], ionizing and non-ionizing radiation [190,191], and anti-cancer drugs [72]. Ionizing radiation is a potent inducer of cell killing by provoking damage to DNA and other cellular components in eukaryotic cells. Following radiation damage, several intracellular events are triggered. The plasma membrane receptors such as IGF1R, epidermal growth factor receptor, and Fas are activated [192-194]. The expression of genes encoding proteins that influence cell survival and cell death pathways such as Fas ligand, tumor necrosis factors, and p53 are changed [195-197]. The DNA repair systems and cell cycle checkpoints that are crucial for maintaining the genomic integrity of cells damaged by radiation are activated [198,199]. Several studies indicate that IGF-1 signaling can modify cellular radio-sensitivity. IGF1R overexpression mediates radio-resistance in breast cancer following radiotherapy [169]. Blocking of IGF1R activity by tyrosine kinase inhibitors or by anti-IGF1R neutralizing antibodies was shown to increase radio-sensitivity of several types of human tumor cell lines [167,200,201]. The level of the IGF1R is often elevated in breast cancer cells and this characteristic has been assumed to be the cause for increased radio-resistance and cancer recurrence in estrogen receptor-positive breast tumors [169,202]. In addition, the IGFs system may also prevent radiation-induced cell death by interfering with proteins involved in the repair or signaling of DNA lesions. It was found that ATM which plays an important role in the DNA damage response pathway is involved in the regulation of IGF1R activation and expression. Indeed, disturbed expression and activation of IGF1R have a major influence on the increased radio-sensitivity in ataxia telangiectasia patient cells [167,187,190].

In this study, the results from clonogenic cell survival upon gamma irradiation clearly showed that IGF-1 has a high potential to rescue gamma irradiation-induced cell death in RPE cells. The negligible effect of IGF-1 on the cell survival in *NBS1* siRNA-transfected cells upon gamma irradiation indicated that increased radio-sensitivity in these cells may be the result, at least in part, from the disturbed IGF-1 signaling cascade. Evidence accumulated in several studies revealed a strong line between the IGFs system and cell killing after exposure to ionizing radiation [169,203-205]. However, the molecular mechanism how the IGFs system influences radio-resistance of the RPE cells is poorly understood. The IGFs system acts at different levels of the apoptotic machinery through different signaling pathways [16]. However, the PI3K/Akt cascade is considered as the canonical pathway involved in inhibition of apoptosis by IGF-1 [206]. Following IGF1R activation, PI3K is phosphorylated and activated, then in turn activates Akt. The activated Akt induces phosphorylation and then inactivation of pro-apoptotic factors, including the Bcl-2 family member Bad, members of the FOXO family, and caspases [16,207]. In addition, activation of Akt by IGF-1 has also been shown to increase expression of anti-apoptotic factors, including Bcl-x [208], and NF-κB [209]. The effects of IGFs on survival are also mediated by activation of the Ras/Raf/MEK/ERK pathway [207]. However, studies in most cell types indicated that the PI3K/Akt pathway was also functioning when Ras/Raf/MEK/ERK pathway was operating. It should be noted that both pathways may have either cooperative actions by objecting identical anti-apoptotic targets [210-212] or synergistic actions by activating different target molecules [213,214]. The data reported here, also showed that in response to IGF-1, activations of PI3K/Akt and Ras/Raf/MEK/ERK were impaired in RPE *NBS1* siRNA-transfected cells. However, which pathway is responsible for the enhanced radio-sensitivity in this cell type remains to be further investigated.

Furthermore, several studies reported that the anti-apoptotic effect of IGFs is independent of the PI3K/Akt or Ras/Raf/MEK/ERK cascades. The additional pathways are meditated through 14-3-3-dependent mitochondrial translocation of Raf and Nedd4. The presence of Raf and Nedd in the mitochondria maintains the mitochondrial integrity, and thus rescues cells from apoptosis [215]. Moreover, activation of IGF1R has been reported to rescue cells from apoptotic signal-regulated kinase 1 (ASK1)-induced cell death independently of PI3K [216]. ASK1 is a MAPK kinase involved in the activation of JNK and subsequent triggering of apoptosis by death-inducing receptors. Activation of IGF-1 signaling induces a complex

formation between activated IGF1R and ASK1 thereby antagonizing ASK1-induced apoptosis. Several studies also reported that additional pathways such as the p38 MAPK and JAK/STAT-3 may participate in the inhibition of apoptosis by IGF-1 [217,218].

As mentioned above, the mechanism underlying enhanced radio-sensitivity of *NBS1* siRNA -transfected cells needes to be clarified. Importantly, it should be considered that the increased radio-sensitivity of *NBS1* siRNA-transfected cells may not be only influenced by the defect in the IGF-1 signaling cascade. A previous study from our group demonstrated that *NBS1* mediates radio-resistance by suppressing the CD95 death receptor-dependent apoptotic pathway in lymphoblasts after gamma irradiation [155]. Therefore, *NBS1* enables survival after gamma irradiation via several mechanisms and the rescue of cells after gamma-irradiation by IGF-1 is only one of them that is influenced by this gene.

The summarizing models for the proposed roles of *NBS1* in the regulation of cell proliferation, cell cycle progression, and cellular radio-sensitivity are shown in Fig. 41.

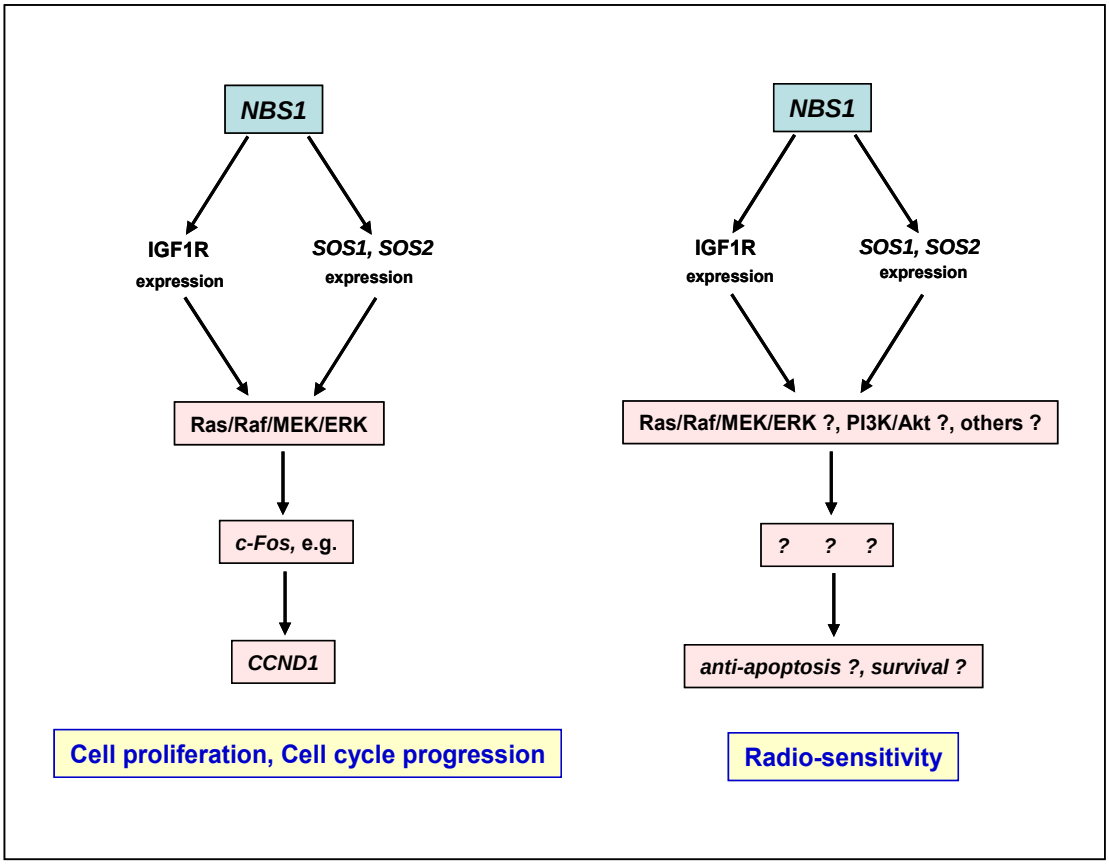


Figure 41. Scheme of the models for the novel roles of *NBS1* in the regulation of cell proliferation, cell cycle progression and cellular radio-sensitivity. The right model; *NBS1* influences cell cycle proliferation and cell cycle progression via its influence on the expressions of IGF1R, *SOS1*, and *SOS2*, which are necessary for the proper activity of the IGF-1 signal transduction cascade that induces the expression of *FOS* and *CCND1*. The left model; the influence of *NBS1* on IGF1R, *SOS1*, and *SOS2* expressions is also important for the anti-apoptotic and survival activities of the IGF-1 signaling system after irradiation–induced damage. These activities are mainly mediated through the Ras/Raf/MEK/ERK and PI3K/Akt pathways. The question marks represent unknown molecules.

4.2 Conclusion

In this study, the RNAi technique was employed to specifically repress *NBS1* levels in RPE cells. This approach is effective for silencing *NBS1* expression and suitable for the analysis of the functional role of Nbs1 without any impact of partial activities of Nbs1 truncated fragments that are expressed in NBS patient cell lines with the common 657del5 mutation and without any changing of protein levels of Mre11 and Rad50.

The impact of *NBS1* on cell cycle progression was clearly demonstrated in *NBS1* siRNA transfected cells by the delayed cell cycle progression and the disturbances in periodical oscillation of cyclin E and cyclin A. The influences of *NBS1* on the mitogen-stimulated cell proliferation and cell cycle re-entry were demonstrated in *NBS1* siRNA-transfected cells by the failure of IGF-1 to increase cell proliferation and expression of *CCND1*. Furthermore, the Ras/Raf/MEK/ERK signaling cascade was identified as a major pathway that is responsible for IGF-1-induced cell proliferation and cell cycle progression in RPE cells. The critical points for this conclusion are considered from the phosphorylation levels and nuclear translocations of ERK1/2, the induction of *FOS*, and the application of the MEK1/2-specific inhibitor (U0126).

The influences of *NBS1* on the IGF1-1 signaling cascade were proven by the findings that the expression of IGF1R, *SOS1*, and *SOS2* were suppressed in *NBS1* siRNA-transfected cells. As a consequence of the reduced IGF1R expression in *NBS1* siRNA-transfected cells, IGF-1 was unable to rescue the cells from radiation-induced cell death. Thus, diminished IGF-1 signaling contributes to enhanced radio-sensitivity of *NBS1*siRNA-transfected cells.

In conclusion, this study provides evidences that *NBS1* is involved in the promotion of cell proliferation, cell cycle progression, and cellular radio-resistance by influencing the IGF-1 signaling cascade.

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Erklärung

Hiermit erkläre ich,

dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskomission vorgelegt worden ist.

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