Functional investigation of the class II tumor suppressor gene *H-REV107-1*

Dissertation

zur Erlangung des akademischen Grades

Doctor rerum naturalium

(Dr. rer. nat.)
im Fach Biologie

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät I
der Humboldt-Universität zu Berlin
von

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geb. am 24. Januar 1975 in Kustanay, Kasachstan

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Tag der Einreichung: 11.06.03
Tag der mündlichen Prüfung: 16.09.03

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List of Abbreviations

A	Adenine
A (Ala)	Alanine
AD	Activation domain
AD fusion library	A cDNA library, constructed in an AD vector such that the protein encoded by the inserts are fused to the AD
AD library plasmid	Plasmid encoding a fusion of the AD and a library insert
AD protein H-REV107-1	A hybrid protein comprised of the AD fused to the H-REV107-1
APS	Ammonium persulphate
BSA	Bovine serum albumin
C	Cytosine
C (Cys)	Cysteine
D (Asp)	Aspartic acid
DABCO	1,4-Diazabicyclo (2,2,2)-octane
DAPI	4',6'-diamidino-2-phenylindole hydrochloride
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytosine Triphosphate
dGTP	Deoxyguanine Triphosphate
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNA-BD	DNA – binding domain
DNA-BD vector	Plasmid encoding the LexA protein (including the DNA-BD)
dNTP	Deoxyribonucleoside Triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymine Triphosphate
E (Glu)	Glutamic acid
G	Guanine
G (Gly)	Glycine
H (His)	Histidine

His^-, or Leu^-, or Trp^-, or Ura^-	Yeast colonies require His, or Leu, or Trp, or Ura in the medium to grow; is auxotrophic for one (or more) of these specific nutrients
I (Ile)	Isoleucine
IPTG	Isopropyl-l-thio-β-D-galactopyranoside
L (Leu)	Leucine
$LacZ^+$	Expressed the *lacZ* reporter gene; is positive for β-
LB	Luria-Bertani
Leu^+	Expressed the *LEU2* reporter gene; does not require Leu in the medium to grow
MBD	Membrane Binding Domain
N (Asn)	Asparagine
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMSF	Phenyl Methyl – Sulfonyl Fluoride
S (Ser)	Serine
SD induction medium	SD medium containing galactose and raffinose as the carbon source; used to induce expression of AD fusion proteins
SD medium	Minimal Synthetic Dropout Medium
SDS	Sodium Dodecyl Sulphate
T	Thymine
T (Thr)	Threonine
Tan	Annealing Temperature
TBST	Tris-Buffered Saline Tween-20
Tm	Melting Temperature
Ura	Uracil
W (Trp)	Tryptophan
X-gal	5-bromo, 4-chloro-3-indol-β-D-galactopyranisode
Y (Tyr)	Tyrosine

Zusammenfassung

Das Klasse II Tumorsuppressor-Gen H-REV107-1, ist in normalen Geweben ubiquitär exprimiert, zeigt jedoch häufig Expressionsverluste, vorzugsweise in Tumoren des Brustgewebes, des Ovars und der Lunge. Das H-REV107-1 Protein wirkt in vitro und in vivo als Wachstumssuppressor. Um den Mechanismus der H-REV107-1 bedingten Wachstumshemmung zu verstehen, haben wir mit Hilfe des LexA-basierten Hefe-2-Hybrid Systems interagierende Proteine identifiziert. Diese Suche wurde mit einem H-REV107-1 Deletionskonstrukt durchgeführt, dem die Membran-bindende Domäne fehlte. Diese Analyse lieferte eine Vielzahl von potentiellen Interaktionspartnern, darunter der Retinsäure Rezeptor RARG, das Calcium-bindende Proteine S100A6, der Translations-Elongationsfaktor ETF und das weitgehend unbekannte Protein p14.5 Die Bindungen des H-REV107-1 Proteins an die beiden potentiellen Kandidaten, den Transkriptionsfactor PC4 und die regulatorische Untereinheit der Protein Phosphatase 2A (PR65), wurden genauer untersucht.

Wir haben dabei einen Proteinkomplex aus H-REV107-1, PC4 und STAT1 (Signal Transducer and Activator of Transcription 1) identifiziert, der vermutlich eine Rolle in der IFNγ - abhängigen Wachstumshemmung in Ovarialkarzinom Zellen spielt. Da sich die Expression des H-REV107-1 Gens durch IFNγ über den Transkriptionsfaktor IRF-1 stimulieren läßt, und in verschiedenen Zelllinien sowohl zur Hemmung des Wachstums, als auch zur Apoptose führt, vermuteten wir verschiedene Mechanismen der Wachstumshemmung durch den IFNγ-Signalweg und H-REV107-1.

Weitere Analysen der H-REV107-1 – vermittelten Apoptose zeigten, daß die Interaktion zwischen H-REV107-1 und PR65 eine wichtige Rolle in diesem Prozeß spielt. Um die Proteindomäne zu identifizieren, welche für die direkte Wechselwirkung von H-REV107-1 mit PR65 verantwortlich ist, wurden H-REV107-1 Mutanten generiert und mittels Co-Immunpräzipitation getestet. Die Prolin-reiche Sequenz am N-Terminus des H-REV107-1 Proteins konnte als verantwortliche Domäne für die Interaktion bestimmt werden.

Die funktionelle Analyse dieser Interaktion zeigte die Hemmung der Protein Phosphatase 2A (PP2A) Aktivität in Ovarialkarzinom Zellen durch H-REV107-1. Der Einsatz der Mutanten im Phosphatase-Aktivitätstest zeigte, daß die selbe Domäne, die die Interaktion vermittelt, auch für die Hemmung der Phosphatase 2A verantwortlich ist. Diese Fakten deuteten auf eine wichtige Rolle der Phosphatase 2A in Ovarialkarzinom Zellen hin, weil sowohl die Verwendung des PP2A Inhibitors (Okadainsäure), als auch die Transfektion der Zellen mit einem H-REV107-1 - Expressionsplasmid zur Apoptose führten. Damit konnten wir zeigen, daß PP2A für das Überleben der Ovarialkarzinomzellen notwendig ist, und die Reaktivierung des H-REV107-1 Proteins durch IFNγ zur Hemmung der Phosphatase und damit zur Apoptose führt.

Schlagwörter: H-REV107-1, PP2A, Apoptose, IFNγ, Tumorsuppressor.

Abstract

The H-REV107-1 class II tumor suppressor gene is ubiquitously expressed in normal tissues and down-regulated in human breast, ovarian and lung tumours. H-REV107-1 has the capacity to suppress growth of tumour cells in vitro and in vivo. To understand the mechanism of H-REV107-1 mediated growth suppression I performed a search for H-REV107-1 interacting proteins using a LexA-based yeast two-hybrid system. I screened a human kidney cDNA library with a truncated form of the H-REV107-1 as a bait. This analysis revealed numerous potential interaction partners. Among them the retinoic acid receptor gamma (RARG), the calcium-binding protein S100A6, the translation termination factor ETF1, and the potential translational inhibitor protein P14.5.

The interaction of H-REV107-1 with the transcriptional co-activator PC4 and with the regulatory subunit A of protein phosphatase 2A (PR65) was analysed in detail. H-REV107-1 binds ectopically expressed and endogenous PC4. In addition, a multiprotein complex between H-REV107-1, PC4 and the signal transducer and activator of transcription 1 (STAT1) was demonstrated. This complex is likely to be involved in IFNγ-mediated growth suppression of ovarian carcinoma cells. Endogenous H-REV107-1 can be induced after application of IFNγ through the IRF-1 transcription factor. This up-regulation of H-REV107-1 leads either to growth suppression via a G1 arrest or to apoptosis depending on the cell line, suggesting different mechanisms of IFNγ-, and H-REV107-1- mediated growth suppression.

Further investigation of the mechanism of H-REV107-1-dependent apoptosis revealed an important role of the interaction between H-REV107-1 and the PR65 protein. The use of several H-REV107-1 mutant proteins generated after disruption of the highly conserved domains identified the proline-rich N-terminal domain responsible for the interaction with PR65 in Co-immunoprecipitation studies. Functional investigation of the H-REV107-1 – PR65 interaction demonstrated that wild-type H-REV107-1 is able to inhibit PP2A activity, however a mutant protein lacking the N-terminal domain was devoid of this function. We sought to identify the functional relevance of the PP2A activity in ovarian carcinoma cells with normally have suppressed the H-REV107-1 gene. Treatment of OVCAR-3 cells with the PP2A inhibitor Okadaic acid and transient transfection of the cells with wild-type H-REV107-1 resulted in the activation of caspase-9, suggesting a role for PP2A in the survival of ovarian carcinoma cells. We suggest, that the down-regulation of H-REV107-1 in ovarian carcinomas is a prerequisite for the PP2A-dependent activation of yet unknown signalling pathways mediating tumour cell survival. Reactivation of H-REV107-1 gene expression via IFNγ leads to the inhibition of PP2A activity and tumour cell death.

Keywords: H-REV107-1, PP2A, Apoptosis, IFNγ, Tumor Suppressor.

1 Introduction

1.1 Multi-Step Progression of Tumors

Experimental approaches, cytogenetic observations and molecular analysis have shown that tumors result from a subversion of diverse mechanisms controlling growth, division, and mortality of cells. Tumor development is generally considered as a multi-step process including consequent dysfunction of genes classified into three categories (Bishop, 1995):

1. proto-oncogenes, which are activated by mutations and become oncogenes. Their acquired oncogenic functions lead to uncontrolled cellular growth and proliferation.
2. tumor-suppressor genes, which normally negatively regulate cell growth and division preventing the development of tumor. Loss or mutational inactivation of these genes leads to the deregulation of cell cycle progression, and other intracellular processes resulting in cancer progression.
3. genes involved in maintaining the genomic stability and genes encoding the DNA-repair system. The “loss-of-function” mutations of these genes result in genetic instability characteristic for tumor cells.

1.1.1 Oncogenes

The oncogenes are genes that are capable of stimulating cellular growth. Their precursors (proto-oncogenes) are present in eukaryotic cells, and promote the normal growth and division of cells. Their oncogenic potential can be activated by one of the following mechanisms (Bishop, 1991). 1 - point mutation or chromosomal rearrangement resulting in an abnormal protein which has a different biological activity, for example, RasV12 (Satoh et al., 1992), and Bcr-Abl (Wang, 1988). 2 - gene amplification increasing the number of copies of a normal proto-oncogene within a cell leading to the activation of its oncogenic potential, like *MYCN* (Schwab, 1990). 3 - viral infection resulting in the control of a proto-oncogene by a more active viral promoter (Lipsick and Wang, 1999).

1.1.2 Tumor Suppressor Genes

The products of tumor suppressor genes normally negatively regulate cell growth (Schwab, 2001). Loss of one or several tumor suppressors is required for the full tumorigenic conversion of a normal cell. Re-expression of these genes in malignant cells leads to the restoration of growth regulation and the reversion of the transforming phenotype (Klein, 1998).

First category of these genes consists of known tumor suppressors down-regulated in transformed cells through chromosomal deletion, loss of heterozygosity (LOH), and mutagenesis. Positional cloning is a classical approach of molecular genetic to identify this kind of genes.

To analyse a difference between normal and tumor cells in more detail, new methods such as differential gene cloning (Lau and Nathans, 1985) or subtractive hybridisation (Scott et al., 1983), and improved second generation like differential display (Lang and Pardee, 1992) subtractive suppression hybridisation, SSH (Diatchenko et al., 1996) were developed. Analysis and comparison of the expression patterns of normal and tumorigenic cells using these methods revealed a new category of genes down-regulated but not mutated in tumor cells. This observation led to the postulation of two classes of the tumor suppressor genes (Sager, 1997).

class I tumor suppressor genes which inactivated by chromosomal rearrangements like a deletion or translocation, and by a mutation of one or both alleles (Hanahan and Weinberg, 2000).

class II tumor suppressor genes which are stably down-regulated but were not found to be mutated in significant subset of cancers and cancer cell lines (Sager, 1997).

1.1.2.1 The Class I Tumor Suppressor Genes

Since the first tumour suppressor was identified, more than 20 other genes have been shown to be mutated or deleted in tumours, inter alia pRB, p53, WT1, BRCA1, BRCA2, APC, NF1, and NF2 (McCormick, 2001; Schwab, 2001). The genes disrupted in a majority of human cancers are the retinoblastoma tumour suppressor gene (*RB1*), and the *TP53* gene. The breast cancer susceptibility genes 1 and 2 (BRCA1 and BRCA2) were demonstrated to play an important role in the heredity of ovarian and breast cancers (Beckmann et al., 1997).

The *RB1* gene was the first tumor suppressor gene to be isolated and cloned (Friend et al., 1988). The product of this gene, the retinoblastoma protein, pRB, is a nuclear phosphoprotein which mediates progression through the first phase of the cell cycle, playing a major role in the control of cell division and differentiation (Cordon-Cardo et al., 1994). Cytogenetic studies of chromosomal alterations in a childhood retinoblastoma, and in breast, lung and pancreatic cancers demonstrated a correlation between tumorigenesis and chromosomal aberrations on chromosome 13q14 where the *RB1* gene is located (Michalova et al., 1982). The inactivation of one of the *RB1* alleles by point mutation or deletion was demonstrated to be often accompanied by loss of heterozygosity (LOH) on chromosome 13 (Lee et al., 1988; Hesketh, 1997). In order to explain the nature of retinoblastoma formation, Knudson suggested the so called “two-hit hypothesis” (Knudson, 1971). He proposed that two inactivating mutations affecting both copies of a gene are necessary for retinoblastoma development. The first could be either a germline or somatic mutation, whereas the second mutation is always somatic.

This hypothesis illustrated how somatic and inherited mutations might collaborate in tumorigenesis, and also proposed that mutations of tumor suppressor genes have a recessive character, behave recursively at the cellular level.

The *TP53* tumor suppressor gene was demonstrated to carry homozygotic somatic alterations in roughly 50% of all human tumours. Mutations in the single copy *TP53* gene are the most frequent genetic changes yet shown in human cancers and occur in 70% of all tumors. Germline mutations of the gene have been shown to be associated with the Li-Fraumeni syndrome (Levine, 1997). Further investigations revealed that in contrast to the pRB protein and Knudsons "two-hit hypothesis" in some tumours the *TP53* gene carries mutations leading to cancer in a dominant negative fashion (Brachmann et al., 1996).

The *TP53* gene encodes a transcription factor activated in response to physical or chemical stress. The p53 protein controls induction of apoptosis, cell cycle progression into G1 and G2 phases, modulation of DNA replication and repair, preventing proliferation of cells with damaged genetic material. Overexpression of wild type TP53 in different cell types leads to growth inhibition (Casey et al., 1991), or to the induction of apoptosis in squamous carcinoma cell lines (Liu et al., 1995). The major down-stream p53 effector participating in the control of the cell cycle check-points is the cyclin dependent kinases inhibitor $p21^{WAF1}$, functioning as a tumour suppressor itself (el-Deiry et al., 1993; Sheikh et al., 1994). Other p53 effectors playing a critical role in apoptosis signalling are the death signalling receptor Apo-1/Fas (el-Deiry, 1998), the repressor of apoptosis Bcl-2, its inhibitor BAX-1 (Sheikh et al, 1994), and the death receptor DR5 (Burn et al., 2001). Several members of the DNA repair machinery, for example, auxiliary subunit of polymerase δ (PCNA), and replication protein A (RPA) were also described as p53 targets (Schwab, 2001).

1.1.2.2 The Class II Tumor Suppressor Genes

The class II of tumor suppressors is represented by genes which, unlike class I, are not mutated during tumorigenesis but rather have sustained a blockage of their expression through diverse mechanisms (Sager, 1997). Interestingly, that some genes exhibit features of class II tumor suppressors in one type of cancer, whereas in other type they are known to belong to the class I tumor suppressors. Thus, allelic loss of *IRF1* occurs frequently in the acute myeloid leukemia, myelodysplastic syndrome (Boultwood et al., 1993), and gastric cancer (Tamura et al., 1996), whereas in ovarian cancer IRF1 is described as a class II tumor suppressor (Sers et al., 2002). Dysfunction of maspin via mutation was identified in prostate cancer (Umekita et al., 1997), whereby down-regulation of this gene is characteristic for many other tumors where the gene is not mutated (Sager, 1990).

An important feature of the class II tumor suppressor genes is that their down-regulation is reversible. The normal genes are present, and their re-expression might be induced by drugs or other treatments, that makes such genes attractive targets for cancer therapy. It is known that inactivation of the expression takes place on the transcriptional and translational levels during cancer progression. But until now the mechanisms of the gene silencing have not yet been elucidated in much detail.

1.1.3 Mechanisms of Gene Silencing

1.1.3.1 DNA Methylation and Deacetylation

One of the important mechanisms of gene silencing is DNA methylation. The nonmethylated CpG islands within promoter regions were demonstrated to be primary targets for the aberrant hypermethylation in tumour cells (Bird, 1995). Loss of expression, associated with the hypermethylation of the promoter CpG islands, was shown for the *RB1* gene in 10% of the patients with the sporadic form of retinoblastoma (Greger et al., 1994). Methylation of *WT1* and calcitonin appears in 68-74% of the analysed colon carcinomas (Hiltunen et al., 1997). The $p21^{WAF1}$, *APC*, *p15/INK4B*, and *p16/INK4* genes were also described to be methylated in tumours (Cameron et al., 1999; Baldwin et al 2000; Roder et al., 2002). Interestingly, for several tumor suppressor genes like *BRCA1*, *RB1*, and *p16/INK4,* methylation was described as an additional mechanism of down-regulation in these types of cancer, where the genes are only rarely mutated (Garinis et al., 2002).

The de novo methylation has two consequences: first, it leads to the inhibition of transcription factors binding (Baylin et al., 1998). Second, methylation attracts other proteins that specifically bind the modified DNA. This blocks an access of the transcription factors required for gene expression to DNA (Bird, 1995), and induces a secondary DNA modification, histone deacetylation, resulting in DNA compaction. This process makes DNA less accessible for the transcriptional machinery (Rountree et al., 2000).

Reactivation of tumor suppressor gene expression often required both de-methylation, and inhibition of histone deacetylation, suggesting that these two processes act synergistically in gene silencing, and may both contribute to oncogenesis and cancer progression (Cameron et al., 1999).

1.1.3.2 Inhibition of Positive Regulators of Transcription

Inactivation of the class I tumor suppressors by mutations or deletions leads to a reduced expression of downstream target genes. The first evidences for such an indirect inactivation of tumour suppressors was described for the $p21^{WAF1}$ gene down-regulated after loss of p53 due to mutations in the *TP53* gene in a variety of human malignancies and cancer cells lines (Shiohara et al., 1994).

Further investigations demonstrated that the loss of p53 results in the down-regulation of many other tumor suppressors, such as the signaling regulator caveolin-1 (Bist et al., 2000), the angiogenesis inhibitor thrombospondin (Dameron et al., 1994), and serine protease inhibitor maspin (Zou et al., 2000). The tumor suppressor gene maspin was originally identified in normal breast epithelial cells (Zou et al., 1994). Further investigation demonstrated down-regulation of maspin in breast, prostate and colon tumors (Zou et al., 2000; Umekita et al., 1997). Investigation of maspin expression revealed a correlation between its down-regulation and p53 inactivation that led to a proposition that maspin might be a target of p53. The hypothesis was confirmed by the fact that over-expression of wild type p53 in prostate and breast cancer cells led to the rapid induction of the maspin expression via direct binding to the promoter sequence (Zou et al., 2000). Thus, tumor development includes the inactivation of class I tumor suppressors such as p53, followed by the down-regulation of their down-stream target genes, the class II tumor suppressors.

1.1.3.3 Inhibition of Expression by Activation of Oncogenic Signaling

Activated oncogenes might also suppress the transcription of negative growth regulators. Thus, the oncogene Myc, activated in a large number of human cancers (Adams and Cory, 1992), was demonstrated to act not only as a transcriptional trans-activator but also to mediate down-regulation of a variety of genes (Kato et al., 1990). The mechanism of a direct transcriptional repression by Myc is poorly understood, but resent data suggest that Myc mediates repression via negative interference with transcriptional coactivators. Thus, expression of the tumor suppressor *p15INK4b* is repressed by Myc through association with Miz-1 transcriptional factor (Staller et al., 2001). The alternative mechanism of Myc-mediated suppression of transcription is an association with transcriptional repressors and recruitment of histone deacetylases to the promoter region of target genes (Satou et al., 2001).

Oncogene Ras negatively regulates expression of a number of genes not via a direct inhibition of transcription but through the activation of its down-stream effectors, such as AP1 transcriptional factor. Activation of AP1 results in the negative regulation of various tumor suppressors, for example $p21^{WAF1}$ (Chang et al., 2002).

To identify genes, expression of which is suppressed via activation of the RAS-downstream signaling, a comparison of the expression patterns of the non-tumorigenic rat fibroblasts 208F and its malignant *HRAS*-transformed derivative, FE-8, was performed (Zuber et al., 2000). Stable down-regulation of a significant subset of class II tumor suppressors was identified. Expression of a fraction of these genes was recovered in the FE-8 cells after inhibition of the MEK1 kinase, a downstream effector of HRAS, via addition of PD 98059. Re-expression of cdc21 (*Mcmd4*), lysyl oxydase (*Lox*), *STAT5a,* and other genes was obtained (Zuber et al., 2000).

Down-regulation of lysyl oxidase in many human tumors has been reported, but mutation of the lysyl oxidase encoded gene (*Lox)* was described only in colorectal tumors, suggesting that lysyl oxidase belongs to the class II tumor suppressors (Csiszar et al., 2002).

Interestingly, inhibition of the lysyl oxidase expression in rat kidney fibroblasts led to the development of the transformed phenotype, activation of the *Ras* oncogene, anchorage independent growth, and tumorigenicity in nude mice (Giampuzzi et al., 2001). Resent investigations demonstrated that overexpression of lysyl oxidase inhibits Ras-mediated transformation by prevention of NF-kappa B activation, highlighting its particular role in controlling Ras activity (Jeay et al., 2003), and confirming its role as a class II tumor suppressor in the reversion of the malignant phenotype.

Rat *H-rev107* was identified as a gene down-regulated in *HRAS* transformed fibroblasts, and up-regulated in revertant and transformation-resistant fibroblasts (Hajnal et al., 1994). Further investigation demonstrated that H-rev107 possesses growth and tumor-inhibitory capacity and, therefore, belongs to the class II tumor suppressors (Sers et al., 1997). Interestingly, similar to the lysyl oxidase gene, down-regulation of *H-rev107* was found to be reversible. Recovery of its expression was obtained in KRAS transformed rat ovarian surface epithelial cells after inhibition of MAP/ERK signalling pathway (Sers et al., 2002). The human *H-REV107-1* gene and its related gene *H-REV107-2/TIG3/RIG1* were cloned several years ago (Husmann et al., 1998; Di Sepio et al., 1998). Both were demonstrated to possess transformation suppressive properties. H-REV107-1 was shown to be implicated in IFNγ signaling, and its expression was recovered in ovarian carcinoma cells after induction with IFNγ (Sers et al., 2002). H-REV107-2/TIG3/RIG1, originally isolated from retinoid-treated cultured epidermal keratinocytes, was demonstrated to participate in the retinoic acid signaling, and negatively-regulate c-Jun N-terminal kinase and p38 mitogen-activated kinase (Huang et al., 2002). In the meantime it is known that an H-REV107-like subfamily of proteins is exists, and consists of 5 members, which were demonstrated to be down-regulated in various human tumors. Their functions have not yet been elucidated in much details, but resent phylogenetic analysis of the NlpC/P60 protein hydrolases demonstrated that H-REV107-like proteins belong to this superfamily (Anantharaman and Aravid, 2003).

1.2 H-REV107-1 is a Member of the NlpC/P60 Protein Superfamily

1.2.1 The NlpC/P60 Protein Superfamily

The H-REV107-like proteins harbour the NlpC/P60 domain specific for bacterial peptidases. Phylogenetic analysis revealed a large superfamily of proteins related to the *E. coli* lipoprotein NlpC, and possessing the so called NlpC/P60 catalytic conservative domain, essential for the hydrolytic activity of these proteins (Anantharaman and Aravind, 2003).

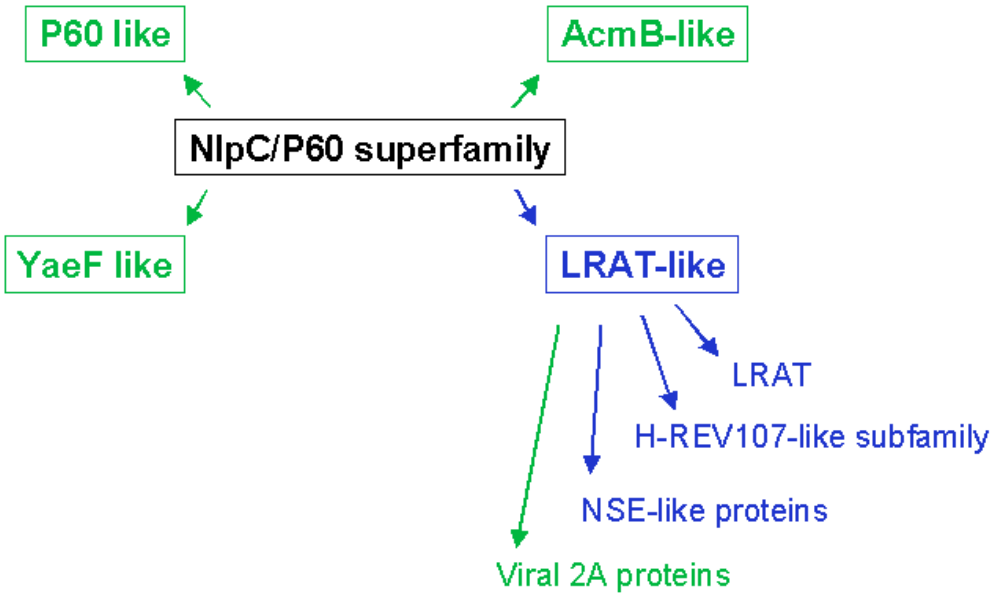

Fig. 1 Schematic presentation of the NlpC/P60 protein superfamily

The NlpC/P60 superfamily consists of four major protein families. Three of them are represented by bacterial and viral proteins (green letters): the p60-like family, the YaeF-like family, and the AcmB-like family. The fourth group, the LRAT-like protein family, contains eukaryotic (blue letters) and viral 2A proteins (green letter).

The superfamily encompasses four diverse groups of proteins: the P60-like family, the Acm/LytN-like family, the YaeF-like family, and the LRAT-like family (Anantharaman and Aravid; Fig. 1). The P60-like family was typified by the P60 protein of Listeria monocytogenes (Pilgrim et al., 2003), and includes bacterial peptidases with an extracellular location. The NlpC/P60 domain has been demonstrated to be essential for their catalytic activity (Pointing et al., 1999). The Acm/LytN-like family is a very divergent family of proteins typified by its two members, the putative peptidoglycan hydrolase, AcmB (Huard et al., 2003), and a novel cell-wall hydrolase LytN (Sugai et al., 1998). This family is represented by extracellular or membrane proteins functioning mostly as cell-wall hydrolases (Anantharaman and Aravind, 2003). The YaeF-like protein family is typified by the *E. coli* protein YaeF, and shows a peculiar phylogenetic distribution being present in bacteria and in poxviruses. A function of these proteins is not known, but a similarity with other members of the NlpC/P60 protein superfamily suggests that they might function as proteases (Anantharaman and Aravind, 2003).

The LRAT family was found only in eukaryotes and animal viruses. The lecithin retinol acyltransferase (LRAT) was identified on the basis of its enzymatic activity, the conversion of all-trans-retinol into retinyl esters, the storage form of retinol (Ruiz et al., 1999). The LRAT ortholog, Egl-26 in *C. elegans,* has been implicated in vulval development (Wendy and Han, 2002). Other members of the LRAT-like family belong to three subfamilies, the H-REV107-like subfamily containing several tumor suppressors, the subfamily of viral 2A proteins, and a novel, NSE-like protein subfamily (Fig. 1).

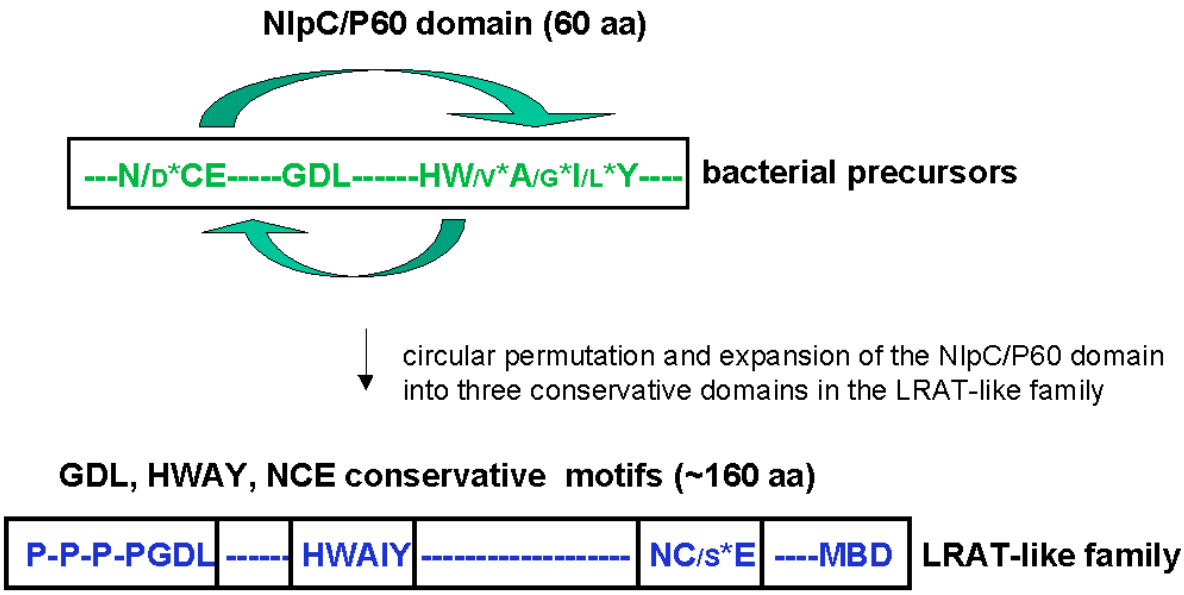

Fig. 2 Circular permutation of the NlpC/P60 conservative domain

The NlpC/P60 domain in the Acm/LytN-like and P60-like families has a length of about 60 aminoacids, and following order of the motifs: NCE, GDL, and HWAY. Circular permutation of the domain leads to the rearrangement of the conservative motifs, and distribution through the whole length of a protein. Thus in the LRAT-like proteins the GDL motif is the most amino-terminal, followed the HWAY, and then the NCE motif at the most C-terminus of the protein.

** alternative aminoacid residues presented in several members of the NlpC/P60 protein superfamily*

The NlpC/P60 domain, shared by all four protein families, contains three highly conservative motifs, the NCE, the GDL, and the HWAY motif (Fig. 2). Remarkably, the members of the P60-like, and Acm/LytN-like families have a canonical distribution of these motifs. Namely, the NlpC/P60 domain has a length of about 60 aminoacids, and the following order of the motifs: NCE, GDL, and HWAY. In contrast, a non-canonical distribution of these motifs was demonstrated in the LRAT-like protein family. A circular permutation of the NlpC/P60 domain, and distribution of the conservative motifs through the whole length of the proteins were revealed. The GDL motif became the most amino-terminal, followed the HWAY, and the NCE motifs (Fig. 2; Anantharaman and Aravind, 2003).

Such a circular permutation of the NlpC/P60 catalytic domain, leading to the rearrangement of the GDL motif to the N-terminus, and NCE motif to the C-terminus, supposes different biochemical activities of the members of the NlpC/P60 protein superfamily. It was hypothesised that the genes were acquired by the eukaryotes through lateral transfer of the bacterial precursor. During evolution, the genes underwent drastic changes resulting in a considerable divergence in biochemical functions between the eukaryotic proteins and the bacterial precursors (Anantharaman and Aravind, 2003).

Protein	Identity	Similarity
H-REV107-1/HRASLS3	100%	100%
HRASLS2 (HRAS like suppressor 2)	60%	82%
H-REV107-2/RIG1 (Retinoid inducible gene 1)	51%	66%
HRLP5 (H-rev107 like protein 5)	51%	64%
HRASLS (HRAS like suppressor)	46%	64%
NSE2	31%	46%
Similar to NSE1	31%	46%
NSE1	23%	42%
LRAT (lecithin retinol acyltransferase)	25%	44%

Table 1 Human proteins belonging to the LRAT-like family of proteins

NCBI BLAST search revealed 9 human proteins with a high homology to the H-REV107-1. The first 5 proteins complete the H-REV107-like subfamily, 3 NSE proteins belong to the novel NSE protein subfamily. All non-redundant GenBank CDS and translations+PDB+SwissProt+PIR+PRF databases were used for the search.

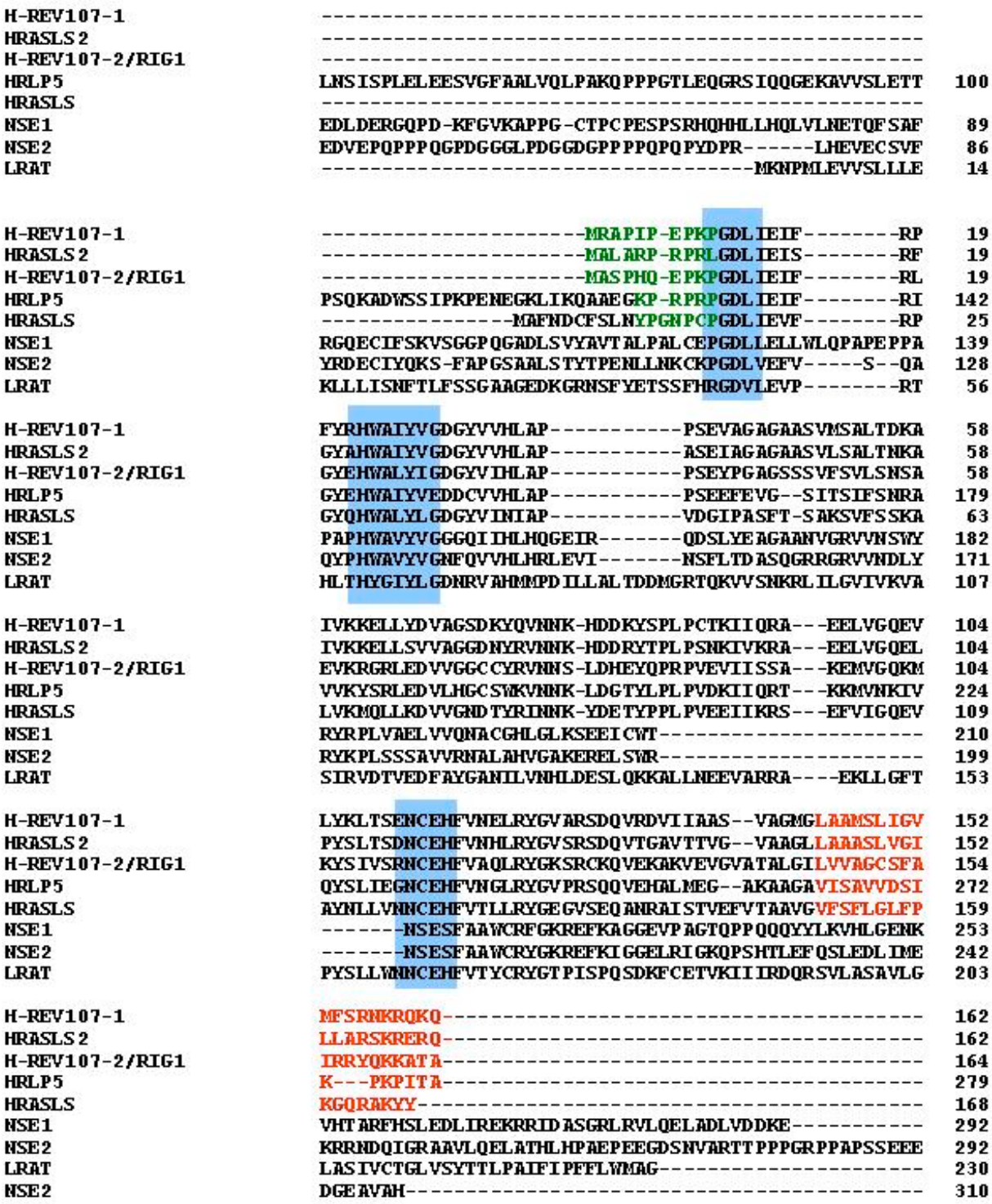
H-REV107-1
HRASLS2
H-REV107-2/RIG1
HRLP5
HRASLS
NSE1
NSE2
LRAT

Fig. 3 Aminoacid sequence alignment of the nine human proteins belong to the LRAT-like protein family

Conserved GDL, HWAY, and NCE motifs found in all NlpC/P60 proteins are shown in blue boxes. A transmembrane domain predicted in several proteins indicated in red letters. Proline-rich sequences characteristic for several members of the family only are indicated in green.

1.2.2 The LRAT-Like Protein Family

The LRAT-like family consists of eukaryotic proteins, and 2A non-structural proteins of picorna, Aichi, and avian encephalomyelitis (AEV) viruses (Hughes and Stanway, 2000). The function of the viral proteins is not completely elucidated. Several 2A non-structural proteins are trypsin-like or cystein proteases involved in polyprotein processing (Ryan and Flint, 1997), the role of others is unclear.

The human members of the LRAT-like family include 9 homologous proteins: LRAT, 5 proteins belonging to the H-REV107-like subfamily, and 3 proteins forming a novel NSE subfamily (Table 1). The result of an alignment of members of the H-REV107-1, the NSE subfamilies, and LRAT is depicted in the Figure 3.

Thus, proteins of the LRAT-like family share four highly conservative motifs: GDL, HWAY, NCE, and a transmembrane domain at the C-terminus. Additionally, several members contain a prolin-rich region at the N-terminus (Fig. 3, green letters). The function of this region is unknown, although such motifs might be important for protein-protein binding (Kay et al., 2000).

The best-characterised member of the LRAT-like family is the lecithin retinol acyltransferase (LRAT). It is an essential enzyme in vitamin A metabolism mediating the conversion of retinol into retinyl ester (Ruiz et al., 1999). The enzyme is found in those tissues known to be involved in the processing and mobilisation of vitamin A, including the retinal pigment epithelium, the liver, and the intestine. It has been demonstrated that a conserved Cys residue within the NCE motif is essential for LRAT catalysis. The nucleophilic Cys residue reacts with lecithin and becomes acetylated to generate a thiolacyl enzyme intermediate. This fatty acyl fraction reacts then with the vitamin A, and generates retinyl esters (Mondal et al., 2000). Further investigation demonstrated that in addition to the Cys, two His residues, distinguished from the His residues in the HWAY motif are important for the catalysis of LRAT (Mondal et al., 2002).

The NSE subfamily is a novel subfamily of proteins. These proteins have one aminoacid exchange in the NCE domain: Cys to Ser, resulting in an NSE domain. The NSE2 protein was identified as a protein associated with the plasma membrane in tumor-derived breast cancer cell lines using a proteomics tool. Its potential role in cancer was predicted because of its unique cancer expression profile and identified protein-binding partners which were demonstrated to be implicated in breast cancer tumorigenesis (Adam et al., 2003).

The members of the H-REV107 subfamily are rather poorly characterised, with the exception of the H-REV107-1 and H-REV107-2/TIG3/RIG1 proteins. The HRASLS mouse homologue, *Ac1*, has been cloned by differential display comparing two mouse cell lines: embryonic fibroblast C3H10T1/2 and chondrogenic ATDC5. The gene is expressed in skeletal muscle, heart, brain, and bone marrow in adult mice. It was demonstrated to posses growth inhibitory capacity, and to revert the phenotype of HRAS transformed NIH3T3 cells, proposing that Ac1 can modulate HRAS-mediated signalling pathways (Akiyama et al., 1999).

The *H-REV107-2/TIG3/RIG1* is an *H-REV107-1* homologous gene (Husmann et al., 1998) which has been identified as a retinoid-responsive gene in primary human keratinocytes (Di Sepio et al., 1998), and as a novel retinoid-inducible gene 1 in human gastric cancer cells (Huang et al., 2000). H-REV107-2/TIG3/RIG1 is a class II tumor suppressor acting as a growth regulator that mediates some of the growth suppressive effects of retinoids. Analysis of truncated forms of this protein demonstrated that the C-terminal hydrophobic domain (Fig. 3) has an important role in determining the intracellular localisation. Both the amino- and carboxy-terminal regions of H-REV107-2/TIG3/RIG1 are required for optimal growth suppression of cells (Deucher et al., 2000). Recently it has been demonstrated that the H-REV107-2/TIG3/RIG1 protein induces apoptosis by negatively regulating extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated kinase (Huang et al., 2002).

Similar to H-REV107-2/TIG3/RIG1, H-REV107-1 belongs to the class II tumor suppressors, and acts as a negative growth modulator (Husmann et al., 1998) by contributing to IFNγ-dependent growth arrest and apoptosis in ovarian carcinoma cells (Sers et al., 2002).

1.3 Purpose of this Work

The H-REV107-1 has been identified as a gene down-regulated in RAS-transformed cells (Hajnal et al., 1994). And was shown to suppress cellular growth (Hajnal et al., 1994; Sers et al., 1997). At the begin of this analysis the H-REV107-1 sequence did not provide any clue to the mechanism of its action. No related proteins were found in the databases at this time.

In view of its down-regulation in tumors and tumor cell lines, and its functioning as a growth suppressor, it was decided to study the mechanism of the H-REV107-1 – mediated anti-proliferative effect. To better understand the mechanism of H-REV107-1 cellular function, I performed a yeast two hybrid screening which resulted in the identification of a number of potential interacting partners.

Interaction with these candidates was tested in COS-7 cells using co-immunoprecipitation. A further intention was to determine the H-REV107-1 protein domains responsible for protein-protein interaction. Most importantly, I aimed to define a role for the identified protein-protein interactions in the H-REV107-1 mediated growth suppression and apoptosis.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used in this work were purchased from Calbiochem, CA, USA; J.T. Baker, Deventer, Holland; Merck, Darmstadt, Germany; R&D Systems Inc., MN, USA; Serva Electrophoresis GmbH, Heidelberg, Germany; Sigma-Aldrich Chemie GmbH, Munich, Germany; Sigma, MS, USA.

MiliQ 18 Ω water was used in all procedures if required.

2.1.2 Kits

QIAprep Spin Miniprep	Qiagen GmbH, Hilden, Germany
Annexin-V-FLUOS Staining Kit	Roche Diagnostics GmbH, Mannheim, Germany
Anti-HA Sepharose conjugate	Sigma, MS, USA
Apoptosis Sampler Kit	Cell Signaling Technology, Inc., MA, USA
Bcl-2 Family Antibody Sampler Kit	Cell Signaling Technology, Inc., MA, USA
Developer RP X-OMAT EX	Eastman Kodak Company, NY, USA
ECL Western Blotting Detection Kit	Amersham Biosciences GmbH, Freiburg, Germany
Fast-Link™ DNA Ligation and Screening Kit	Biozym Diagnostik GmbH, Oldendorf, Germany
Fixer RP X-OMAT LO	Eastman Kodak Company, NY, USA
Immunoprecipitation Kit (Protein G)	Roche Diagnostics GmbH, Mannheim, Germany
PhosphoPlus Stat1 Antibody Kit	Cell Signaling Technology, Inc., MA, USA
Protease inhibitors Cocktail	Roche Diagnostics GmbH, Mannheim, Germany
QIAGEN Plasmid Midi and Maxi	Qiagen GmbH, Hilden, Germany
QuikChange Mutagenesis Kit	Stratagene, CA, USA
RediPack GST Purification Module	Pharmacia Biotech Inc., CA, USA
SequaGel XR	National diagnostics, GE, USA
SequiTherm EXCEL™ II DNA Sequencing Kit	Biozym Diagnostik GmbH, Oldendorf, Germany
Ser/Thr Phosphatase Assay Kit 1	Upstate Biotechnology, NY, USA
Western Blot Recycling Kit	Alpha Diagnostic International, TX, USA

2.1.3 Enzymes

Lyticase	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Ampli Taq DNA Polymerase	Perkin Elmer, MA, USA
Ampli Taq Gold DNA Polymerase	Perkin Elmer, MA, USA
Restriction Endonucleases: EcoRI, BamHI, HindIII, XbaI,	Promega, Mannheim, Germany

2.1.4 Antibodies

Anti-BRCA1 (H-1000)	Santa Cruz Biotechnology, Inc., CA, USA
Anti-cleaved caspase-3	Cell Signaling Technology, Inc., MA, USA
Anti-cleaved caspase-9	Cell Signaling Technology, Inc., MA, USA
Anti-GST antibody	Cell Signaling Technology, Inc., MA, USA
Anti-HA	Sigma, MS, USA
Anti-HA Sepharose Conjugate	Sigma, MS, USA
Anti-Histone 3	Cell Signaling Technology, Inc., MA, USA
Anti-H-rev107, rat (08B6)	C. Sers, Charité, Berlin, Germany; (Sers et al., 1997)
Anti-H-REV107-1 (315)	C. Sers, Charité, Berlin, Germany; (Sers et al., 2002)
Anti-IRF1	Santa Cruz Biotechnology, CA, USA
Anti-IRF2	Santa Cruz Biotechnology, CA, USA
Anti-p14.5,	G. Schmitz, University of Regensburg, Germany (Schmiedeknecht et al., 1996)
Anti-p21	Santa Cruz Biotechnology, CA, USA
Anti-pan-actin	Chemicon, CA, USA
Anti-PC4	R. Heilbronn, Free University Berlin, Germany (Weger et al., 1999)
Anti-PR65 (6F9)	Covance Research Products Inc., CA, USA
Anti-STAT1	Cell Signaling Technology, Inc., MA, USA
Anti-V5	Invitrogen, CA, USA
peroxidase-conjugate goat anti-mouse	Dianova, Hamburg, Germany
peroxidase-conjugate goat anti-rabbit	Cell Signaling Technology, Inc., MA, USA
peroxidase-conjugate goat anti-rabbit	Dianova, Hamburg, Germany

2.1.5 Fluorophore-Labelled Antibodies

AlexaFluor 488 fragment of goat anti-mouse IgG (H+L)	MoBiTec, Göttingen, Germany
AlexaFluor 594 fragment of goat anti-mouse IgG (H+L)	MoBiTec, Göttingen, Germany
AlexaFluor 546 fragment of goat anti-rabbit IgG (H+L)	MoBiTec, Göttingen, Germany
AlexaFluor 594 fragment of goat anti-rabbit IgG (H+L)	MoBiTec, Göttingen, Germany

2.1.6 cDNA Library

Pre-made human kidney LexA cDNA library cloned into the pJG4-5 vector containing AD, and carrying the yeast *TRP1* transformation marker for selection in Trp^- yeast (Clontech, San Diego, TX, USA).

2.1.7 Mammalian Cell Lines

A27/80	Human Ovarian carcinoma cell line, European Cell Culture Collection
COS-7	African green monkey kidney fibroblasts, American Type Culture
FE-8	HRAS-transformed derivative of the immortalised non-tumorigenic rat fibroblasts 208F (Griegel et al., 1986)
FE-8 H-rev107	FE-8 cells harbouring *H-rev107* cDNA under tetracycline-inducible promoter (Sers et al., 1997)
FE-8 pUHD	FE-8 cells harbouring an empty vector containing tetracycline-inducible promoter (Sers et al., 1997)
OVCAR-3	Human Ovarian carcinoma cell line, American Type Culture Collection

2.1.8 E. coli *Strains*

KC8	*pyrF, leuB600, trpC, hisB463*	BD Biosciences, Clontech, CA, USA
XL2-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 suoE44 relA1 lac* [F' proAB $lacI^qZ\Delta M15$ Tn10(Tet^r) Amy Cam^r]^a	Stratagene, La Jolla, Canada
Sure 2	*e14$^-$ (McrA$^-$)Δ(mcrCB-hsdSMR-mrr)171 endA1 supE44 gyrA96 thi-1 hsdR17 relA1 lac recB recJ*	Stratagene, La Jolla, Canada
B21	E. coli B F^- *dem omp T hsdS(r_B-m_B-) gal*	Pharmacia Biotech Inc., CA, USA

2.1.9 Yeast Strains (OriGene Technologies, Inc., MD, USA)

EGY48	MATα *trp1 his3 ura3 leu*2::6 LexAop-LEU2 (high sensitivity)
RFY206	MATa *trp1Δ::hisG his3Δ200 ura3-52 lys2Δ201 leu2-3* (mating strain)

2.1.10 Plasmids and Expression Constructs

ΔC107-ΔN	The expression construct was generated by PCR-amplification of a 375-bp fragment of ΔCH-REV107-1HA, using the ΔN-fw and ΔN-rv primers, followed by insertion of the PCR-product into the BamHI sites of a pcDNA3.1 plasmid
ΔC107-HWAY	The expression construct was generated using the QuikChange Mutagenesis Kit from the ΔCH-REV107-1HA expression vector, with the hway-fw and hway-rv primers
ΔC107-NCE	The expression construct was generated using the QuikChange Mutagenesis Kit from the ΔCH-REV107-1HA expression vector, with the nce-fw and nce-rv primers
ΔCH-REV107-1HA	The ΔCH-REV107-1HA fragment was created by PCR-amplification of 454-bp of *H-REV107-1* cDNA including 49 bp of 5'-untranslated region, and 405 bp of a coding region. The 107HA-fw, and 107HA-rv reverse primer containing sequence encoding HA-epitope were used. The amplified fragment was cloned into the BamHI and XbaI sites of a pcDNA3.1 plasmid
107-GST	The ΔCH-REV107-1 fragment was obtained from H-REV107-1 expression vector using PCR-amplification with 107-TH-fw and 107-TH-rv primers. PCR product was cloned into the BamHI sites of a pGE-2TK plasmid
BRCA1	Full length *BRCA1* cDNA cloned into pcDNA3.1 expression vector was kindly provided by T. Ouchi, The Mount Sinai School of Medicine, NY, USA
EGFP (enhanced green fluorescent protein)	Clontech, San Diego, TX, USA
H-REV107-1 full length expression vector	pcDNA3.1 expression plasmid contains the complete open reading frame, 62 bp of 5'-untranslated region and 174 bp of 3' untranslated region of the *H-REV107-1* cDNA (Husmann et al., 1998)

H-REV107-1V5	The expression construct was generated by PCR-amplification of the full length *H-REV107-1* cDNA using 107-HA-fw and 107-rv primers. The amplified fragment of 415 bp was ligated into the BamHI sites of a pEF6/V5 vector
pEF6/V5	Invitrogen, CA, USA
p8op-lacZ, *URA3*, Amp^R	Clontech, San Diego, TX, USA
PC4-V5	The PC4 expression vector containing *PC4* full length cDNA fused with V5 epitope, was purchased from GeneStorm Collection (Invitrogen, CA, USA)
PcDNA3.1	Invitrogen, CA, USA
ETF1-HA	The *ETF1* full length cDNA was PCR-amplified from the yeast expression cDNA library, using pJG-Hindfw and BCO3-Hindrv primers. The fragment was ligated into the HindIII sites of a pcDNA3.1 expression plasmid
p14.5	The *p14.5* full length cDNA was PCR-amplified from the yeast expression cDNA library, using pJG-Hindfw and BCO3-Hindrv primers. The fragment was ligated into the HindIII sites of a pcDNA3.1 expression plasmid
PcDNA3.1/GS	GeneStorm Collection (Invitrogen, CA, USA)
pEG202, *HIS3*, Amp^R	OriGene Technologies Inc, MD, USA
pEG202-107	ΔCH-REV107-1 yeast expression plasmid containing 405 bp of the human H-REV107-1 cDNA with a deletion of 81 bp encoding a C-terminal membrane binding domain. The H-REV107-1 fragment was generated by PCR-amplification of H-REV107-1 cDNA using 107-TH-fw and 107-TH-rv primers, and cloned into the BamHI sites of a pEG202 plasmid
pGE-2TK	Pharmacia Biotech Inc., CA, USA
pJG4-5, *TRP1*, Amp^R	OriGene Technologies Inc, MD, USA
PR65-V5	The PR65-expression vector containing full length PR65α-encoded cDNA fused with V5 epitope, was purchased from GeneStorm Collection (Invitrogen, CA, USA)

RARG-V5	The RARG-expression vector containing full length of RARG cDNA fused with V5 epitope was purchased from GeneStorm Collection (Invitrogen, CA, USA)
S100A6HA	The *S100A6* full length cDNA was PCR-amplified from the yeast expression cDNA library, using pJG-Bamfw and BCO3-Xbarv primers. The fragment was ligated into the BamHI and XbaI sites of a pcDNA3.1 expression plasmid
STAT1	The STAT1-expression vector was kindly provided by S. Vinkemeier, Institute of Molecular Pharmacology, Berlin, Germany

2.1.11 Oligonucleotides (MWG-Biotech, Ebersberg, Germany)

Oligonucl.	Labelling	Sequence
ΔN-fw		5' – CGG GAT CCC GAA GAT GGG AGA CC GAT TGA GAT TTT TCG – 3'
ΔN-rv		5' – CGG GAT CCC GTT AGG CAT AAT CAG GGA CGT CAT AAG G – 3'
107HA-fw		5' – CGG GAT CCA TGC GTG CGC CCA TTC CAG AG – 3'
107-HA-rv		5' – TTA GGC ATA ATC AGG GAC GTC ATA AGG ATA AGG ATA GAT GAT GAC ATC TCT GAC CTG G – 3'
107-rv		5' – CGG GAT CCT TAG ATG ATG ACA TCT CTG ACC TGG – 3'
BCO3	IRD800	5' – GTC AAG TCT CCA ATC AAG GTT – 3'
BCO3-Hindrv		5' – TCC GAA GTC AAG TCT CCA ATC AAG GTT – 3'
BCO3-Xbarv		5' – AGA TCT GTC AAG TCT CCA ATC AAG GTT – 3'
BCO5	IRD800	5' – TAA CGA TAC CAG CCT CTT GC – 3'
BCO5-5	IRD800	5' – CGA GGA GTG CAA TGC – 3'
hway-fw		5' – CGC CCT TTC TAC AGA GCC TGG GCC GCC TAT GTT GTT GGC GAT GG – 3'
hway-rv		5' – CCA TCG CCA ACA TAG GCG GCC CAG GCT CTG TAG AAA GGG CG – 3'

nce-fw		5’ – CCA GTG AGA ACA GCG AGC ACT TTG TGA ATG AGC – 3’
nce-rv		5’ – GCT CAT TCA CAA AGT GCT CGC TGT TCT CAC TGG – 3`
pEF6-rv		5’ – CTA GAA GGC ACA GTC GAG GC – 3’
pGE-fw	IRD800	5’ – GGG CTG GCA AGC CAC GTT TGG TG – 3’
pGE-rv	IRD800	5’ – CCG GGA GCT GCA TGT GTC AGA GG – 3’
pJG-Bamfw		5’ – CGG GAT CCG AAG ATG GTC TAC CCT TAT GAT GTG CC – 3’
pJG-Hindfw		5’ – AAG CTT GCC ACC ATG GTC TAC CCT TAT GAT GTG CCA G – 3’
Sp6	IRD800	5’ – CGA TTT AGG TGA CAC TAT AG – 3’
T3	IRD800	5’ – AAT TAA CCC TCA CTA AAG GG – 3’
T7	IRD800	5’ – TAA TAC GAC TCA CTA TAG GG – 3’

2.2 Methods

2.2.1 Yeast Two-Hybrid System

2.2.1.1 Yeast Expression Vectors and General Procedure

To identify H-REV107-1 interacting proteins, a LexA-based Yeast Two-Hybrid system was used. We screened a human kidney cDNA library with a truncated form of the H-REV107-1 protein. For this purpose, a pEG202-107 expression vector was generated. We subcloned 405 bp of human *H-REV107-1* cDNA, encoding the H-REV107-1 protein without 27 C-terminal aminoacids, into the pEG202 yeast expression vector in frame with the DNA-binding domain (DNA-BD). The pEG202 vector carries a yeast *HIS3* marker for selection on HIS^- medium. The DNA-BD was provided by the prokaryotic LexA protein, which normally functions as a suppressor of *SOS* genes in *E.coli* when it binds to LexA operators (Ebina et al., 1993). A premade cDNA library, purchased from Clontech, contained inserts cloned into the pJG4-5 vector. This vector carries a yeast *TRP1* transformation marker for selection on the TRP^- medium and a transcriptional activation domain under the control of a *GAL1* promoter. The transcriptional activation domain (AD) was an 88-residue acidic *E.coli* peptide (B42) (Ma and Ptashne, 1987). To activate transcription from the *GAL1* promoter, transformants must be grown in medium containing galactose (Gal) and raffinose (Raf) as the carbon source. Interaction between a target library-encoded protein fused with the AD, and H-REV107-1 fused with the DNA-BD resulted in the reconstitution of a novel transcriptional activator with binding affinity for LexA operators (Gyuris et al., 1993). Two reporter genes with up-stream LexA operators, the *LEU2*, integrated in the EGY48 genome, and the lacZ, located on the p8op-lacZ reporter plasmid, made an interaction phenotypically detectable. If the proteins did not interact with each other, the reporter genes were not transcribed (Fig. 4).

The EGY48 yeast host strain was first transformed with the p8op-lacZ reporter plasmid carrying the *lacZ* reporter gene and stored in SD/-Ura (Table 2) medium/25% glycerol for further experiments. These transformants were then used for the co-transformation with pEG202-107 and pJG4-5 library plasmids. After library transformation, cells were plated on a minimal synthetic dropout (SD) non-induction medium that selected for both pEG202-107 and the AD/library plasmid, but not for the interaction directly, to maximise plasmid copy number in each cell. After this step colonies were plated on the SD induction medium lacking leucine and containing X-gal for detecting protein-protein interactions (Fig. 5). Individual blue colonies were isolated by restreaking on the same medium and stored as master plates.

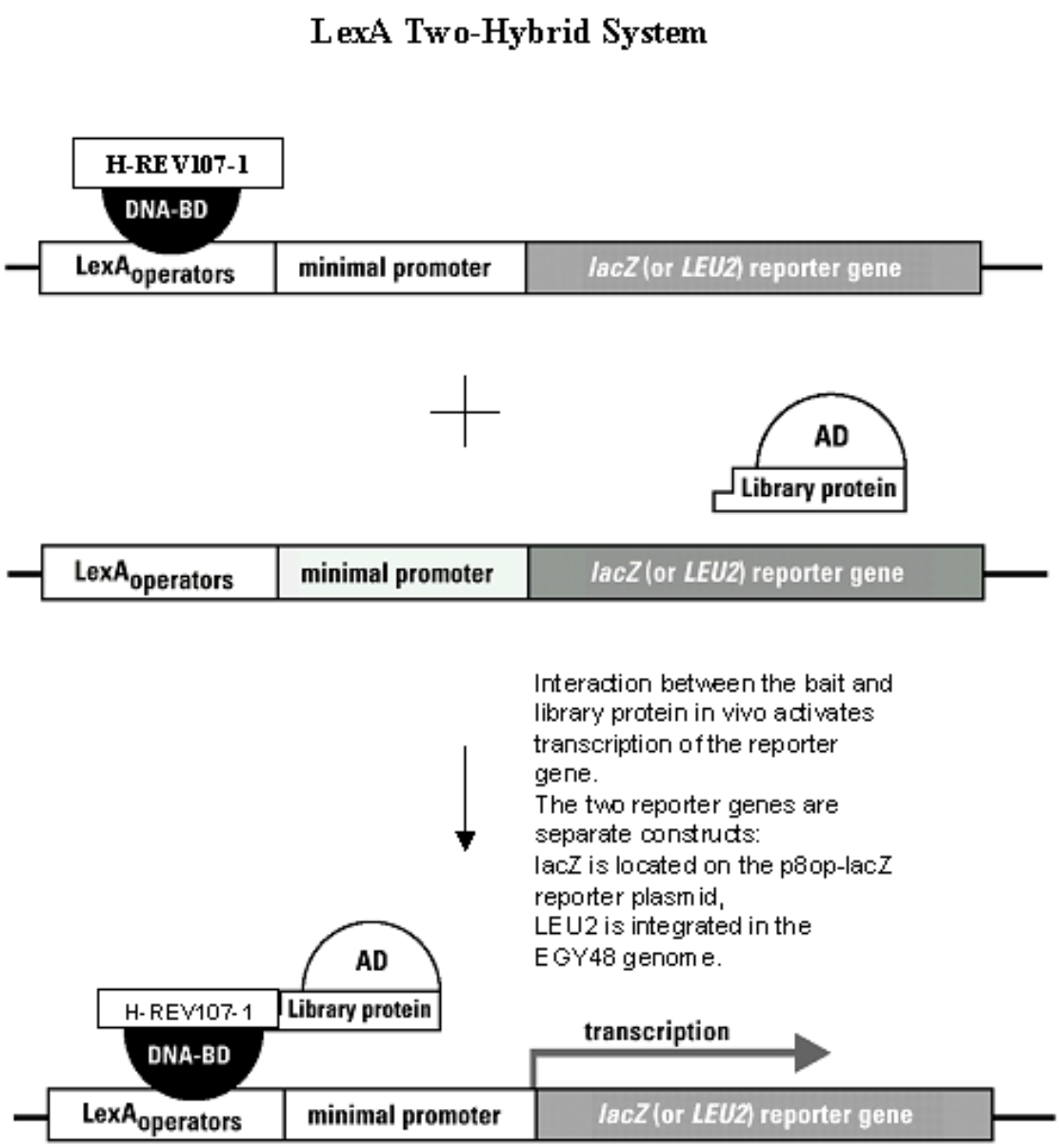

Fig. 4 Schematic diagram of the LexA Two-Hybrid System (BD Biosciences, Clontech, CA, USA)

The bait, the H-REV107-1 protein fused to the DNA-BD, and the pray, library proteins fused to the AD cannot activate transcription of the reporter genes by themselves. The Interaction between the bait and a pray leads to the close proximity of the binding and activation domains, and activation of transcription of the reporter genes.

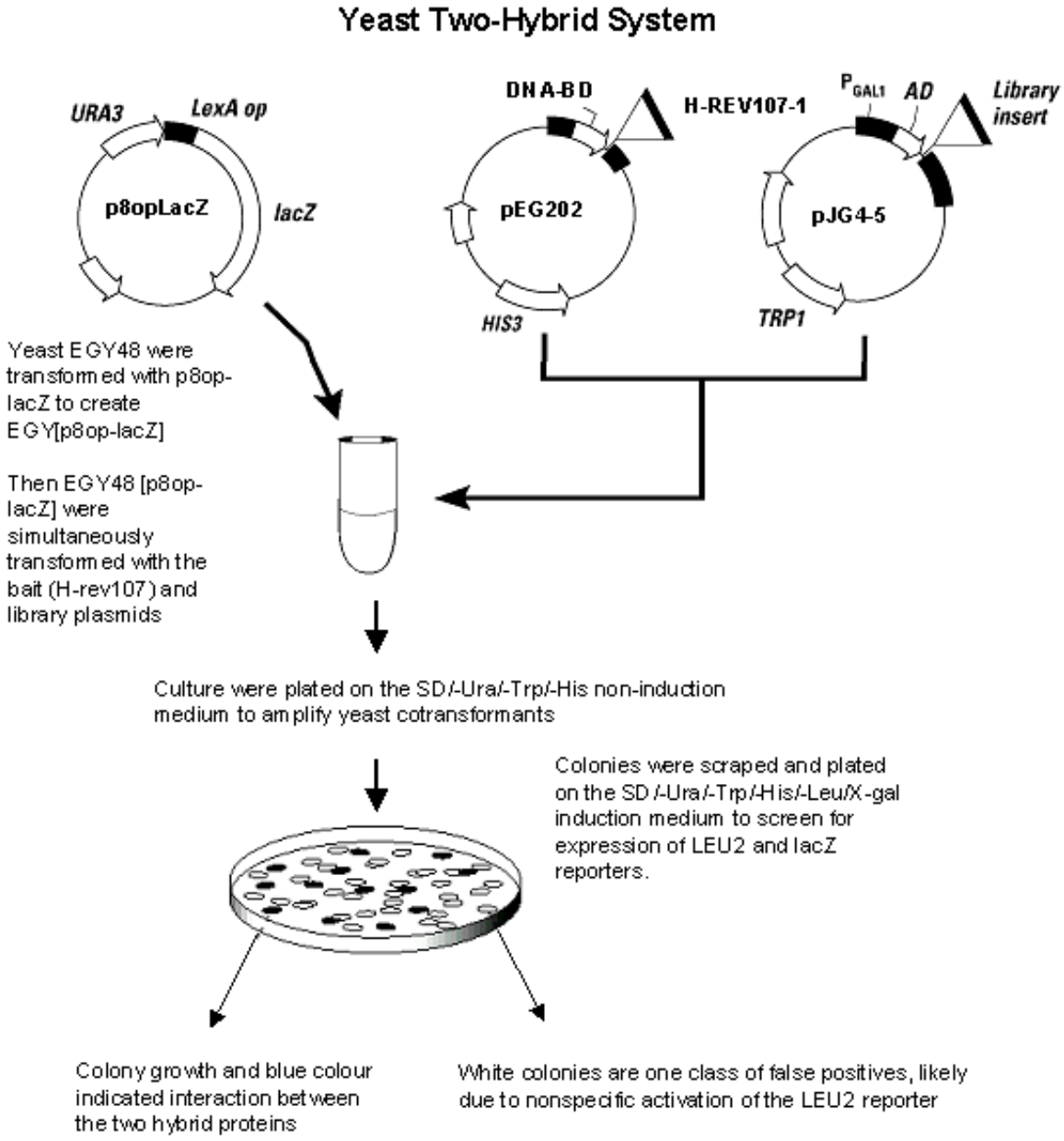

Fig. 5 Screening of a AD fusion library for proteins that interact with H-REV107-1 (BD Biosciences, Clontech, CA, USA)

Table 2 Generated yeast strains and respective selective media

Plasmids used for the transformation of the yeast strain EGY48	**Media used to select transformants**
p8op-lacZ	SD/-Ura
pEG202-107	SD/-His
pJG4-5	SD/-Trp
pEG202-107, pJG4-5, p8op-lacZ	SD/-His/-Trp/-Ura
	Media used to activate reporter genes
EGY48[pEG202-107, pJG4-5, p8op-lacZ]	SD/-His/-Leu/-Trp/-Ura to activate the *LEU2* reporter gene
EGY48[pEG202-107, pJG4-5, p8op-lacZ]	SD/Gal/Raf/-His/-Trp/-Ura/X-gal to activate the *LacZ* reporter gene
EGY48[pEG202-107, pJG4-5, p8op-lacZ]	SD/Gal/Raf/-His/-Leu/-Trp/-Ura/X-gal to activate both the *LEU2* and *lacZ* reporter genes

2.2.1.2 Yeast Strain Storage and Culturing

2.2.1.2.1 Storage

To prepare a yeast glycerol stock, a single colony was scraped from the agar plate. Then the cells were resuspended in 200–500 μl of YPD medium (or the appropriate SD medium) in a 1.5-ml microcentrifuge tube. After dispersing the cells by vortexing, sterile 50% glycerol was added to a final concentration of 25%. The vials were frozen at –70°C. Transformed yeast strains were stored in the appropriate SD dropout medium to keep selective pressure on the plasmid.

To recover frozen yeast, a small portion of the frozen glycerol stock was streaked onto a YPD (or appropriate SD) agar plate. The plate was incubated at 30°C until yeast colonies reach ~2 mm in diameter (this took 3–5 days). These colonies were used as a working stock. The plates were sealed with parafilm and stored at 4°C for up to two months.

2.2.1.2.2 Culturing

Fresh (<2-months old) colonies from the working stock plate were used. One large (2–3-mm diameter) colony or several small colonies were inoculated in 5 ml of medium, and vigorously vortexed for ~1 min to disperse the cells. The Suspension was incubated at 30°C for 16–18 hr with shaking at 230–270 rpm. This yielded a stationary phase culture (OD_{600} > 1.5). To obtain a mid-log phase culture, the overnight culture was transferred into fresh medium and incubated at 30°C for 3–5 hr with shaking (230–250 rpm) to produce an OD_{600} = 0.2–0.3.

2.2.1.3 Yeast Transformation

2.2.1.3.1 Preparation of Fresh Competent Yeast

Several colonies, 2–3 mm in diameter were inoculated in 1 ml of YPD or SD, and vortexed vigorously for 5 min to disperse any clumps. Then the cells were transferred into a flask containing 50 ml of YPD or the appropriate SD medium and incubated at 30°C for 16–18 hr with shaking at 250 rpm to yield a stationary phase culture (OD_{600}>1.5). 30 ml of this overnight culture were transferred to a flask containing 300 ml of YPD and incubated at 30°C for 3 hr with shaking (230 rpm) until the OD_{600} reached 0.4–0.6.

Cells were placed in 50-ml tubes and centrifuged at 1,000 x g for 5 min at room temperature (20–21°C). Cell pellets were resuspended in H_2O, then pooled into one tube (final volume 25–50 ml) and Centrifuged again at 1,000 x g for 5 min at room temperature. The cell pellet was resuspended in 1.5 ml of freshly prepared, sterile 1X TE/1X LiAc.

2.2.1.3.2 Transformation

For small scale transformation 0.1 μg of p8op-lacZ plasmid DNA and 0.1 mg of salmon testes carrier DNA were added to a fresh 1.5-ml tube and mixed.

For simultaneous co-transformation of pEG202-107 and library plasmids a large scale transformation was performed. The following amounts of DNA were used: 50 μg of pEG202-107 expression vector, 25 μg of library plasmid, and 2 mg of Salmon testes carrier DNA.

0.1 ml of yeast (1 ml for a large scale transformation) competent cells was added to each tube containing plasmid and salmon testes DNA and mixed well by vortexing. Then 0.6 ml of sterile PEG/LiAc solution (6 ml for a large scale transformation) were added to each tube and vortexed at high speed for 10 sec to mix, and incubated at 30°C for 30 min with shaking at 200 rpm. After the addition of 70 μl of DMSO (700 μl for a large scale transformation) the culture was mixed by gentle inversion.

A heat shock was performed for 15 min in a 42°C water bath and afterwards cells were chilled on ice for 1–2 min. Cells were centrifuged for 5 sec at 14,000 x g at room temperature (5 min 1000 x g for a large scale transformation). The cell pellets were resuspended in 0.5 ml of sterile 1X TE buffer (5 ml for a large scale transformation). 100 μl of the suspension (volume plated in large scale transformation) were than plated on SD agar plates 10 x 10 cm (15 x 15 cm for large scale transformation), that selected for the desired transformants.

For a small-scale transformation with the p8op[lacZ] plasmid only, the transformation was spread on the SD/-Ura plates. For large scale transformation the yeast suspension was spread on the SD/-His/-Trp/-Ura plates. Additionally, 100 μl of a 1:1000, 1:100, and 1:10 dilution were plated on 10 x 10 cm SD agar plates.

These plates were used as controls for transformation efficiency. Plates were incubated up-side-down, at 30°C until colonies appeared (2–4 days). To calculate the co-transformation efficiency, the colonies (cfu) growing on the dilution plate were counted (optimal 30-300 colonies on the plate with the dilution 1:100).

cfu x total suspension vol. (μl) / Vol. plated (μl) x dilution factor x amount of used DNA (μg)* = cfu/ /μg DNA

Sample calculation:

100 colonies grew on the 1:100 dilution plate (dilution factor = 0.01);

plating volume: 100 μl ; resuspension volume = 0.5 ml; amount of limiting plasmid = 0.1 μg

100 cfu x 0.5 ml x 10_3 μl/ml / (100 μl x 0.01 x 0.1 μg) = 5 x 10^5 cfu/μg

If a small scale transformation was performed, for example to create an EGY48[p8op-lacZ] yeast strain, the largest colonies grown after transformation were picked and restreaked on the same selection medium for master plates. Then they were sealed with parafilm and stored at 4°C for 3–4 weeks. Alternatively they were stored at -70°C in SD/-Ura/25% glycerol.

If large scale transformation was performed the transformants were harvested as follows:

Plates were placed at 4°C for 3-4 hours to harden. Then 1 ml TE buffer was added to the surface of each plate. Colonies were scraped into the liquid using a sterile Pasteur pipette. All liquids were combined into a single sterile 50-ml tube and vortexed to resuspend the cells.

A Glycerol stock of the amplified yeast library was created by adding an equal volume of sterile 65% glycerol/$MgSO_4$ solution. This stock can be stored at 4°C for one week or at -70°C up to 1 year.

2.2.1.4 β-Galactosidase Assay

To screen transformants for expression of a lacZ reporter, β-galactosidase assay was performed. We used two different methods: an in vivo assay and a colony-lift filter assay.

2.2.1.4.1 In Vivo Plate Assay Using X-gal – Containing Medium

Colonies from the master plates were replica plated on selection medium containing X-gal and BU salts, and incubated at 30°C for 4 days. Plates were checked every 12 hr (up to 96 hr) for the development of blue colour.

2.2.1.4.2 Colony-Lift Filter Assay

In this assay fresh colonies (i.e., grown at 30°C for 2–4 days), 1–3 mm in diameter were used. A sterile Whatman filter was placed onto the surface of the plate, and gently rubbed, to improve attachment of the colonies to the filter. Three holes were poked through the filter in an asymmetric way to mark the orientation of the filter on the agar plate. When the filter was evenly wetted, it was carefully lifted off the agar plate with forceps and transferred (colonies facing up) to a pool of liquid nitrogen. Using the forceps, the filter was completely submerged for 10 sec. After the filter was frozen completely (~10 sec), it was removed from the liquid nitrogen and left to thaw at room temperature.

For each plate of transformants to be assayed, a fresh sterile Whatman filter was pre-soaked by placing it in 2.5–5 ml of Z buffer/X-gal solution in a clean 15 x 15 cm plate. (Avoid trapping air bubbles under or between the filters).

Then the first filter was carefully placed, colony side up, onto the pre-soaked filter to allow the X-Gal solution to get into contact with the colonies. The appearance of blue colonies was checked periodically.

The ß-galactosidase-producing colonies were identified by aligning the filter to the agar plate using the orientation marks. Corresponding positive colonies were picked from the original plates to fresh medium and incubated for 1–2 days to re-grow the colony.

2.2.1.5 Secondary Test of Positives Colonies

Positive colonies were re-tested at least once on the SD/-His/-Trp/-Ura plates. After incubation at 30°C for 4-6 days, colonies were replica plated on the SD/Gal/Raf/-His/-Leu/-Trp/-Ura induction medium to verify that they maintained the correct phenotype. Restreaked and re-tested colonies were collected on SD/-His/-Trp/-Ura plates, incubated at 30°C for 4-6 days. After colonies have grown, the plates were sealed with Parafilm and stored at 4°C for up to 4 weeks. For long term storage, a glycerol stock was prepared.

Recipes for Solutions and Buffers

YPD (rich medium), pH 5.8

20 g/l	Tryptone
10 g/l	Yeast extract
20 g/l	Agar (for plates only)
20 g/l	Glucose

SD-ura-his-leu-trp (selective medium)

6.7 g/l	Yeast nitrogen base w/o amino acids
20 g/l	Agar (for plates only)
0.6 g/l	-his-ura-trp-leu dropout mix
20 g/l	Galactose
20 g/l	Raffinose
20 g/l	Agar (for plates only)
80 mg/l	X-Gal
100 ml/l	10 x BU salts

All media were sterilised by autoclaving 120°C 20 min. The galactose, raffinose, X-Gal, and 10 x BU salts solutions were filter sterilised and added after autoclaving.

10 x BU salts (100 ml), pH 7.0

7 g	Sodium phosphate (dibasic)
3 g	Sodium phosphate (monobasic)

To prepare other SD selective media, for example lacking one of the aminoacid, following stock solutions were used:

Trp	10 ml of 4 mg/ml stock per litter of medium (0,04 mg/ml final concentration)
Ura	5 ml of 4 mg/ml stock per litter of medium (0,02 mg/ml final concentration)
Leu	15 ml 4 mg/ml stock per litter of medium (0,06 mg/ml final concentration)
His	5 ml of 4 mg/ml stock per litter of medium (0,02 mg/ml final concentration)

Salmon testes carrier DNA

Salmon testes carrier DNA (sodium salt) was dissolved in water (10 mg/ml), and the solution was stirred on a magnetic stirrer for 2-4 hours at the room temperature. Then the solution was extracted with phenol and with phenol : chloroform (1:1). The aqua phase was transferred into a new tube and sheared by passing 12 times rapidly through a 17-gauge hypodermic needle. The DNA was precipitated by adding 2 volumes of ice-cold ethanol.

DNA was recovered by centrifugation, and re-dissolved at a concentration of 10 mg/ml in water, boiled and stored in small aliquots at –20°C. Just before use, the solution was heated for 5 minutes in a boiling water bath and quickly chilled on ice.

PEG/LiAc solution

	Final Conc.	10 ml solution
PEG 4000	40%	8 ml 50% PEG
TE buffer	1X	1 ml 10X TE
LiAc	1X	1 ml of 10X LiAc

Z buffer (pH 7.0), sterilised by autoclaving

16.1 g/l	Sodium phosphate (dibasic)
5.50 g/l	Sodium phosphate (monobasic)
0.75 g/l	Potassium chloride
0.25 g/l	Magnesium sulphate

Stock solutions:

50% PEG3350 prepared with H_2O

10 x TE: 0,1M Tris-HCl, 10 mM EDTA, pH 7.5, autoclaved

10 x LiAc: 1M lithium acetate (pH 7.5 , adjusted with acetic acid), autoclaved

65% glycerol/MgSO4 solution (sterilised by autoclaving)

	Final Conc.
Glycerol	65% v/v
$MgSO_4$	100 mM
Tris-HCl, pH 8.0	25 mM

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was dissolved in DMF (N,N-dimethyformamide) at a concentration of 20 mg/ml and stored in the dark at – 20°C.

2.2.1.6 Mating Test

To test positive clones isolated in a library screening for interaction with H-REV107-1 a, mating assay was performed. Two yeast strains of opposite mating type, EGY48 – MATa, and RFY206 – MATα were used. Mating occurred when the haploid cells of opposite mating type came into a contact and fused into a diploid yeast strain.

The EGY48 cells were transformed with the p8opLacZ plasmid and with either the pJG4-5 vector without insert or with the pJG4-5 vector containing candidate inserts chosen after sequencing analysis. Transformants were selected on the SD/-Trp/-Ura plates. The RFY206 cells were transformed with pEG202 or pEG202-107. Transformants were selected on the SD/-His plates. Then the two strains were mixed on SD/-His/-Trp/-Ura/-Leu/X-gal plates, where they formed diploid cells in which H-REV107-1 and one of the potential interacting partners have the opportunity to interact and to activate the reporter genes.

For each candidate of the library plasmid the follow matings were done:

Plasmid in EGY48	**Plasmid in RFY206**	**LacZ phenotype for a true positive**	**Leu2 phenotype for a true positive**
pJG4-5 without insert	pEG202 without insert	White	no growth
pJG4-5 with library insert	pEG202 without insert	white	no growth
pJG4-5 with library insert	pEG202-107	Blue	growth of colonies
pJG4-5 with insert	pEG202-107	White	no growth

Mating procedure:

One colony (2-3 mm) of each type used for the mating analysis was picked and placed together in a 1.5-ml microcentrifuge tube containing 0.5 ml of YPD medium, vortexed and incubated at 30°C with shaking at 250 rpm overnight. An aliquot (20μl) of the mating culture was spread on 100-mm, SD/-His/-Trp/-Ura plates and incubated at 30°C for 3-5 days to allow diploid colonies to form visible colonies. Then the diploid transformants were replica plated on the SD/Gal/Raf/-His/-Leu/-Trp/-Ura induction plates to assay for *LEU2* an *LacZ* expression. True positives were those library clones which exhibited activation of the reporter gene expression only in presence of pEG202-107 plasmid.

2.2.1.7 Yeast Plasmid Isolation

A large (2-4 mm) fresh (2-4-day old) yeast colony was inoculated into 0,5 ml of the appropriate SD liquid medium and vortexed vigorously to resuspend the cells. The culture was incubated at 30°C overnight with shaking at 230-250 rpm. Then cells were spun down by centrifugation at 14,000 x g for 5 min, the supernatant was poured off and pellets were resuspend in the residual liquid (total volume ~ 50 μl) by vortexing or pipetting up and down.

10 μl of lyticase (5 units/μl in TE buffer) was added to each tube, and cells were thoroughly resuspended by vortexing or pipetting up and down. Tubes were incubated at 37°C for 30-60 min with shaking at 200-250 rpm. Then 10 μl of 20% SDS was added to each tube, and vortexed for 1 min to mix. All samples were incubated for 15 min at –20°C, than thawed and vortexed again to ensure complete lysis of the cells. (If necessary, samples were stored frozen at –20°C). The volume of each sample was brought up to 200 μl in TE buffer (pH 7.0).

Then 200μl of phenol : chloroform : isoamyl alcohol (25:24:1) was added and vortexed for 5 min. After centrifugation at 14,000 x g for 10 min, the upper phase was transferred to a fresh tube, 8 μl of 10 M ammonium acetate and 500 μl of 95-100% Ethanol were added. The solution was mixed carefully and placed at –70°C for 1 hour. Afterwards the tubes were centrifuged at 14,000 x g for 10 min. The supernatant was discarded, the pellet was dried and resuspend in 20 μl of H_2O. This solution was used for transformation into competent E.coli KC8.

2.2.1.8 Preparation of Electrocompetent E. coli KC8

To prepare electrocompetent E. coli KC8 cells 1l of a 2x YT medium was inoculated with 100 ml of a fresh overnight culture, and incubated at 37°C with shaking until the OD_{600} reached 0.5–0.7. Then the flask was chilled for 15-30 min on ice, and centrifuged at 3,000 x g for 10 min in a pre-chilled rotor. The cells were washed in half of the original volume of sterile ice cold 1 mM Hepes pH 7.0, then spun down by centrifugation at 3,000 x g for 10 min in a pre-chilled rotor, and washed a second time with 50 ml of sterile ice cold water. The cells were centrifuged, and the pellet was resuspended in 2 ml of sterile ice cold water. The prepared cells were used directly for electroporation.

2.2.1.9 Transformation of the Electrocompetent E. coli KC8 with Yeast Plasmids

To transform E. coli KC8 with yeast plasmids, 40 μl of electrocompetent cells were mixed with 3 μl of yeast plasmid DNA, and transferred into a pre-chilled micro-electroporation chamber. For electroshock the following conditions were used: 1700 Volts, 200 Ohms, pulse control units were set to 25 μF. Directly after the shock, 1 ml of freshly prepared SOC medium was added to every sample and incubated with shaking for 1 hour at 37°C. Then 200 μl bacterial culture from every transformation were plated on M9 Agar plates to select transformants containing a yeast library plasmid.

<u>2 x YT medium</u>

16 g/l bacto-tryptone
10 g/l bacto-yeast extract
5 g/l NaCl
pH was adjusted to 7.0 and sterilised by autoclaving

<u>SOC medium</u>

20 g/l bacto-trypton
5 g/l bacto-yeast extract
0.58 g/l NaCl
0.19 g/l KCl
pH was adjusted to 7.0 with NaOH, and sterilised by autoclaving. Then 1 ml of 2 M $Mg2^+$ stock and 1 ml of 2 M Glucose was added to 98 ml of autoclaved medium.

Stock solutions

2 M $Mg2^+$ stock (for 100 ml solution):

20.33 g $MgCl_2$ $6H_2O$
24.65 g $MgSO_4$ $7H_2O$
H_2O was added to 100 ml and autoclaved.

2 M Glucose : 36.04 g of glucose was diluted in 100 ml of H_2O and filter sterilised.

M9 minimal medium

200 ml 5 x M9 salts
2 ml 1M $MgSO_4$
20 ml 20% glucose
0.1 ml 1M $CaCl_2$
20 g/l agarose (for plates only)

The medium was sterilised by autoclaving for 15 min at 120°C, and cooled down to 50°C. Then the following components were added per 1 l of the M9 medium:

1 ml ampicillin 50 mg/ml
1 ml thiamine-HCl solution
100 ml 10 x /-Trp supplement dropout (SD)

5 x M9 salts stock solution

64 g/l NaH_2PO_4 $7H_2O$
15 g/l KH_2PO_4
2.5 g/l NaCl
5.0 g/l NH_4Cl

2.2.1.10 Yeast Protein Isolation

For each clone to be assayed 5 ml of overnight culture were prepared in the appropriate SD selection medium. As a negative control untransformed yeast was cultured in 5 ml of YPD medium. The overnight cultures were inoculated in 50 ml of YPD medium and incubated at 30°C with shaking at 250 rpm until OD_{600} reached 0.4-0.6 (4-8 hours).

Each culture was chilled by pouring it into a pre-chilled 100-ml tube halfway filled with ice, and centrifuged for 5 min at 1000 x g by 4°C. The supernatant was poured off, and pellets were resuspend in 50 ml of ice-cold water. Cells were again centrifuged for 5 min at 1000 x g by 4°C, and frozen immediately by placing them in liquid nitrogen or dry ice. The cells where either be stored at -70°C, or used directly for further preparation.

The cell pellets were thawed and resuspend in 100 µl of pre-warmed (60°C) cracking buffer. Because PMSF degrades rapidly, every 7 minutes 1 µl of 100 mM PMSF was added to each sample. The suspensions were transferred into 1,5-ml microcentrifuge tubes containing 80 µl of glass beads. Then the samples were heated for 10 min at 70°C, vortexed vigorously for 1 min, and centrifuged for 5 min at 14,000 x g by 4°C.

Supernatants were saved on ice (supernatant I), the pellets were incubated for 3-5 min at 100°C, vortexed, and centrifuged again. The supernatant II was combined with supernatant I of each sample. Proteins were boiled for 1 minute and immediately loaded on a SDS-PAGE. Alternatively proteins were stored at -70°C.

PMSF 100 mM stock solution

0.17 g of PMSF was dissolved in 10 ml isopropanol

Protease inhibitor solution (688 µl were prepared freshly before use)

Inhibitor	Stock solution	Final concentration
Pepstatin A	66 µl of 1 mg/ml stock solution	0.1 mg/ml
Leupeptin	2 µl of 10,5 mM stock solution	0.03 mM
Benzamidine	500 µl of 200 mM stock solution	145 mM
Aprotinin	120 µl of 2.1 mg/ml stock solution	0.37 mg/ml

Cracking buffer stock solution (100 ml)

48 g	Urea	8M
5 g	SDS	5% v/v
40 mg	Bromphenol blue	0.4 mg/ml
4 ml	1M Tris-HCl, pH 6.8	40 mM
20 µl	0.5 M EDTA	0.1 mM

Cracking buffer complete (prepared before use)

1 ml	Cracking buffer stock solution
10 µl	β-mercaptoethanol
70 µl	Protease inhibitor solution
50 µl	PMSF stock solution

2.2.2 Bacterial Culture

2.2.2.1 Routine Culturing and Storage Conditions

The *E. coli* cultures were routinely grown at 37°C on Luria-Bertani (LB) agar or in LB broth, containing Ampicillin (75 µg/ml), Kanamycin (30 µg/ml), or Zeocine (25 µg/ml).

For long-term storage, a fresh overnight culture was prepared: bacteria were streaked onto an agar plate of the respective selective medium, and incubated at 37°C overnight. Several colonies were transferred separately into a 10 ml? Falcon tube containing 2 ml liquid media, and incubated for 14-16 hours at 37°C with shaking at 180-200 rpm. Then 800 µl of bacterial culture were transferred into the storage tube and sterile glycerol was added to a final concentration of 20%. The vials were snap-frozen in liquid nitrogen, and stored at –70°C until use. To recover a bacteria culture, a small proportion of the frozen glycerol stock was streaked onto an LB agar plate, containing the appropriate antibiotic.

The plate was incubated overnight at 37°C. These colonies were used as a working stock. The plates were sealed with parafilm and stored at 4°C for up to four weeks. Alternatively a small portion of the frozen glycerol stock was transferred in 2 ml of LB broth containing the appropriate antibiotic, and incubated overnight at 37°C with shaking at 180-200 rpm.

LB Broth (Agar)

10 g/l	NaCl
10 g/l	Tryptone
5 g/l	yeast extract
20 g/l	agar (for plates only)

pH was adjusted to 7.0 with NaOH and H_2O was added to a final volume of 1 litter.

To prepare agar plates, the LB agar was cooled to 50°C, appropriate antibiotics were added and petri dishes were poured (~15 ml/100-mm plate). If a β-galactosidase assay was done, LB agar plates were prepared with 80 µg/ml X-gal (5-bromo, 4-chloro-3-indol-β-D-galactopyranisode), and 20 mM IPTG (isopropyl-l-thio-β-D-galactopyranoside). IPTG was prepared in sterile water, and X-gal in dimethylformamide (DMF).

2.2.2.2 Transformation

Competent E. *coli* XL2-blue or Sure2 were purchased from Stratagene. Transformation was performed according to the protocol supplied by the manufacturer. Competent cells were thawed on ice and mixed gently. For each transformation, 100 µl of the cells were transferred into a microcentrifuge tube and 2 µl of β-mercaptoethanol were added. After incubation on ice for 10 min, 10 ng of plasmid DNA were added, swirled gently and incubated on ice for 30 min.

NZY^+ broth was pre-heated to a 42°C. Tubes with bacteria were heat-pulsed in a 42°C water bath for 30 seconds. Then the tubes were incubated on ice for 2 min, 900 μl of the pre-heated NZY^+ broth were added to every tube and incubated at 37°C for 1 hour with shaking at 225-250 rpm. 100 μl of each transformation mixture was plated on the appropriate plates using a sterile spreader and incubated overnight at 37°C.

2.2.2.3 Mini-Preparation of Plasmid DNA

For mini-preparation of plasmid DNA from bacteria a QIAprep Spin Miniprep Kit from Qiagen was used. For isolation of ~10 μg of plasmid DNA, 2 ml overnight culture of *E. coli* in LB medium was prepared. Plasmid DNA was isolated according to the manufacturer's recommendations. DNA was eluted in 30 µl of the EB buffer (10 mM Tris-HCl, pH 8.5) and stored at -20°C.

2.2.2.4 Large-Scale Preparation of Plasmid DNA

For a large-scale plasmid isolation a QIAGEN Plasmid Midi and Maxi Kit was used. Plasmid DNA was isolated according to the supplier's manual, a 200 ml *E.coli* overnight culture was prepared for each experiment. An additional purification step was included in the protocol. The pellet, resuspended in 400 µl of EB buffer was precipitated with 0,7 volumes of 4 M NaAc and 2,5 volumes of ethanol, and incubated for 1 hour at –70°C.

Then the solution was centrifuged at 14,000 x g for 30 min at 4°C, the pellet was washed with 1 ml ice-cold 70% ethanol, and centrifuged a second time at 14,000 x g for 5 min at 4°C, dried and resuspend in 200 µl of EB buffer.

2.2.2.5 Measurement of DNA Concentration

Concentration of DNA was measured using a UV-Spectrophotometer (Hitachi) by the wave length of 260 nm. The Amount of DNA was calculated regarding that OD_{260} = 1 corresponds to 50 μg/ml double-stranded DNA.

2.2.3 Enzymatic Manipulation and Analysis of DNA

2.2.3.1 Digestion of DNA with Restriction Endonucleases

Digestion of DNA with restriction endonucleases was performed according to the recommendations of the manufacturer. For the digestion of 5-10 µg of DNA, 10U of enzyme were added with an appropriate buffer to the DNA in a final volume of 15 ul. Reactions were incubated for 2 hours if PCR-fragments were digested, or 4-6 hours if plasmid DNA was digested.

2.2.3.2 DNA Ligation

For the ligation of cDNA fragments into plasmid vectors, a Fast-Link DNA Ligation and Screening Kit purchased from Biozym was used. Experiments were performed according to a protocol by the supplier. For ligations, 200 ng of a digested plasmid DNA were used. The amount of insert was calculated according to a molar ration 3:1 (insert : vector). The reactions were incubated for 1 hour at room temperature. To inactivate the *Fast-Link* Ligase the reaction was transferred to 70°C for 15 min. To determine the efficiency of ligation, 5 µl of the reaction mix were run on an agarose gel and visualised with ethidium bromide. If the ligation was successful, 1-5 µl of the reaction mix was used for transformation of *E.coli*.

2.2.3.3 Vector Dephosphorylation

Prior to ligation of a digested plasmid DNA with an insert, the protruding 5'-end of the vector was dephosphorylated using a HK Thermolabile Phosphatase (Biozym). The reaction was performed according to the recommendations of the supplier. For dephosphorylation of 10 µg plasmid DNA 10 units of the phosphatase were used.

Digestion proceeded for 2 hours at 30°C, then the phosphatase was deactivated at 65°C for 15 min.

2.2.3.4 Polymerase Chain Reaction (PCR)

The PCR reactions were performed in a PCR Thermal Cycler (Techne). Amplification of DNA was performed according to the following protocol:

Template DNA	20,0 ng
10 x PCR buffer (including 15 mM $MgCl_2$)	2.50 µl
dNTP (each 10 nM)	0.25 µl
5'- and 3' – oligonucleotide primers	1.00 µl (each)
H_20 filled up to	24.0 µl
Ampli*Taq* Gold (1U)	0.25 µl

The Following program was used to amplify inserts:

Activation of the AmpliTaq polymerase	95°C	12 min	
Denaturation of double stranded DNA	95°C	30 sec	30 cycles
Annealing of oligonucleotides to the template DNA	Tan	30 sec	
Elongation	72°C	60 sec	
Extension	72°C	5 min	
Chilling	4°C	1 hour up to overnight.	

Annealing temperatures were calculated using further formula: Tan = Tm - 4°C, where Tm is the melting temperature of the primers used for amplification . Tm of each oligonucleotide primer was calculated: Tm = 4 x (G + C) + 2 x (A + T), where G – guanine, C – cytosine, T – thymine, A – adenine.

2.2.3.5 Purification of PCR-Amplified Fragments of DNA

For purification of PCR-amplified DNA fragments a QIAquick PCR Purification kit from Qiagen was used. The purification was performed according to the supplier's protocol. Purified PCR fragments were controlled using agarose gel electrophoresis.

2.2.3.6 Site-Directed Mutagenesis

To generate the ΔC107-NCE and ΔC107-HWAY mutants of the H-REV107-1 protein, *in vitro* site-directed mutagenesis was performed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). The basic procedure utilised the ΔCH-REV107-1HA-expression plasmid and two oligonucleotide primers (sense and antisense) harbouring the desired mutations.

The primers were annealed to the denatured template DNA (ΔCH-REV107-1HA-expression plasmid) and subsequently extended during temperature cycling using a *PfuTurbo* DNA polymerase. The incorporation of the primer sequence resulted in the generation of a single stranded, mutated plasmid containing a staggered nick. To digest the parental DNA template, the *Dpn I* endonuclease, specific for methylated and hemimethylated DNA, was used. Afterwards, the doublestranded plasmid containing the mutated base pairs was transformed into competent bacteria and amplified.

Two mutated H-REV107-1 proteins were generated. The first contained the His/Ala exchange at position 23 (the ΔC107-HWAY mutant), the second contained the Cys/Ser exchange at position 112 (the ΔC107-NCE mutant). For each mutagenesis a primer pair (sense and antisense) of 30-35 bp was used. For the His/Ala exchange, primer pair 1 contained mutated nucleotides G and C (wild type C and A, respectively). For the Cys/Ser exchange, primer pair 2 contained mutated nucleotide A (wild type T).

1sense (His/Ala) 5' – CCT TTC TAC AGA **G**$_{/C}$**C**$_{/A}$C TGG GCC ATC TAT GTT GGC – 3'

1antisense (His/Ala) 5' – GCC AAC ATA GAT GGC CCA G**G**$_{/T}$**C**$_{/G}$ TCT GTA GAA AGG –3'

2sense (Cys/Ser)5' – CCA GTG AGA AC**A**$_{/T}$ GCG AGC ACT TTG TGA ATG AGC – 3'

2antisense (Cys/Ser) 5' – GCT CAT TCA CAA AGT GCT CGC **T**$_{/A}$GT TCT CAC TGG – 3'

The reaction was set the following way

5 μl – 10 x reaction buffer
1 μl – template DNA (10 ng)
1 μl – oligonucleotide primer 1ps/2ps (125 μg)
1 μl – oligonucleotide primer 1ns/2ns (125 μg)
1 μl – dNTP mix
H_20 filled to 50 μl; Then 1 μl of *PfuTurbo* DNA polymerase (2.5 U/μl) was added.

Cycling parameters:

Activation of the *PfuTurbo* DNA polymerase	95°C	30 sec	
Denaturation of double stranded DNA	95°C	30 sec	16 cycles
Annealing of oligonucleotides to the template DNA	55°C	60 sec	
Elongation	68°C	12 min	
Chilling	4°C	1 hour up to overnight	

Amplification was checked by electrophoresis of 10 μl of the product on a 1% agarose gel. If a band was visible on the gel, a digestion with the *Dpn I* restriction enzyme was performed to digest the parental non-mutated supercoiled double stranded DNA. 1 μl of the enzyme was directly added to each amplification reaction and incubated for 1 hour at 37°C.

For transformation of the amplified, mutated cDNA, 1 μl of the reaction mix was used. Several colonies were selected for plasmid preparation and sequencing analysis to control that selected cloned contained the desired mutations.

2.2.3.7 Sequencing

Sequencing analysis was performed on a LI-COR automated DNA sequencer using fluorescent primers labelled with the tricarbocyanine dye IRD800 at their 5'-end.

Gel components were pre-mixed the following way

30 ml	Sequagel XR (Biozym)
7.5 ml	Sequagel buffer (Biozym)
400 μl	DMSO
300 μl	10% APS

and polymerised for 45 min.

Cycle sequencing reactions were done using a SequiTherm ExcelTM II DNA Sequencing Kit-LC (Biozym) according to a protocol of the supplier:

The reactions were pre-mixed in the following way

2 μl	2 pmol IRD800-labelled primer
100-250 fmol	DNA template
7.2 μl	3.5X SequiTherm EXCEL II Sequencing Buffer
1 μl	SequiTherm EXCEL II DNA Polymerase (5 U/μl)

H_20 filled to 17 μl of a total reaction volume

The pre-mix was distributed in four tubes, 4 μl in each, 2 μl of the SequiTherm EXCEL II-LC Termination Mix A/C/G/T was added to each tube.

The following parameter were used for a cycle-sequencing:

30 sec	95°C	
15 sec	Tan	30 cycles
1 min	70°C	

Each reaction was stopped with 3 µl of a Stop/Loading Buffer. Samples were then analysed by electrophoresis or stored at -20°C. The annealing temperature of the primers was used equal to their melting temperature, and calculated as described in 2.2.3.5.

The reaction tubes were heated for 3-5 minutes at ≥ 70°C to denature the samples, and chilled on ice. Then 1,2 µl/well were loaded onto a sequencing gel, and run for 5-6 hours. Results were analysed using a Datalogger user program (LI-COR).

2.2.3.8 Electrophoretic Separation of DNA

Gel electrophoretic separation of DNA was performed in 1,0 – 1,5% agarose gels run at 30-50V with 1 x TBE as a running buffer. Gels contained 0,2 µg/ml ethidiumbromid, samples were run with 6 x Blue Loading Dye (Promega).

10 x TBE

108 g/l	Tris Base
55 g/l	Boric Acid
20 ml/l	0.5M EDTA

2.2.3.9 Elution of DNA Fragments from a Gel

To elute DNA fragments from a gel, a QIAquick Gel Extraction-Kit (QIAGEN) was used. The DNA fragments were cut from the gel on UV-light, and handled as recommended by the supplier. DNA was eluted in 30 μl of elution buffer.

2.2.4 Culturing of Mammalian Cells

2.2.4.1 Routine Culturing

All mammalian cell lines were maintained at 37°C and 95% humidity in the presence of 5% CO_2. Human epithelial ovarian carcinoma cell lines A27/80 and OVCAR-3, African green monkey kidney fibroblasts COS-7, and FE-8 cells (*HRAS*-transformed derivative of 208F rat fibroblasts described by (Griegel et al., 1986), were cultivated in Dulbecco's modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum and 2 mM glutamine. Tet-inducible FE-8 transformants (Sers et al., 1997) were cultured in DMEM medium supplemented with 10% tetracycline-free fetal calf serum and 2 mM glutamine either in the presence or in the absence of 2 μg/ml doxycycline.

For sub-culturing of the cells, the medium was removed, and the cells were rinsed with 1 x PBS buffer. Then the buffer was removed and 2 ml of trypsin-EDTA solution was added per 75 cm^2 culture flask. The flask was incubated for a few minutes at 37°C until the cells detached. 8 ml of a fresh culture medium was added, aspirated and dispensed into new culture flasks. Split ratio**:** 1:10 for A27/80, COS-7, FE-8, and OVCAR-3 cells, and 1:2 for the Tet-inducible FE-8 cells. Fresh medium was added every 2 to 3 days.

Trypsin-EDTA 10 x stock solution (Biochrom AG) contained 0,5 % Trypsin and 0,2 % EDTA. For use in cell culture the solution was diluted 1:10 with PBS, filter-sterilised, and frozen at -20°C in 80 ml aliquots. Before use, an aliquot was thawed in a water bath at 37°C and used as described above.

PBS 10 x stock solution (pH 7.2)

2,3 g/l	NaH_2PO_4
11,5 g/l	Na_2HPO_4
87,5 g/l	NaCl

2.2.4.2 Freezing and Thawing Procedure

Cells were allowed to grow until they were 70% confluent, then medium was removed, and the cells were rinsed with 1 x PBS buffer, and 2 ml of trypsin-EDTA solution was added per 75 cm^2 culture flask. The flask was incubated for a few minutes at 37°C until the cells detached, then they were transferred into a 15 ml Falcon tube and centrifuged at 800 x g for 3 min at 25°C. The cell pellet was resuspended in 900 μl of culture medium, and 100 μl of DMSO was added, mixed carefully and transferred into a cryo-tube and placed into a cryo-container with fresh isopropanol. The cells were kept at –70ºC for 24 hours to allow them to freeze slowly. After this time, they were stored in liquid nitrogen.

Thawing of cells was performed quickly. Frozen cells were thawed in a 37ºC water bath for 1-2 minutes and transferred immediately in a 15 ml Falcon tube. Ten ml of pre-warmed culture medium were added drop-wise and cells were resuspended. Then the tube was centrifuged at 800 x g for 3 min at 25°C to remove DMSO. The cell pellet was resuspended in 10 ml of fresh medium, transferred into a 75 cm^2 culture flask, and cultured as described above.

2.2.4.3 Cell Treatments

OVCAR-3 cells were allowed to grow in their culture medium until they were 70% confluent. Stock solutions of 100 nM OA, and 500 μM LY294002 were prepared in DMSO. The cells were treated with 0,5 nM and 10 nM OA, and 50 μM LY294002, respectively for 48 hours in their culture medium. Cells were treated for 1 hour with 2 nM OA for the phosphatase assay. Control plates were treated with the vehicle, DMSO.

2.2.4.4 Transfection of Mammalian Cells

COS-7 and OVCAR-3 cells were transfected using FuGENE 6 transfection reagent (Roche) according to a protocol of the supplier. For transfection in 6-well plates 5 x 10^4 cells were seeded. Twenty four hours later, cells were transfected with 0,5 μg plasmid DNA and 1.2 μl FuGENE reagent per well. For transfection in 10 mm dishes 3 x 10^5 cells were seeded and cultured for 24 hours, then transfected with 3 μg of total plasmid DNAs and 7 μl FuGENE reagent.

To control the transfection efficiency, cells were transfected with an EGFP plasmid (enhanced green fluorescent protein; Clontech) and viewed on UV light 48 hours after transfection. The number of green fluorescent cells was visually estimated.

2.2.4.5 Colonie Formation Assay

The colony formation assays, was performed by Jacqueline Hellwig (Institute of Pathology, Berlin, Germany) as described by Sers et al., (2002). Shortly, 2x10^5 cells per well of a 6 well plate were transfected with 1.5 μg plasmid DNA using FuGENE 6 transfection reagent (Roche). Forty-eight hours post transfection, G418 was added at 0.6 mg/ml to the culture medium. Selection was continued for 10 to 14 days until colonies were visible. Colonies were fixed in methanol, stained in methylen blue and counted.

2.2.5 Apoptosis Assays

We used two different assays to measure apoptosis. One assay was based on the altered nuclear morphology visible in apoptotic cells, the second assay measured the expression of Annexin V on the outer cellular membrane during apoptosis.

2.2.5.1 DAPI-Staining of Apoptotic Nuclei

For immunofluorescence cells were grown on the glass coverslips. After fixation with 3% paraformaldehyde in 1 x PBS for 20 min.

Cells were permeabilised using 0.2% Triton X-100 in 1 x PBS for 1.5 min. Coverslips were incubated with 100 ng/ml DAPI in 1 x PBS/1% BSA for 30 min, washed 3 times with 1 x PBS and mounted with 2.3% DABCO.

The cells were viewed using a confocal microscope TCS (Leica), and photographed. A number of cells exhibiting a nuclear morphology characteristic for apoptosis was calculated.

PBS 10 x stock solution (pH 7.2)

2.3 g/l	NaH_2PO4
11.5 g/l	Na_2HPO_4
87.5 g/l	NaCl

DAPI (4',6'-diamidino-2-phenylindole hydrochloride) stock solution

100 μg/ml diluted in sterile H_2O and stored at -20°C.

DABCO (1,4 – 1,4-Diazabicyclo (2,2,2)-octane) solution

2.3% w/v DABCO diluted in Glycerol/0,2 M Tris pH 8.0, ratio 9:1.

2.2.5.2 Flow Cytometric Analysis of Annexin V

OVCAR-3 cells were harvested 48 and 72 hours after incubation with 10 nM OA or the vehicle DMSO by trypsin-EDTA treatment and washed twice with ice-cold PBS. Apoptotic cells were detected using an Annexin-V-FLUOS Staining Kit (Roche) based on the dual staining technique with Annexin V, specifically staining apoptotic cells, and propidium iodide, staining all damaged cells. The experiment was performed according to the recommendations of the supplier. In a FACScan automate (Becton Dickinson), 10 000 cells were analysed in channels FL-1 for detection of the Propidium iodide stained cells, and FL-3 to detect Annexin V stained cells. The experiment was performed by Cornelia Giseler (Institute of Pathology, Charité, Berlin, Germany). The fraction of apoptotic cells was calculated using CellQuest software.

2.2.6 Analysis of Proteins

2.2.6.1 Protein Isolation from Mammalian Cells

Cells were grown in 10 cm dishes, and harvested when they reached confluency of 70%. The Medium was aspirated and cells were washed 3 x with ice-cold PBS. Then the cells were incubated with TNE lysis buffer for 30 min on ice. The lysed cells were scraped off the plates, transferred into microcentrifuge tubes and centrifuged for 15 min at 14,000 x g at 4°C. The Supernatants were removed, and pellets were discarded. An equal volume of 2 x SDS Sample buffer was added to each sample, the samples were boiled for 10 min, then chilled on ice and microcentrifuged for 5 min. Aliquots were stored at –20°C until use.

TNE lysis buffer

10 mM	Tris-HCl, pH 7.8
150 mM	NaCl
1%	NP-40
1 mM	EDTA
1/20 ml	protease inhibitor complete ^{TM} tabs

2 x SDS Sample buffer

50 mM	Tris-HCl, pH 6.8
100 mM	DTT
2% w/v	SDS
10% w/v	Glycerol
0.1% w/v	bromophenol blue

Prior to Western Blot analysis of caspases, an alternative protocol was used for the preparation of cellular extracts. The cells were allowed to grow for 48 hours then the medium was aspirated, transferred into pre-chilled Falcon tubes, and kept on ice. The cells were washed 3 x with ice-cold PBS and scraped into PBS. Then the tubes with medium and cells in PBS were centrifuged for 5 min at 1,000 x g. The supernatants were discarded, and CHAPS cell extract buffer (one volume of cell pellet) was added to the cell pellets.

The cells were resuspended in CHAPS buffer, frozen and thawed three times to lyse them, and centrifuged at 14,000 x g for 15 min at 4°C. The Supernatants were transferred into fresh tubes, an equal amount of SDS-Sample buffer was added, boiled for 10 min, and chilled on ice. The aliquots were stored at -20°C until use.

Chaps cell extract buffer

50 mM	Pipes/NaOH, pH 6.5
2 mM	EDTA
0.1%	Chaps
5 mM	DTT
20 μg/ml	Leupeptin
10 μg/ml	Pepstatin
10 μg/ml	Aprotinin
1 mM	PMSF

2.2.6.2 Subcellular Fractionation

Cells were seeded in 10 cm dishes and allowed to grow in their culture medium until they were 70% confluent. The medium was aspirated, cells were washed 3 x with ice-cold PBS, and incubated with hypotonic-lysis buffer (1 ml per 10 cm plate/2 x 10^6 cells) for 5-10 min on ice. Samples were transferred into Dounce Homogenizers, pre-chilled on ice and homogenised by repeated strokes to disrupt the cells. To pellet the nuclei cells were centrifuged at 1000 x g for 10 min at 4°C. Supernatants were transferred into Beckman tubes (13 x 15 mm), the pellets containing nuclei were saved on ice.

To recover membrane-associated proteins, the supernatants were centrifuged in a TLA 100.3 rotor at 100 000 x g for 30 min at 4°C. The supernatants containing cytoplasmic proteins were removed and transferred into Corex tubes, pellets were dissolved in 50µl ice-cold PBS and saved on ice.

To collect cytoplasmic proteins, a methanol/chloroform precipitation was used (Wessel and Flugge, 1984). To each supernatant prepared in the previous step, 4 ml pre-cooled methanol was added, then 1 ml pre-cooled chloroform was added and mixed, then 3 ml pre-cooled sterile water was added and mixed. The solution was centrifuged at 9 000 x g for 15 min at 4°C. The upper phase was discarded, and 3 ml methanol were added to each sample, mixed and centrifuged again 9 000 x g for 15 min at 4°C. The pellets were dried and dissolved in SDS sample buffer.

SDS-sample buffer was added to all fractions, samples were boiled for 10 min, chilled on ice, and stored at -20°C until use.

Hypotonic lysis buffer

10 mM	Tris-HCl, pH 8.0
0.1 mM	DTT
1 tab/20 ml	protease inhibitor complete ™ tabs

2.2.6.3 Determination of Protein Concentration

The protein concentration was determined using the BSA Protein Assay (PIERCE) according to the recommendations of the supplier.

2.2.6.4 One-Dimensional SDS Gel Electrophoresis (PAGE)

Protein samples were separated by polyacrylamide gel electrophoresis (PAGE). 50 µg of protein extract for detection of H-REV107-1 protein, and 30 µg for detection of caspases were loaded on a 12 % gel, run 30 min at 65V, and 2,5 hours at 98V in 1 x running buffer.

Separating gel: 10 ml of a 12% gel

3.35 ml	H_2O
2.5 ml	1,5 M Tris-HCl, pH 8,6
4 ml	30% acrylamide/bisacrylamide
100 µl	10% SDS
50 µl	APS
5 µl	TEMED

Stacking gel: 10 ml of 4% gel

6.1 ml	H_2O
2.5 ml	1,5 M Tris-HCl, pH 8,6
1.33 ml	30% acrylamide/bisacrylamide
100 µl	10% SDS
50 µl	APS
10 µl	TEMED

5 x running buffer

15.1 g/l	Tris-base
75 g/l	Glycine
5 g/l	SDS

2.2.6.5 Western Blot Analysis

After gel electrophoresis was finished, the stacking gel was discarded, and the separating gel was immersed in transfer buffer for 15 min. A PVDF-membrane was immersed in methanol for 5 sec, then in water for 2 min with shaking on a rotor platform, then the membrane was incubated 15 min in transfer buffer. On a Bio-Rad Transblot 3 sheets of Whatman paper pre-wetted in transfer buffer, the PVDF membrane, the gel, 3 sheets of pre-wetted Whatman were assembled.

Proteins were transferred to the PVDF membrane during 35 min at 16 V. After the transfer was finished, the membrane was washed briefly in TBST buffer, and blocked for 1 hour in 15 ml of blocking solution at room temperature with shaking on a rotor platform.

The gel was stained in Comassie blue solution to control for equal loading of protein amounts and efficient transfer. Afterwards the gel was photographed and discarded.

After the blocking procedure, the membrane was washed 3 x 10 min in 15 ml TBST, and incubated overnight with the primary antibody in blocking solution. The next day the membrane was washed 3 x 10 min in 15 ml TBST, and incubated for 1 hour with the corresponding secondary antibody.

The membrane was washed again 3 x 10 min in TBST, and the signal were developed using an ECL kit (Amersham), according to the supplier's recommendations.

To strip a membrane, a Western Blot Recycling kit (Alpha Diagnostic) was used. Membranes were incubated in stripping solution for 45 min up to 1 hour, and rinsed with blocking solution supplied with the kit 2 x 5 min. After an additional washing in TBST for 10 min the membrane was ready for re-probing with other antibodies.

Primary antibodies, corresponding secondary antibodies, and their dilutions

Primary antibody	**Dilution**	**Secondary antibody**	**Dilution**
Anti-cleaved caspase-3 (Cell Signaling Technology, Inc., MA, USA)	1:1000	peroxidase-conjugate goat anti-rabbit (Cell Signaling Technology, Inc., MA, USA)	1:2000
Anti-cleaved caspase-9 (Cell Signaling Technology, Inc., MA, USA)	1:1000	peroxidase-conjugate goat anti-rabbit (Cell Signaling Technology, Inc., MA, USA)	1:2000
Anti-HA (Sigma Aldrich, Inc., MO, USA)	1:10 000	peroxidase-conjugate goat anti-mouse (Dianova, Hamburg, Germany)	1:10 000
Anti-Histone 3 (Cell Signaling Technology, Inc., MA, USA)	1:1000	peroxidase-conjugate goat anti-rabbit (Cell Signaling Technology, Inc., MA, USA)	1:2000
Anti-H-rev107, rat (C. Sers, Charité, Berlin, Germany; Sers et al., 1997)	1:500	peroxidase-conjugate goat anti-rabbit (Dianova, Hamburg, Germany)	1:50 000
Anti-H-REV107-1 (C. Sers, Charité, Berlin, Germany; Sers et al., 2002)	1:500	peroxidase-conjugate goat anti-rabbit (Dianova, Hamburg, Germany)	1:50 000
Anti-GST antibody (Cell Signaling Technology, Inc., MA, USA)	1:1000	peroxidase-conjugate goat anti-mouse (Dianova, Hamburg, Germany)	1:10 000
Anti-IRF1 (Santa Cruz Biotechnology, CA, USA)	1:1000	peroxidase-conjugate goat anti-rabbit (Dianova, Hamburg, Germany)	1:50 000
Anti-IRF2 (Santa Cruz Biotechnology, CA, USA)	1:1000	peroxidase-conjugate goat anti-rabbit (Dianova, Hamburg, Germany)	1:50 000
Anti-p14.5, obtained from C. Kerkhoff, University of Regensburg, Germany (Schmiedeknecht et al., 1996)	1:150	peroxidase-conjugate goat anti-rabbit (Dianova)	1:50 000

Anti-p21 (Santa Cruz Biotechnology, CA, USA)	1:1000	peroxidase-conjugate goat anti-rabbit (Dianova, Hamburg, Germany)	1:50 000
Anti-pan-actin (Chemicon, CA, USA)	1: 5000	peroxidase-conjugate goat anti-mouse (Dianova, Hamburg, Germany)	1:10 000
Anti-PC4, obtained from R. Heilbronn, Free University Berlin, Germany (Weger et al., 1999)	1: 500	peroxidase-conjugate goat anti-rabbit (Dianova, Hamburg, Germany)	1:50 000
Anti-PR65 (Covance Research Products, Inc., CA, USA)7	1:1000	peroxidase-conjugate goat anti-mouse (Dianova, Hamburg, Germany)	1:10 000
Anti-STAT1 (Cell Signaling Technology, Inc., MA, USA)	1:1000	peroxidase-conjugate goat anti-rabbit (Cell Signaling Technology, Inc., MA, USA)	1:2000
Anti-V5 (Invitrogen, CA, USA)	1 : 5000	peroxidase-conjugate goat anti-mouse (Dianova, Hamburg, Germany)	1:10 000

Transfer buffer (pH 8.5)

25 mM	Tris-Base
0.2 M	Glycine
20 %	Methanol

TBST (pH 7.4)

8.8 g/l	NaCl
0.2 g/l	KCl
3 g/l	Tris-Base
500 µl/l	Tween-20

Blocking buffer 5% non-fat dry milk in TBST

Commasie blue 0.1% Comassie blue R-250 dissolved in 40% Methanol, 10% acetic acid.

2.2.7 Protein interaction analysis

2.2.7.1 Glutathione-S-Transferase Fusion System

2.2.7.1.1 Production of GST-Fusion Protein in Bacteria

The GST gene fusion system (Pharmacia Biotech, Inc., CA, USA) was used to express an H-REV107-1 – GST fusion protein in *E.coli* (107-GST). For this purpose the *H-REV107-1* cDNA encoding a truncated H-REV107-1 protein lacking the membrane binding domain (the ΔCH-REV107-1) was ligated into the pGE-2TK bacterial expression vector in frame with the Glutathione S-transferase. The *E. coli* B21 bacterial strain recommended and purchased by Pharmacia Biotech was used for the expression of the fusion protein. To maintain the pGE-2TK plasmid and the 107-GST – expression vector the *E.coli* Sure 2 strain was used as recommended by the manufacturer.

Preparation of competent B21 cells

A single colony of a fresh overnight culture was inoculated in 50 ml LB and incubated at 37°C with shaking at 250 rpm. Cells were grown to an OD_{600} of 0,4 – 0,5, this took approximately 2.5 – 3 hours. Cells were then centrifuged at 2500 x g for 15 min at 4°C. The cell pellet was gently resuspended in 5 ml of ice-cold TSS buffer, placed on ice and used for transformations within the next 30 min.

TSS buffer (Recipe for 100 ml), pH 6.5, filter sterilised

1 g	Tryptone
0.5 g	yeast extract
0.5 g	NaCl
10 g	Polyethylene Glycol (MW 3350)
5 ml	DMSO
5 ml	1 M $MgCl_2$

Transformation of competent B21 cells

One ml of competent cells was mixed with 10 ng of the pGE-2TK plasmid or 10 ng of the 107-GST expression vector, and incubated on ice for 45 min. All samples were transferred into a water-bath and incubated for 2 min at 42°C, then chilled briefly on ice. Afterwards, 100 µl of the transformation mix were transferred into a microcentrifuge tube with 900 µl LBG medium (LB-medium containing 20 mM Glucose) and incubated for 1 hour at 37°C with shaking at 250 rpm. 100 µl of the transformed cells were plated onto LBG plates containing 100 µg/ml Ampicillin and incubated overnight at 37°C.

Screening of pGE-recombinant transformants for expression of the fusion protein

Several colonies of *E.coli* transformants were picked into separate tubes containing 2 ml 2xYTA medium. Liquid cultures were grown to an OD_{600} of 0,6-0,8 (3-5 hours) with shaking at 30°C. Expression of the fusion proteins was induced by adding 2µl of 100 mM IPTG to each sample. Incubation was continued for an additional 2 hours. Liquid cultures were transferred into fresh tubes and centrifuged for 5 sec in a microcentrifuge. Pellets were resuspended in 300 µl of ice-cold PBS, 10 µl were analysed on an SDS-PAGE. To visualise the GST-protein (made in control cells carrying the parental pGE-2TK vector) and the fusion protein (made in cells carrying the 107-GST – expression vector), the gel was stained with Commassie Blue. Alternatively, a Western blot analysis with an anti-GST antibody was performed. The GST-protein has a size of 29 kDa, the 107-GST protein of 35 kDa. Several transformants, synthesising the recombinant protein of the predicted size were stored as a glycerol stock, as described in chapter 2.2.2.1.

2.2.7.1.2 GST Pull-Down Assay

The interaction between bacterially expressed 107-GST fusion protein and PR65-V5, over-expressed in COS-7 cells, was analysed under cell-free conditions using a GST pull-down assay.

Preparation of the recombinant 107-GST and GST proteins

To prepare recombinant 107-GST and GST, overnight cultures containing *E.coli* clones harbouring the pGE-2TK plasmid, and the 107-GST expression vector were grown. The overnight cultures were diluted 1:100, and 100 ml of every sample were incubated during 2-5 hours with shaking at 250 rpm at 30°C. Then, expression of the fusion proteins was induced by the addition of 100 mM IPTG to a final concentration of 0,2 mM. Incubation was continued for 3 hours. Cells were pelleted by centrifugation at 8000 x g for 10 min at 4°C and resuspended in 10 ml PBS. To lyse the cells, they were sonicated, and 20% Triton X-100 solution was added to a final concentration of 1%. Cell debris was pelleted at 12000 x g for 10 min at 4°C. Supernatants were transferred into fresh tubes, and 100 µl of 50% freshly prepared slurry of Glutathione Sepharose 4B were added to each supernatant.

The required amount of 50% Glutathione Sepharose 4B was prepared according to the recommendations of the supplier. 2 ml of a Sepharose were used for the purification of 250 µg protein.

For binding of recombinant proteins to Sepharose beads, samples were incubated at room temperature with gentle agitation for 30 min. Afterwards the suspensions were centrifuged at 500 x g for 5 min, and supernatants were removed. The Glutathione Sepharose beads with the bound proteins were washed 3 times with ice-cold PBS and added to a freshly prepared COS-7 cell protein extract.

GST-pull down assay

For this purpose COS-7 cells were transiently transfected with a PR65-V5 expression plasmid and incubated for 48 hours post-transfection. The cells were lysed in GST-lysis buffer, cell lysates were clarified by centrifugation, and saved on ice in 1 mg aliquots. To each aliquot were added purified recombinant 107-GST, or as a negative control GST proteins bound to Glutathione Sepharose beads, prepared as described above. The samples were incubated overnight at 4°C on a rocking platform. Then they were washed 3 times with GST-lysis buffer and resolved in SDS-PAGE, following Western blot analysis with an anti-GST, and an anti-V5 antibodies.

GST-lysis buffer

50 mM	Tris-HCl, pH 7.5
150 mM	NaCl
1 x (per 10 ml)	protease inhibitor complete tabs, EDTA free
1%	Nonidet P40
10%	Glycerol

2.2.8 Co-Immunoprecipitation

COS-7 cells were transiently transfected as described in chapter 2.2.4.4 and incubated for 42 hours post-transfection. The cells were lysed in the appropriate lysis buffer for 5 min, transferred to a pre-chilled Dounce homogeniser, and homogenised by repeated strokes on ice to disrupt the cells. Homogenised suspensions were centrifuged at 12,000 x g for 10 min at 4°C. Equal amounts of the supernatant from each sample were transferred into microcentrifuge tubes, optimal volume 1 ml. To each sample were added 5 µl of anti-V5, alternatively, 5 µl of anti-HA antibody, and gently rocked for 4 hours at 4°C. Then 50 µl of protein G-agarose (Roche, Mannheim, Germany) were added, and incubated overnight on a rocking platform at 4°C.

Protein complexes were collected by centrifugation at 12,000 x g for 20 seconds in a microcentrifuge, and washed 3 times with lysis buffer. Traces of lysis buffer after the final wash were removed, and 30 µl of SDS Sample buffer were added to each sample, heated for 5 min at 100°C, and frozen at -20°C. Precipitated protein complexes were analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

Lysis buffer (for co-immunoprecipitation of H-REV107-1 with P14.5, ETF1, PC4, and PR65).

50 mM	Tris-HCl, pH 7.5
150 mM	NaCl
1 x (per 10 ml)	protease inhibitor complete tabs, EDTA free
1%	Nonidet P40
10%	Glycerol

For precipitation of S100A6/Calcyclin $CaCl_2$ and $ZnCl_2$ were added to a final concentration of 2 nM.

Lysis buffer for co-immunoprecipitation of H-REV107-1 with RARG (NET buffer)

20 mM	Tris-HCl, pH 8.0
200 mM	NaCl
1 mM	EDTA
0.1%	Nonidet P40
10%	Glycerol
1 x (per 20 ml)	protease inhibitor tabs complete

The following components were used to enhance binding of the RARG protein to H-REV107-1. They were added to the cell lysates and incubated for 30 min on ice prior to the addition of the antibody.

1 μg/ml	double stranded DR5 RARE or DR5M oligonucleotides
1 μM	ATRA
1 μM	TTNPB

2.2.9 Immunofluorescence Analysis and Confocal Microscopy

For immunofluorescence analysis cells were grown on glass coverslips. After fixation with 3% paraformaldehyde in 1 x PBS for 20 min, cells were permeabilised using 0,2% Triton X-100 in 1 x PBS for 1,5 min, washed 3 x 5 min with PBS and incubated with the primary antibody.

The H-REV107-1 antiserum was diluted 1:2000, and anti-V5, anti-PR65, anti-PR36 antibodies were diluted 1:300 in PBS/1% BSA. Coverslips were incubated with primary antibodies for 2 hours at room temperature, then washed 3 x 5 min in PBS and incubated with the secondary antibodies. A goat anti-rabbit antibody AlexaFluor 594 (Molecular Probes, Eugene, OR) at 1:500, and a goat anti-mouse antibody AlexaFluor 488 (Molecular Probes, Eugene, OR) at 1:200 were used. For nuclear staining, cell were incubated with diamidinophenylindole (DAPI) and analysed using confocal microscopy (Leica TCS).

Colocalisation of H-REV107-1 with PR65, and ΔNΔC107-1 with PR65 was analysed using a Leica confocal microscope TCS SL. Colocalisation was semi-quantitatively evaluated using the standard application LCS program (Version 2.585), and the LCS Multicolor-Software (Leica) by Kerstin Lehmann (MetaGen, Berlin, Germany).

2.2.10 Phosphatase Assay

Phosphatase activity was measured by using para-nitrophenyl phosphate (p-NPP) as a substrate with the Phosphatase Assay Kit (Upstate Biotechnology, NY, USA). To investigate a potential effect of the H-REV107-1 protein, OVCAR-3 cells were transfected with H-REV107-1 expression plasmid or the control pcDNA3.1 vector without insert, and then starved for 24 hours. OVCAR-3 cells were pre-treated with or without 2 nM OA for 1 hour at 37°C. Then the cells were lysed in lysis buffer used for co-immunoprecipitation of H-REV107-1 with PR65. Cell lysates were clarified by centrifugation at 12.000 x g for 10 min. The clarified supernatants were incubated with the anti-PR65 antibody (Covance Research Products. Inc., CA, USA) and protein G-agarose (Roche, Mannheim, Germany) for 2 hours at 4°C. The immunoprecipitates were washed 2 times with lysis buffer and 1 time with assay buffer, resuspended in assay buffer containing 2.5 mM $NiCl_2$, and 900 μg/ml pNPP, and incubated for 30 min at 37°C. The amount of para-nitrophenol produced by dephosphorylation was determined by measuring the absorbance at 405 nm.

Assay buffer

50 mM	Tris-HCl, pH 7.0
0.1 mM	$CaCl_2$

3 Results

3.1 Identification of Proteins Interacting with H-REV107-1

3.1.1 Screening of a Human Kidney cDNA Library to Identify Potential Interacting Partners of the H-REV107-1 Protein

To understand the mechanism of H-REV107-1 – mediated growth suppression (Sers et al., 1997), a search for its potential interacting partners using a Lex-A Yeast Two-Hybrid system (Fields et al., 1989) was performed. In this approach an H-REV107-1 recombinant protein, pEG202-107 (bait), fused with a prokaryotic DNA-binding domain of the LexA protein was generated. The pEG202-107 protein and library cDNA inserts, fused with the *E.coli* B42 activation domain, were co-expressed in the yeast cell line EGY48 carrying the *LexA* and *GAL4* reporter genes. Yeast transformants grew on a selection medium, in which activation of the reporter genes made an interaction phenotypically detectable. The approach is described in detail in chapter 2.2.1.

As a bait, a truncated form of the human H-REV107-1 protein, lacking the membrane binding domain that hampered the transport of the hybrid protein into the yeast nucleus where the interaction takes place, was used. To screen a human kidney cDNA library available from Clontech, three independent large scale co-transformations were performed. More than two hundred preliminary positive clones were identified (Fig. 6).

Although the yeast two-hybrid system is a convenient tool for detection of protein-protein interactions in vivo, one disadvantage of this approach is the abundance of false positives. To verify putative interacting partners of the H-REV107-1 protein, the following strategy has been chosen:

Positive colonies were restreaked and re-tested on a selective medium to verify stability of the interactions.

Inserts of positive colonies were amplified using PCR to find out whether some of the colonies contain more then one cDNA library plasmid.

The cDNA library plasmids were isolated from yeast and transformed into *E. coli* KC8, the plasmids isolated from the clones confirmed in previous steps were sequenced.

Common yeast two-hybrid false positives were excluded from further analysis. The mating assay was applied to confirm interactions with the candidates chosen in the previous steps.

The interacting partners confirmed in the mating step were tested using co-immunoprecipitation in mammalian cells and an *in vitro* binding assay. Confirmed interactions were analysed functionally.

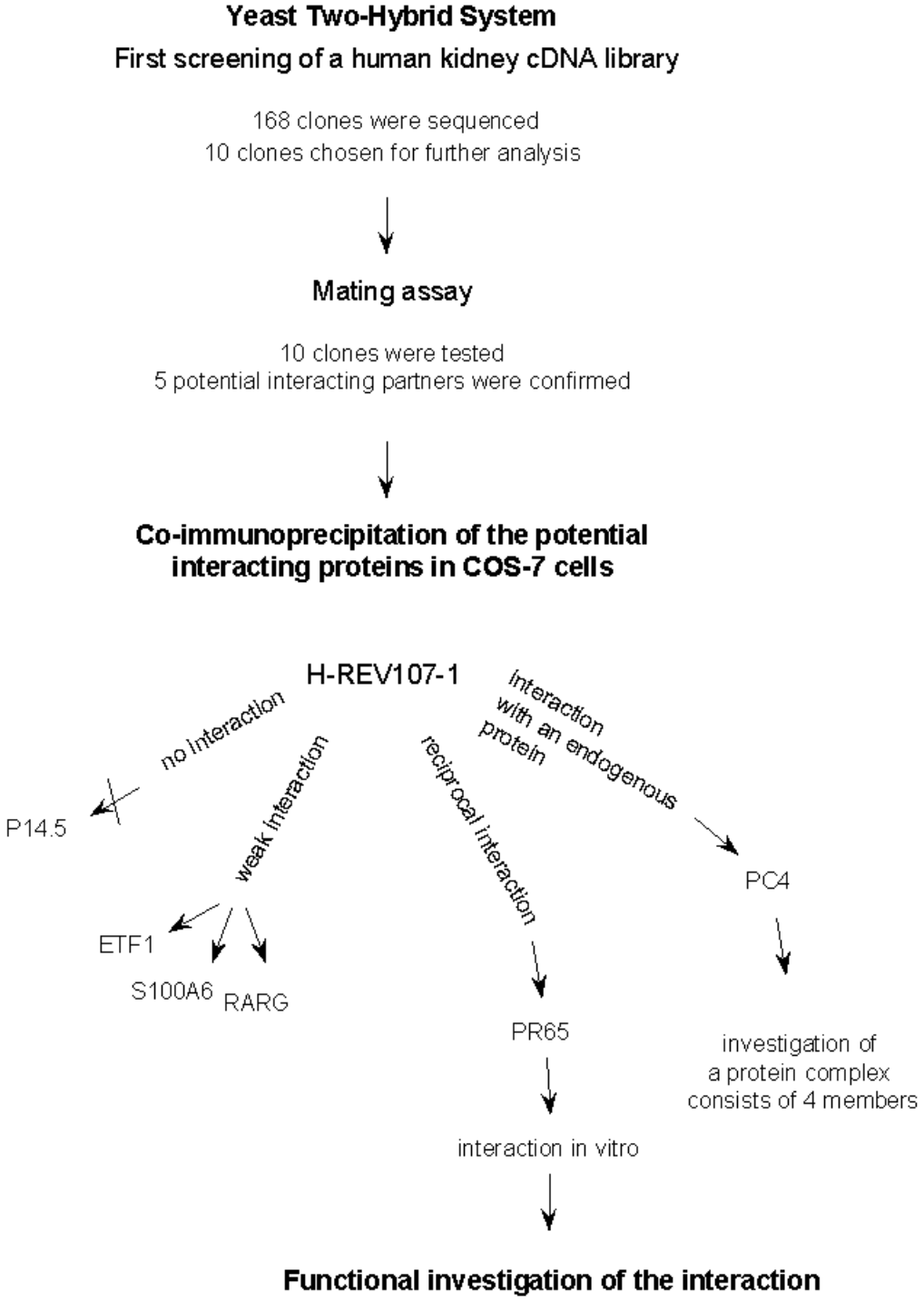

Fig. 6 Yeast Two Hybrid screen for H-REV107-1 interacting proteins, and strategy for their validation

3.1.2 Sequencing Analysis of Clones Encoding Putative Interaction Partners of the H-REV107-1 Protein

Screening of the cDNA library and exclusion of colonies containing more than one cDNA plasmid resulted in the selection of 168 clones appropriate for a sequencing analysis. cDNA library plasmids were isolated from the *E.coli* KC8 transformants, sequenced, compared to each other for elimination of duplicates, and analysed using NCBI Data Base http://www.ncbi.nlm.nih.gov/BLAST/ in order to determine which genes are present. The genes encoding potential interacting partners of the H-REV107-1 protein are listed in the Table 3. In addition, 37 yeast open reading frames, 33 mitochondrial genes, and 45 unknown genes were identified and excluded from further analysis.

Table 3 List of genes encoding putative interacting partners of the H-REV107-1 protein

cDNA	Human Homologue	Accession N.
1	23kD highly basic protein (HS23KD)	X56932
2	Activated RNA polymerase II transcription cofactor 4 (PC4)	NM_006713
3	Alpha-amylase	BAA14130
4	BAG-family molecular chaperone regulator-3 (BAG-3)	AF095193
5	Beta-2-microglobulin (B2M)	NM_004048
6	Calcyclin (PRA, S100A6)	NM_014624
7	Carboxypeptidase D (CPD)	NM_001304
8	Cathepsin D, lysosomal aspartyl protease (CTSD)	NM_001909
9	Cystatin C, amyloid angiopathy and cerebral hemorrhage	NM_000099
10	Enoyl Coenzyme A hydratase 1, peroxisomal (ECH1)	NM_001398
11	Fasciculation and elongation protein zeta 2 (zyginII,FEZ2)	U69140
12	Ferritin L-chain	Y09188
13	Heat shock protein 90 (Hsp90)	NM_005348
14	L apoferritin (exons 3 and 4)	X03743
15	Leukocyte common antigen (CD45)	AJ006102
16	Na, K-ATPase beta subunit (ATP1B)	NM_001677
17	Pancreatic lipase (PNLIP)	NM_000936
18	Plasma membrane Na+/H+ exchanger isoform (NHE3)	U28043
19	Proteasome subunit HC2	D00759
20	Proteasome subunit, alpha type, 2 (PSMA1)	NM_148976
21	Protein phosphatase 2A regulatory subunit A (PR65/PPP2R1A)	NM_014225
22	Putative DNA-directed RNA polymerase III C11 subunit	AF126531

23	Putative permease-related protein (SLCA8)	Y18483
24	Retinoic acid receptor gamma (RARG)	NM_000966
25	Retinoid inducible gene 1 (RIG1/TIG3/H-REV107-2/RARRES3)	AF092922
26	Reversion-induced LIM protein (RIL)	P50479
27	Ribosomal protein S14	BC006784
28	RNA-binding protein regulatory subunit (DJ-1)	NM_007262
29	Small EDRK-rich factor 2 (SERF2)	NM_005770
30	Sterol carrier protein-2 (SCP-2)	M55421
31	Syndecan 1 (SDC1)	NM_002997
32	TATA element modulatory factor	L01042
33	Translation termination factor gene (ETF1)	NM_004730
34	Translational inhibitor protein (p14.5)	NM_005836

3.1.3 Verification of Specificity of Interactions Using the Mating Test

The mating test was applied to ensure that the proteins identified in the primary yeast two-hybrid screening activate the reporter genes only due to interaction with H-REV107-1 and not through unspecific interactions with one of the reporter genes (Russell et al., 1998). To choose candidates for a mating test, the results from the library screenings were compared with the list of common false positives, the result of 100 yeast two-hybrid library screenings done by E. Golemis, Fox Chase Cancer Centre, Philadelphia, PA, USA.(Estojak et al., 1995).

http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html

The following genes were noticed there as common false positives:

Table 4 List of known false positives found in yeast two-hybrid screenings

16 heat shock proteins	2 cytoskeletal proteins
14 ribosomal proteins	ferritin
5 cytochrome oxydase	lamin
3 collagen-related proteins	tRNA synthase
3 mitochondrial proteins	ubiquitin
3 zink finger proteins	vimentin

After excluding these genes, the following candidates were selected for the mating assay:

Table 5 List of the genes encoding potential H-REV107-1 interacting partners chosen for the mating test

Activated RNA polymerase II transcription cofactor (PC4)
Beta-2-microglobulin (B2M)
Calcyclin (PRA, S100A6)
Cathepsin D (CTSD)
Cystatin C (CST3)
Protein phosphatase regulatory subunit A (PR65/PPP2R1A)
Retinoic acid receptor gamma (RARG)
Retinoid inducible gene 1 (RIG1/TIG3/H-REV107-2/RARRES3)
Syndecan 1 (SDC1)
Translation termination factor gene (ETF1)
Translational inhibitor protein P14.5 (P14.5)

In this test the specificity of interaction was examined by activation of both the Leu2- and LacZ- reporter genes in the diploid yeast strain, generated by fusion of the EGY48 and RFY206 haploid yeast strains. For every interaction three independent mating tests were performed. Interactions obtained as specific in at least two tests were chosen for further analysis. As a negative control, I analysed whether these proteins are able to activate the reporter genes without H-REV107-1. These negative controls are indicated with "-" in Fig. 7. A PDZ domain, shown to activate both reporter genes, was kindly provided by E Cuppen, (Institute of Cellular Signaling, Nijmegen, The Netherlands), and used as a positive control.

Interaction of the H-REV107-1 protein with ETF1, PC4, and S100A6 was shown to be specific but relatively weak, because activation of the *LacZ* reporter gene resulted in a light blue colour of colonies (Fig., upper panel). Interaction with SDC1, CST3, p14.5, and RARG was strong, although p14.5 and SDC1 activated the Leu reporter gene in one of the negative control (Fig. 7). Interaction with B2M, CTSD, and H-REV107-2/RIG1 was demonstrated as unspecific. The B2M and CTSD were able to activate the *Leu* reporter gene in two negative control tests, and H-REV107-2/RIG1 activated the *Leu* reporter gene in three negative control tests, independent of H-REV107-1 presence (white colonies in the negative control tests indicated with "-" in the Fig. 7).

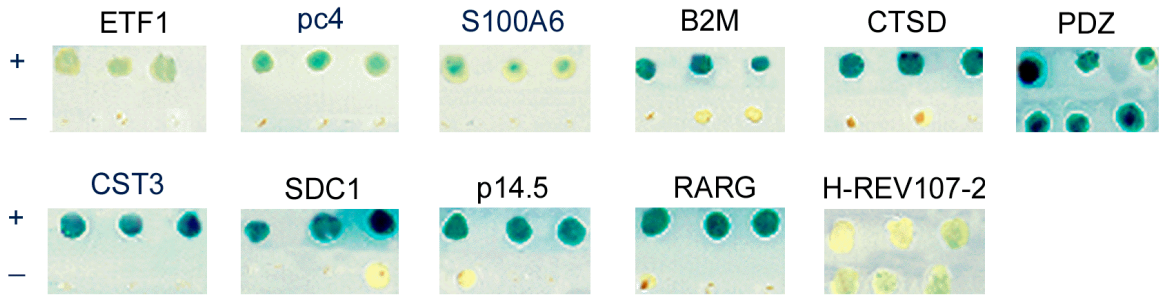

Fig. 7 Interaction between H-REV107-1 and potential binding proteins found in the yeast two-hybrid screening is verified using the mating assay

Diploid yeast colonies were grown on Leu – deficient, X-Gal – containing medium. Stability of an interaction between H-REV107-1 and one of the tested proteins was monitored through the intensity of the blue colour of the colonies. The more stable interactions resulted in the development of a dark blue colour (CST3, SDC1, p14.5, RARG). Weak or transient interactions resulted in growth of lightly coloured colonies (ETF1, PC4, and S100A6). Colonies in the upper lanes signed with "+" expressed H-REV107-1 and one of the interacting proteins. Colonies in the lower lanes signed with "-" do not contain H-REV107-1, but only the activation domain and the library cDNA insert. Three mating tests for every target are depicted.

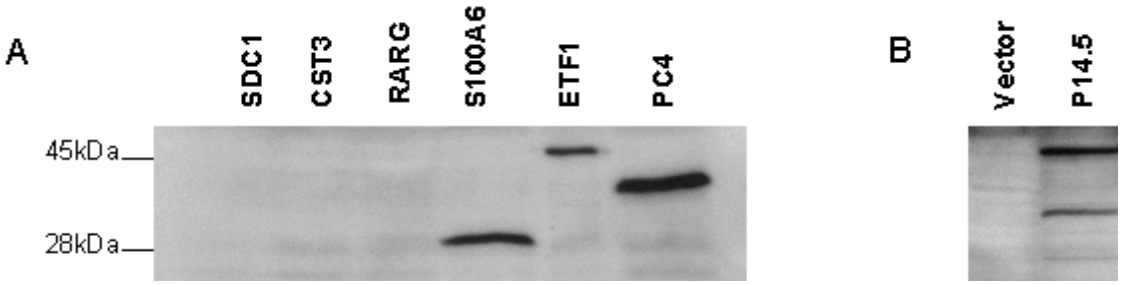

Fig. 8 Expression of the S100A6, ETF1, PC4, and P14.5 proteins is confirmed in yeast

Protein extracts were prepared form the yeast colonies used in the mating test. 10μg of the protein lysate was subjected to SDS-PAGE and analysed by Western blotting. A: analysis with an anti-HA antibody. The S100A6, ETF1, and PC4 proteins were detected. B: analysis with an antibody against P14.5.

3.1.4 Examination of Protein Expression in Yeast

To choose candidates for further analysis in mammalian cells, the expression of the protein encoded by the cDNA library inserts in yeast colonies, verified by mating assay, was examined. All cDNA inserts used in the interaction trap were fused to a sequence encoding an HA-epitope at their 5'-ends. To control the expression of the recombinant proteins, lysates from yeast expressing the genes of interest were analysed by Western blotting using antibodies recognising an HA-epitope or a recombinant protein. The S100A6, ETF1 and PC4 proteins were detected with an anti-HA antibody (Fig. 8 A), and the P14.5 protein was detected with an antibody against P14.5, kindly provided by C. Kerkhoff, Institute of Clinical Chemistry and Laboratory Medicine, Regensburg, Germany (Schmiedeknecht et al., 1996; Fig. 8 B). No signals were detected in the analysis of the yeast expressing SDC1, CST3, and RARG, possibly, because the conformation of these proteins hampered binding of the HA-antibody to N-terminal epitopes.

The PC4, ETF1, S100A6, and P14.5 proteins were potential interacting partners of H-REV107-1 complying the criteria of "true putative positives" of the yeast two-hybrid system. They were confirmed in the mating test, and expression of the recombinant proteins was demonstrated in yeast (Fig. 8).

To verify the interaction between proteins identified by yeast two-hybrid system, co-immunoprecipitation from cellular extracts is the standard procedure. Therefore I used recombinant mammalian expression vectors to co-express H-REV107-1 and the putative interacting partners in COS-7 cells.

In addition to the proteins validated by mating assay, co-immunoprecipitation was done with the RARG and PR65 proteins. Retinoic Acid Receptor Gamma (RARG) is an important mediator of the retinoic acid cellular response (Holmes et al., 2000). Retinoic acid regulates cell differentiation and proliferation. Furthermore, it has been demonstrated to inhibit growth in several tumor cell lines (Ogata et al., 1994). Therefore, the interaction between H-REV107-1 and RARG was tested by co-immunoprecipitation despite the fact that no RARG protein has been detected in Western Blot analysis (Fig. 8)

The second potential interaction partner of H-REV107-1 was the PR65 protein, the regulatory subunit of the protein phosphates 2A. The PR65 encoding cDNA clone identified in the original yeast two-hybrid screening was lost. However, PR65 is a known regulator of numerous cellular events appearing to play a privileged role in the regulation of cell growth (Janssens and Goris, 2001). The catalytic subunit of PP2A was demonstrated to interact with the ETF1 protein (Andjelkovic et al., 1996), a potential interacting partner of H-REV107-1 found in this yeast two-hybrid screening. It was possible that the interaction with PR65 mediates growth suppressive properties of the H-REV107-1

3.1.5 Generation of the H-REV107-1V5 and ΔCH-REV107-1HA Expression Vectors.

For co-immunoprecipitation in COS-7 cells H-REV107-1 recombinant proteins fused to HA-, and V5-epitopes were designed. These two epitopes were used because we wished to test which antibody is better appropriate for the immunoprecipitation of protein complexes. Full length and truncated H-REV107-1 recombinant proteins were generated to estimate if the H-REV107-1 membrane binding domain might influence binding capacity of the H-REV107-1 protein. Thus, the ΔCH-REV107-1HA recombinant protein consisted of a truncated H-REV107-1 lacking the membrane binding domain, and fused at its C-terminus to an HA-epitope (Fig. 9 A). The H-REV107-1V5 protein consisted of full length H-REV107-1 fused at its C-terminus to an V5-epitope (Fig. 9 B).

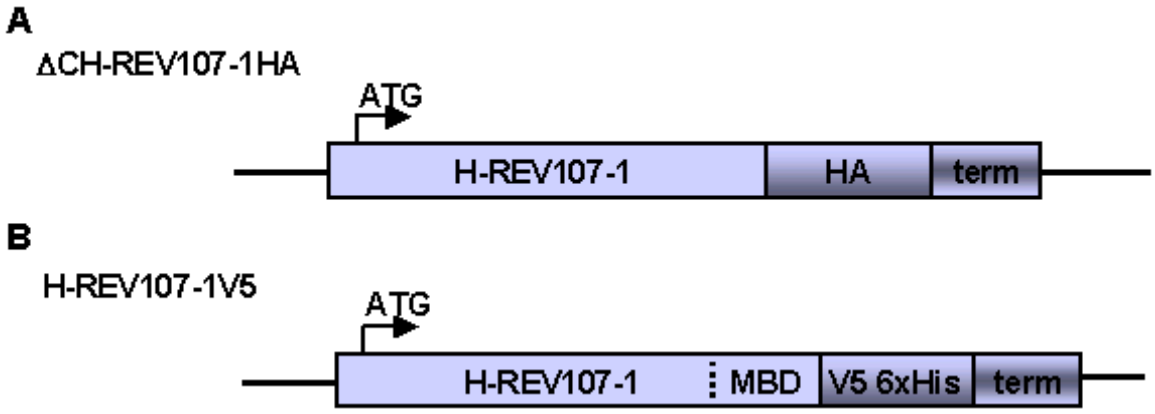

Fig. 9 The H-REV107-1 HA and V5 fusion proteins

A: ΔCH-REV107-1HA, cDNA without membrane binding domain was amplified using PCR, and subcloned in frame with the HA-Tag into the pcDNA3 vector.

B: H-REV107-1V5, H-REV107-1 full length cDNA was amplified using PCR, and ligated into the pEF6/V5 vector in a way that the V5-encoded sequence was located in frame at the 3'-end behind the membrane-binding domain. Abbreviations: MBD – membrane binding domain, term – termination of transcription (stop codon).

3.2 PC4

3.2.1 H-REV107-1 Interacts with PC4 in COS-7 Cells

The PC4 protein has been isolated as a RNA-polymerase II transcriptional coactivator (Ge et al., 1994). A mammalian expression vector containing full length *PC4* cDNA fused to a V5-epitope (PC4-V5) was purchased from the GeneStorm collection of Invitrogen. To examine whether PC4 interacts with the H-REV107-1 protein, COS-7 cells were transiently transfected with the ΔCH-REV107-1HA and PC4-V5 expression vectors. The PC4-V5 protein was detected in the precipitated complex containing ΔCH-REV107-1HA, however the interaction was very weak (Fig. 10, lane 3, arrow). These results were reproduced several times.

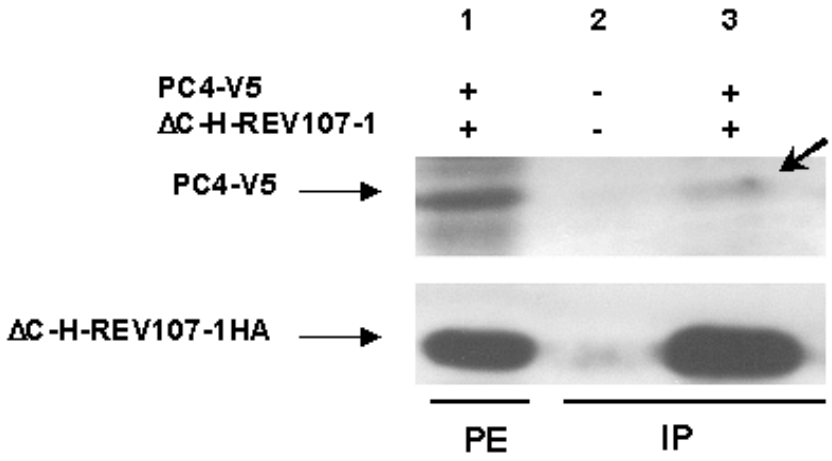

Fig. 10 The PC4-V5 and ΔCH-REV107-1HA proteins interact with each other in COS-7 cells

COS-7 cells were transiently transfected with PC4-V5 and ΔCH-REV107-1 expression vectors. As a negative control epitope – containing plasmids without inserts were used. Cell lysates were harvested 48 hours after transfection and used for immunoprecipitation with a HA-conjugated Sepharose. The immunocomplexes were subjected to SDS-PAGE and analysed using Western Blot with anti-V5 and anti-HA antibodies.

Lane 1: both proteins are present in the whole protein extract used for the immunoprecipitation. Lane 2: negative control. Lane 3: ΔCH-REV107-1HA precipitated with an HA-conjugated Sepharose, and co-precipitated PC4-V5 detected with an anti-V5 antibody.

3.2.2 Examination of the Intracellular Localisation of the Ectopically Expressed H-REV107-1 and PC4 Proteins

The PC4 protein is a transcriptional coactivator localised in the nucleus (Ge et al., 1994), whereas H-REV107-1 was described as a non-nuclear protein (Sers et al., 1997). To identify potential intracellular compartments where the proteins might be colocalised, immunofluorescence staining of COS-7 cells transiently transfected with the PC4-V5 and H-REV107-1 expression plasmids was performed. The H-REV107-1 expression vector containing full length cDNA was used because the role of the membrane binding domain has been shown to be important for the cellular distribution of the protein (Sers et al., 1997). PC4-V5 was localised mainly in nucleus, and in some cells in the cytoplasm. The H-REV107-1 protein was detected only in the cytoplasm (Fig. 11). Thus, the preferential localisation of PC4 in the nucleus prevented efficient interaction with the H-REV107-1 protein.

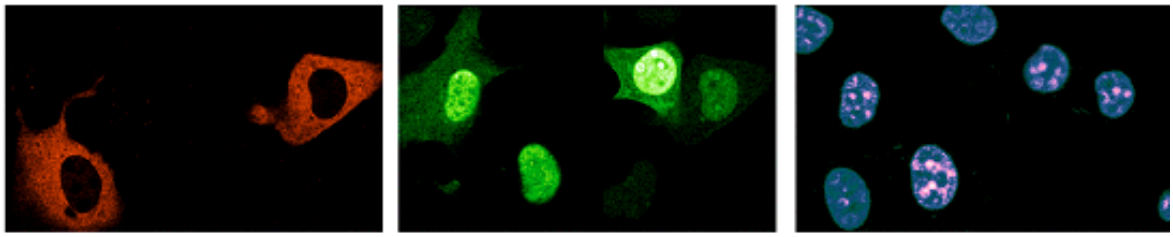

Fig. 11 The H-REV107-1 protein is distributed through the cytoplasm in COS-7 cells, whereas the PC4-V5 protein is localised preferably in the nucleus

COS-7 cells were transiently transfected with the PC4 and H-REV107-1 full length expression vectors. Immunofluorescence staining and subsequent confocal laser microscopy was done 48h post transfection. Cells were stained with an anti-H-REV107-1 and anti-V5 primary antibodies, and anti-mouse AlexaFluor 488 nm, and anti-rabbit AlexaFluor 594 nm secondary antibodies. DAPI was used for DNA – staining.

To define the intracellular localisation of H-REV107-1 more precisely, we performed a subcellular fractionation of the H-REV107-1 protein in COS-7 cells transiently transfected with the H-REV107-1 expression vector. H-REV107-1 was revealed in the cytoplasmic and in the nuclear fractions (Fig. 12). The presence of two bands was explained by possible post-translational modification of the protein, like it was described for the rat H-rev107 protein (Sers et al., 1997).

Thus, the intracellular localisation of H-REV107-1 and PC4 partially overlapped in the nucleus and in the cytoplasm. This result would explain the weak interaction demonstrated in the co-immunoprecipitation experiments (Fig. 10).

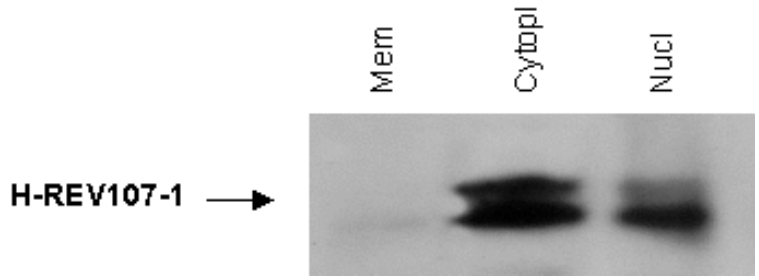

Fig. 12 The H-REV107-1 protein is distributed through the nuclear and cytoplasmic fractions of the transiently transfected COS-7 cells

COS-7 cells were transiently transfected with the H-REV107-1 expression plasmid. Proteins were harvested 48 hours after transfection and fractionated. For SDS-PAGE 15μg of protein extracts from every fraction were loaded. Immunoblotting was performed against a polyclonal anti-H-REV107-1 antibody. A specific H-REV107-1 band was detected in the cytoplasmic (Cytopl) and nuclear fractions (Nucl), but not in the membrane fraction (Mem).

3.2.3 H-REV107-1 Interacts with Endogenous PC4 in COS-7 Cells

A further proposition explaining the weakness of the PC4-V5 – ΔCH-REV107-1HA binding was that the C-terminated epitope of one of the proteins can handicap the interaction. An interaction of ΔCH-REV107-1HA with other proteins was demonstrated in previous experiments. The PC4 protein carried at its C-terminus a single-strand DNA-binding domain, that has been already shown to play a decisive role in a protein-protein binding (Weger et al., 1999). To prove whether the C-terminal epitope of PC4-V5 has an inhibitory effect, the interaction between ΔCH-REV107-1HA and endogenous PC4 was investigated in further experiments.

PC4 expression in COS-7 cells was examined by Western Blot analysis using an anti-PC4 antibody, a generous gift of Professor R. Heilbronn, Free University, Berlin, Germany (Weger et al., 1999). PA1 cells already described as PC4-positive were used as a positive control (Kannan et al., 1999). Equal expression of PC4 protein in PA-1 and COS-7 cell lines was demonstrated (Fig. 13 A). Co-immunoprecipitation with a HA-conjugated Sepharose was done in COS-7 cells transiently transfected with the ΔCH-REV107-1HA and PC4-V5 expression vectors. More stringent washing of the precipitated protein complex were performed to check whether the endogenous PC4 or the recombinant PC4-V5 protein interacts more stably with ΔCH-REV107-1HA.

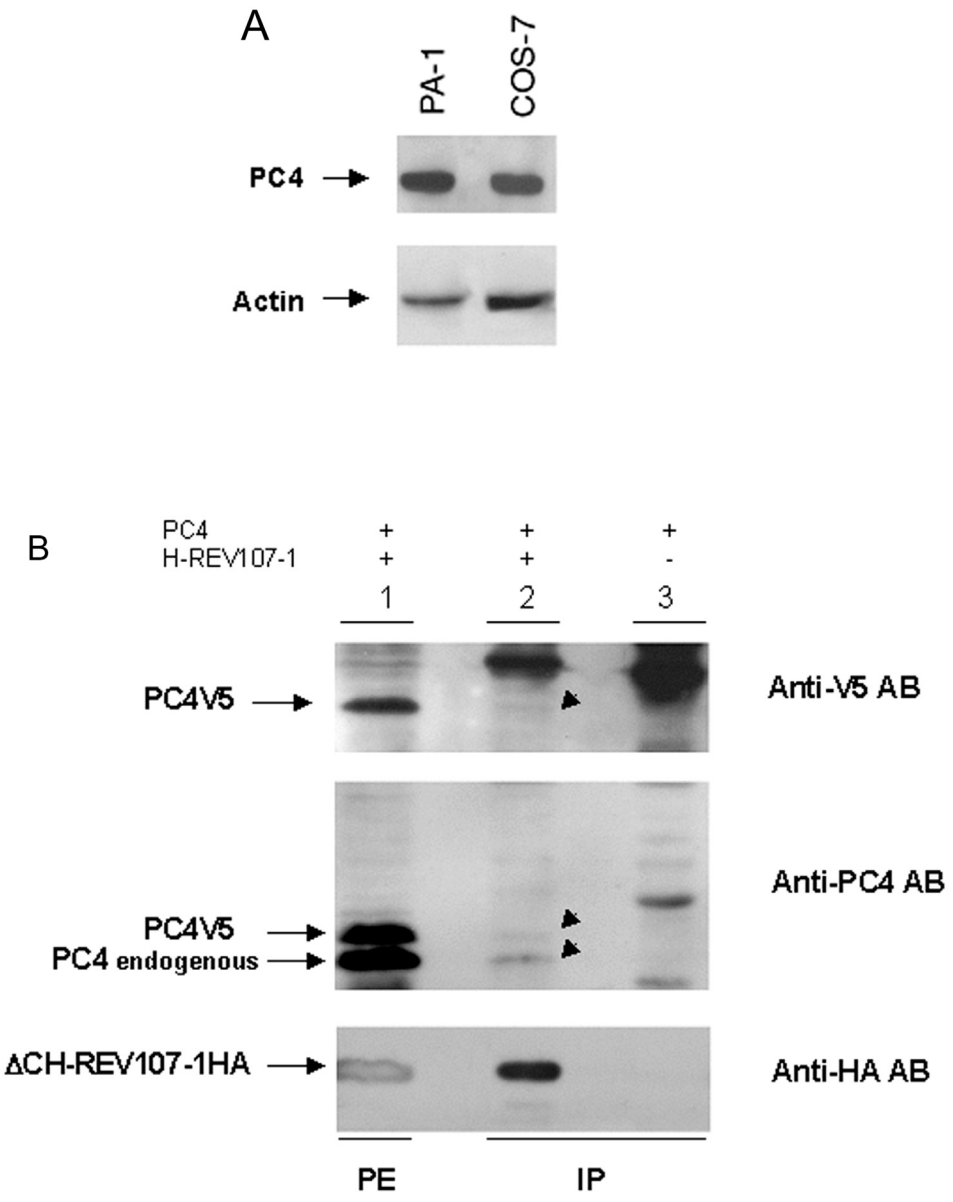

Fig. 13 H-REV107-1 interacts with the endogenous PC4 protein

A: COS-7 and PA1 cells were lysed in SDS-Sample buffer. 15μg of the lysate was subjected to SDS-PAGE. Upper panel - Western blotting against anti-PC4 antibody, bottom panel – actin was used as a loading control.

B: COS-7 cells were transiently co-transfected with PC4-V5 and ΔCH-REV107-1HA expression vectors, or with an empty vector containing HA-epitope only as a negative control. Forty eight hours after transfection cells were lysed and immunoprecipitated with a HA-conjugated Sepharose. The whole protein extract (10μg), and the immunoprecipitated complex were resolved by SDS-PAGE, and analysed via Western blotting. Middle panel – Western blot analysis with an anti-PC4 antibody. Lane 2 - the immunoprecipitated endogenous and V5-tagged PC4 proteins were revealed (arrowheads). Upper panel – the membrane was stripped and incubated with an anti-V5 antibody. Bottom panel – after second stripping the membrane was incubated with an anti-HA antibody. Lane 1 – protein extracts, lane 2 – co-immunoprecipitation , lane 3 – negative control.

Western Blot analysis using the anti-PC4 antibody revealed two bands in the protein extract. The precipitated protein complex contained endogenous PC4 and very low amount of the ectopically expressed PC4-V5 protein (Fig. 13 B, middle panel, lane 2, arrowheads). The V5-tagged PC4 protein had a slightly lower mobility in electrophoresis gel due to the V5-epitope (Fig. 13 B, middle panel, lane 1, 2). Immunoblotting with the anti-V5 antibody revealed a single band in the protein extract (Fig. 13 B, upper panel, lane 1). Incubation with the anti-HA antibody exhibited ΔCH-REV107-1HA protein, expressed in the COS-7 cells, and precipitated with a HA-conjugated Sepharose (Fig. 13 B, bottom panel, lane 1 and 2, accordingly). This experiment demonstrated that H-REV107-1 interacts more stably with the endogenous PC4 protein.

Thus, interaction with the endogenous protein suggested that the H-REV107-1 – PC4 binding takes place in vivo. A potential role of this interaction in H-REV107 function was investigated in further experiments.

3.2.4 H-REV107-1, PC4, and STAT1 Form a Protein Complex related to IFNγ-signaling

The PC4 protein participates in the regulation of RNA-polymerase II activity, and interacts with a variety of other transcriptional regulators (Wu and Chiang, 2001). It has been demonstrated that PC4 enhances transcriptional activity of the BRCA1 protein (Haile and Parvin, 1999), which is involved in IFNγ - dependent growth control (Ouchi et al., 1999). A differential display from rat astrocytoma cells treated with IFNγ recovered the *H-rev107* – sequence (Kuchinke et al., 1995), indicating that this gene is an IFNγ- target. This prompted us to suppose a potential role of the H-REV107-1 – PC4 complex in IFNγ - signaling.

Therefore, we investigated an effect of IFNγ on the *H-REV107-1* expression in human cells. H-REV107-1 was demonstrated to be down-regulated in most tumor cell lines and in 50% of human ovarian carcinomas as compared to normal tissue. Increase of *H-REV107-1* mRNA expression under IFNγ-treatment was shown in human ovarian carcinoma cell lines: A27/80 described as IFNγ-sensitive, and OVCAR-3 demonstrated to be resistant to the IFNγ-treatment. Further investigation revealed that H-REV107-1 is a direct target of interferon regulatory factor 1, IRF1 (Sers et al., 2002), a tumor suppressor and a mediator of the IFNγ-signalling (Tanaka and Taniguchi, 2000).

The increase of *H-REV107-1* mRNA expression upon IFNγ treatment in OVCAR-3 and A27/80 cells suggested a potential involvement of the gene in IFNγ-mediated growth suppression (Sers et al., 2002). To prove this hypothesis, the restoration of H-REV107-1 expression upon IFNγ-induction was examined at the protein level.

OVCAR-3 and A27/80 cells were treated for 9, 12, 24, 36, and 48 hours with IFNγ, and analysed using Western blotting. A low but specific H-REV107-1 signal was detected in OVCAR-3 cells after 36 hours of treatment, which further increased during the following 12 hours (Fig. 14, upper panel). In A 27/80 cells the protein was below detectable level (data not shown), although up-regulation of mRNA expression was demonstrated (Sers et al., 2002).

To understand the mechanism of the IFNγ -dependent growth suppression, and to pursue a role of the H-REV107-1 protein in this process, the phenotype of A27/80 and OVCAR-3 cells treated with IFNγ was investigated using immunofluorescence analysis. In A27/80 cells no considerable phenotypic changes were detected after 48 hours of induction (data not shown). In a small fraction of OVCAR-3 cells (below 5%) the H-REV107-1 protein was detected. Most importantly, up-regulation of endogenous H-REV107-1 correlated with a nuclear morphology typical for apoptotic cells, and in a fraction of the cells, which underwent apoptosis, H-REV107-1 was localised in the nuclei (Fig. 15, red arrowheads). This suggested involvement of H-REV107-1 in the IFNγ-mediated cell death, and supposed that nuclear localisation of the protein might be important for its growth suppressive properties.

The small fraction of OVCAR-3 cells sensitive to IFNγ-treatment was probably not detected in growth analysis performed earlier. Therefore, OVCAR-3 cells were assumed to be resistant to IFNγ-treatment. In contrast, A27/80 cells showed considerable growth inhibition up to 50% (Sers et al., 2002), but no phenotypic changes were obtained in this cell lines, suggesting different down-stream mechanisms of the IFNγ-signaling in A27/80 and OVCAR-3 cell lines. To determine how IFNγ-responses diverse in OVCAR-3 and A27/80 cells, expression of the major down-stream IFNγ-effectors was investigated in these cell lines. Among them are the signal transducer and activator of transcription, STAT1 (Schindler and Darnel, 1995), and its down-stream targets involved in growth inhibitory effects, interferon regulatory factor 1 (IRF1) and cyclin-dependent kinase inhibitor, $p21^{WAF1}$ (Naldini et al., 2001). The STAT1 and $p21^{WAF1}$ proteins were shown to be induced after IFNγ-exposure in several ovarian cancer cell lines (Burke et al., 1999). The increase of IRF1 expression upon IFNγ-induction in OVCAR-3 and A27/80 cells has been described (Sers et al., 2002; Fig. 14).

The expression of the STAT1 and $p21^{WAF1}$ proteins after incubation of OVCAR-3 and A27/80 cells with IFNγ was investigated using Western blot analysis. Up-regulation of STAT1 was detected after 24 hours and increased up to 48 hours in both cell lines (Fig. 16, upper panel). Immunoblotting with an anti- $p21^{WAF1}$ antibody revealed a specific band in A27/80 cells after 24 hours of IFNγ-treatment. The amount of protein was stable during the following 72 hours. The $p21^{WAF1}$ protein was not detected in OVCAR-3 cells (Fig. 16, middle panel).

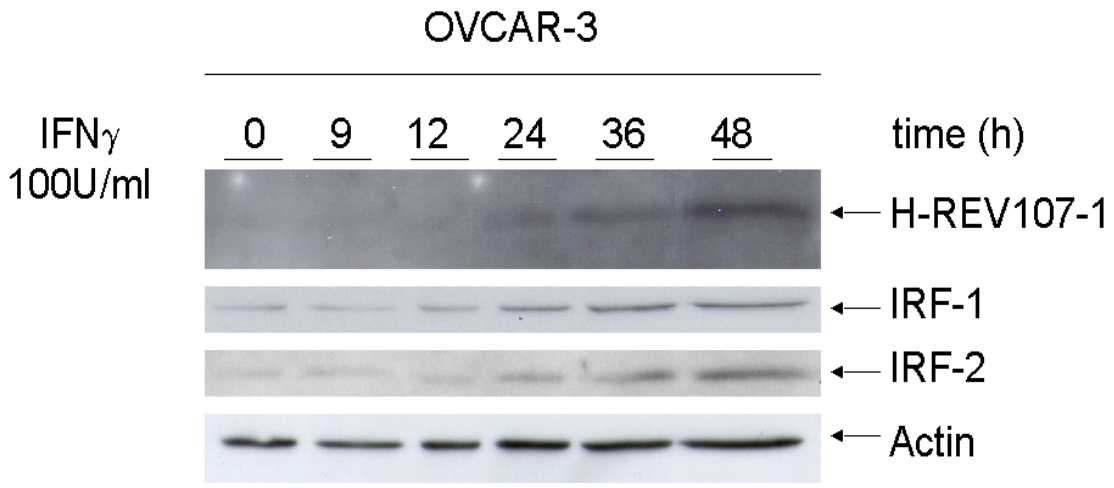

Fig. 14 Up-regulation of H-REV107-1 expression in OVCAR-3 cells after IFNγ-induction

Western blot analysis of H-REV107-1 expression in OVCAR-3 cells was performed after incubation with 100 U/ml of IFNγ. H-REV107-1 was detected with a specific anti-H-REV107-1 antibody. Then the membrane was incubated with an antibody against IRF1, and after the next stripping with an anti-IRF2 antibody. Actin was used as a loading control.

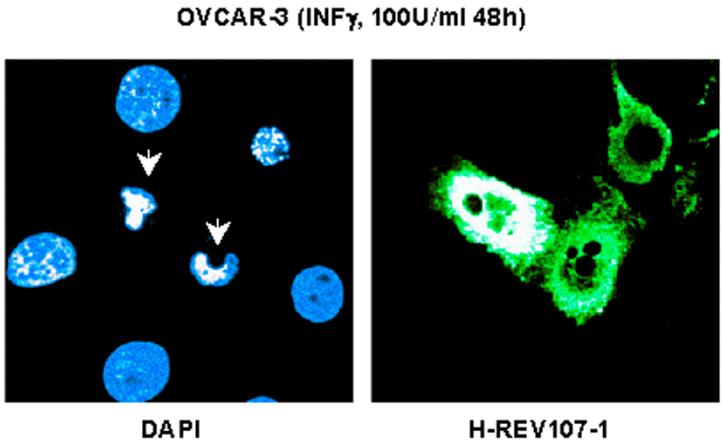

Fig. 15 Induction of the H-REV107-1 expression upon IFNγ - treatment leads to cell death

Immunofluorescence analysis of OVCAR-3 cells treated for 48 hours with 100U/μl of IFNγ. Cells were incubated with a primary anti-H-REV107-1 antibody, and secondary anti-rabbit AlexaFluor546 antibody. Nuclei were stained with DAPI. Only cells expressing H-REV107-1 showed an altered morphology of the nucleus, indicative of apoptosis (white arrowheads).

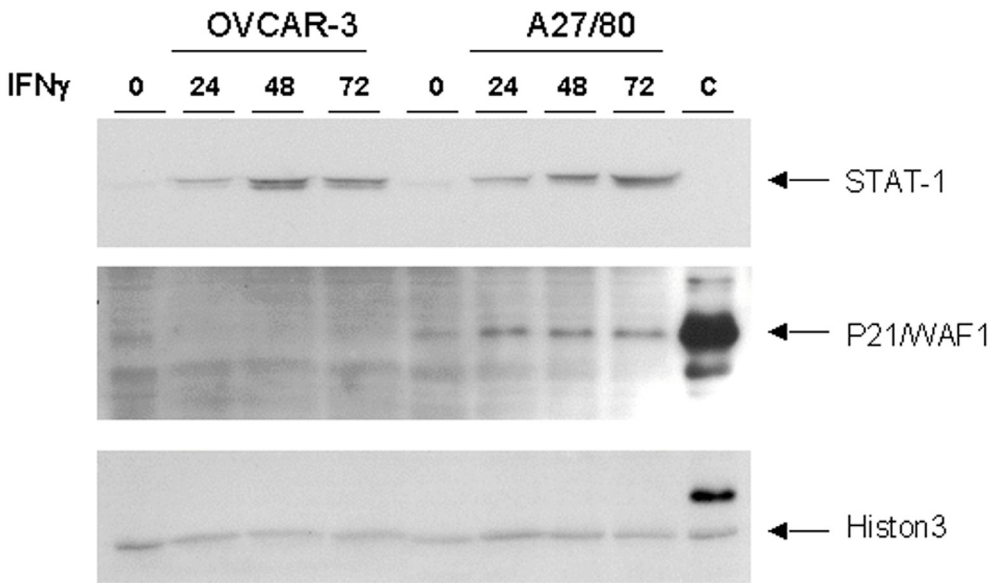

Fig. 16 STAT1 and P21^{WAF1} expression after IFNγ - induction in OVCAR-3 and A27/80 cells.

OVCAR-3 and A27/80 cells were treated with 100 and 1000 Units of IFNγ, respectively for 24, 48, and 72 hours; 10 μg of nuclear extracts were subjected to SDS-PAGE followed Western Blot analysis using antibodies against p21^{WAF1} and STAT-1. Histone 3 was used as a loading control. C – p21^{WAF1} control protein.

Thus, up-regulation of different STAT1-targets was demonstrated in OVCAR-3 and A27/80 cell lines in response to IFNγ-treatment. In A27/80 cells, the IFNγ-dependent growth suppression correlated with the up-regulation of p21^{WAF1} expression. In contrast, induction of apoptosis in OVCAR-3 cells was p21^{WAF1} independent, but correlated with up-regulation of the IRF-1 and H-REV107-1 proteins.

Two different STAT1-downstream pathways were described earlier (Ouchi et al., 2000). The first led to IRF1 up-regulation. The second, an IRF-1 independent pathway, led to the activation of a STAT1 – BRCA1 complex, and up-regulation of their target genes, including p21^{WAF1} (Ouchi et al., 2000). This pathway seemed to be activated in A27/80 cells. The tumor suppressor, breast cancer susceptibility gene 1 (BRCA1) was demonstrated to bind STAT1 in IFNγ-induced cells, thereby enhancing STAT1 activity (Ouchi et al., 2000). Furthermore, BRCA1 has been demonstrated to act as a transcription factor (Shuai et al., 1994).

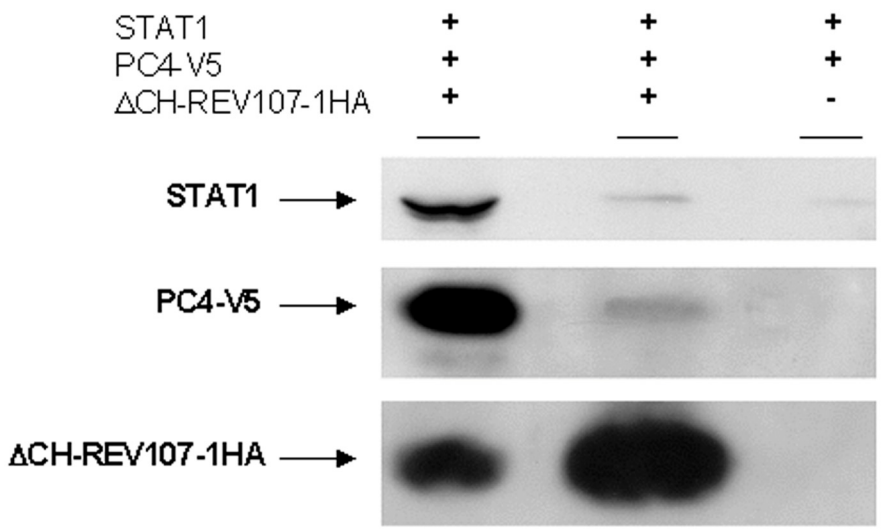

Fig. 17 STAT1 and PC4 proteins interact with H-REV107-1

COS-7 cells were transiently transfected with the STAT1, PC4-V5, and ΔCH-REV107-1HA expression plasmids, or with the STAT1, PC4-V5, and pcDNA3-HA as a negative control. Protein extracts were harvested 42 hours after transfection. The clarified supernatant was incubated with the HA-Sepharose for 10 hours. The immunoprecipitated protein complex was subjected to SDS-PAGE and analysed by Western blotting.

Lane 1 – protein extracts, lane 2 – immunoprecipitation, lane 3 – negative control. Upper panel – incubation of the membrane with STAT1 antibody revealed the specific band in the primary protein extract used for the precipitation (lane 1), and a weak signal in the immunocomplex with the H-REV107-1 protein (lane 2). Middle panel – the PC4-V5 protein was detected with the epitope-specific anti-V5 antibody. Bottom panel – ΔCH-REV107-1HA detected with an anti-HA antibody.

Experiments in vitro showed that the transcriptional activation by BRCA1 is maximal in the presence of the PC4 coactivator, although their direct interaction has not been confirmed (Haile and Parvin, 1999). Therefore, it was possible that the H-REV107-1 protein, interacting with PC4, could be a link between PC4 and the STAT1 – BRCA1 complex. This suggested the existence of a protein complex consisting of STAT1, PC4, H-REV107-1, and BRCA1.

The interaction between these proteins was tested using co-immunoprecipitation. To ensure their high expression level, COS-7 cells were simultaneously transfected with either ΔCH-REV107-1HA, PC4-V5, STAT-1, and BRCA1, or ΔCH-REV107-1HA, PC4-V5, and STAT-1 expressing plasmids. As a negative control, a transfection with PC4-V5, and STAT1 expression vectors, and a plasmid containing the HA-epitope only was done. Immunocomplexes were purified with a HA-conjugated Sepharose. The precipitated proteins were analysed using Western blotting. Incubation with an anti-STAT1 antibody revealed a specific band of approximately 90 kDa in the protein extract and in the immunocomplex precipitated with the ΔCH-REV107-1HA protein (Fig. 17, lane 1, 2 respectively). The PC4-V5 protein was detected with an anti-V5 antibody (Fig. 17, lane 1, 2). The BRCA1 protein was detected neither in the protein extract, nor in the precipitated protein complexes, suggesting that overexpression of this protein failed (data not shown).

Thus, the interaction between H-REV107-1, STAT-1 and PC4 proteins ectopically expressed in COS-7 cells was demonstrated. Participation of the BRCA1 protein in this complex is still a question to be answered. A potential role of this interaction in IFNγ-signalling has to be further investigated.

3.3 PR65

3.3.1 H-REV107-1 Interacts with PR65 in COS-7 Cells

PR65 is the regulatory subunit A of the protein phosphatase 2A (PP2A). PP2A is a highly conserved serine/threonine phosphatase essential for a number of cellular functions including signal transduction, translational control, and cell cycle regulation (Millward et al., 1999; Sontag, 2000; Janssens and Goris, 2001). PP2A was shown to act as a pro-apoptotic phosphatase (Klumpp and Krieglstein, 2002). The H-REV107-1 protein was demonstrated to possess growth suppressive properties (Sers et al., 1997), and to induce apoptosis in human ovarian carcinoma cells (Sers et al., 2002). Therefore, the possible interaction between these two proteins was analysed.

Mammalian expression vector containing full length PR65 cDNA fused to a V5-epitope (PR65-V5) was purchased from the GeneStorm collection of Invitrogen. To examine the interaction between H-REV107-1 and PR65, co-immunoprecipitation in COS-7 cells was performed. The cells were transiently transfected with the PR65-V5 and ΔCH-REV107-1HA expression vectors. Protein complexes were purified with an HA-conjugated Sepharose, and analysed with the anti-V5 antibody (Fig. 18 A). A PR65-V5 – specific band was present in the protein complex together with ΔCH-REV107-1HA (Fig. 18 A, upper panel, lane 2, arrow), but not in the control precipitation with the expression vector containing a HA-epitope only (Fig. 18 A, upper panel, lane 3). To ensure a presence of the ΔCH-REV107-1HA protein, Western blotting with an anti-HA antibody was done (Fig, 18 A, bottom panel).

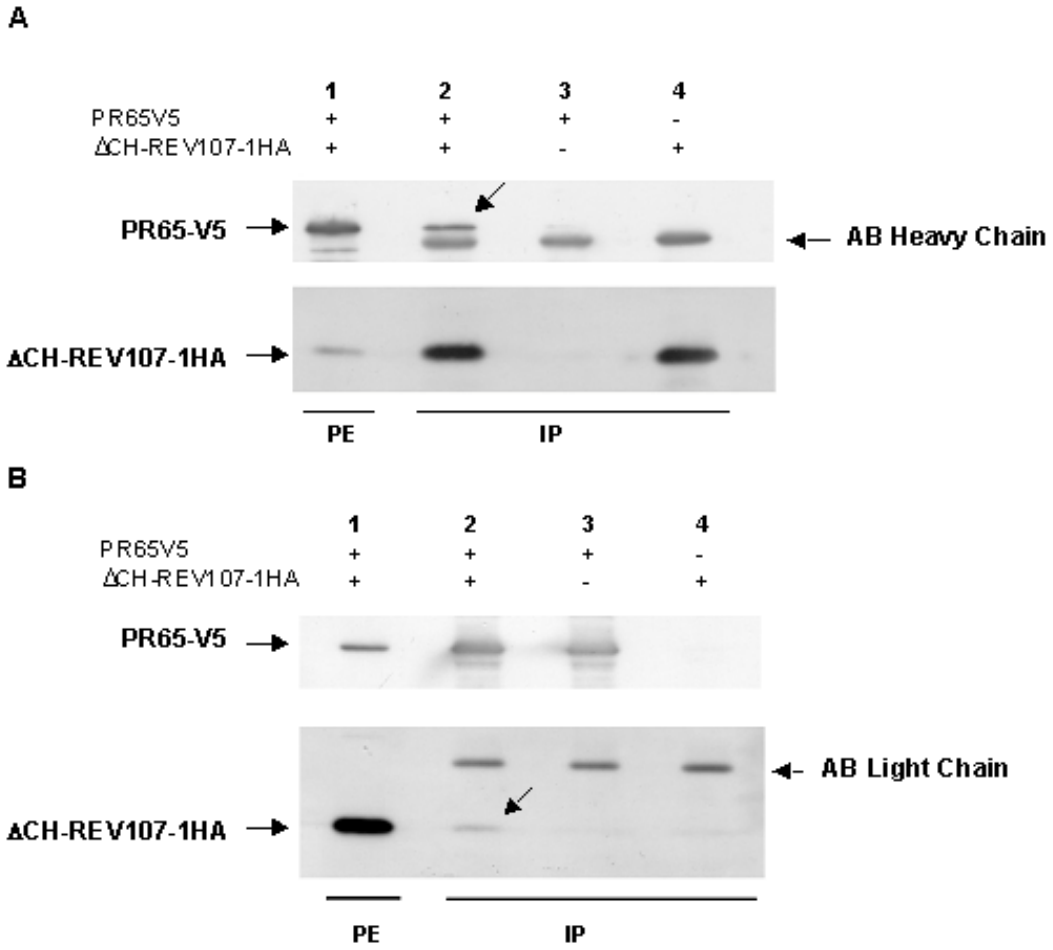

Fig. 18 Co-immunoprecipitation of ΔCH-REV107-1HA and PR65-V5 in COS-7 cells

COS-7 cells were transiently transfected with the PR65-V5 and ΔCH-REV107-1HA expression plasmids. Protein extracts were harvested 48 hours after transfection, and co-immunoprecipitated with V5 and HA-antibodies. A: Protein complexes precipitated with the anti-HA antibody. Upper panel – co-precipitated PR65-V5 protein was detected with the anti-V5 antibody, bottom panel – precipitated ΔCH-REV107-1HA protein was detected with the anti-HA antibody. Lane 1 – expression of both ΔCH-REV107-1HA and PR65-V5 proteins in COS-7 cells used for the co-immunoprecipitation. Lane 2 – co-immunoprecipitation of PR65-V5 with ΔCH-REV107-1HA. Lane 3 and 4 – control immunoprecipitation where either PR65-V5 or ΔCH-REV107-1HA are present. B: Protein complex was precipitated with the anti-V5 antibody. Bottom panel – co-precipitated ΔCH-REV107-1HA protein detected with the anti-HA antibody; upper panel – precipitated PR65-V5 revealed with the anti-V5 antibody. Lane 2 - co-immunoprecipitation of the ΔCH-REV107-1HA protein with PR65-V5. Lanes 3, 4 – negative controls.

The reverse experiment, precipitation with the anti-V5 antibody, confirmed that the ΔCH-REV107-1HA – specific band precipitated together with the PR65-V5 protein (Fig. 18 B, bottom panel, lane 2, arrow).

3.3.2 H-REV107-1 and PR65 are Co-Localised in COS-7 Cells

In addition to co-immunoprecipitation, the intracellular localisation of the H-REV107-1 and PR65 proteins was examined using immunofluorescence analysis of COS-7 cells transiently transfected with the PR65-V5 and H-REV107-1 expression vectors. Staining was analysed using a confocal laser microscopy (Fig. 19). Ectopically expressed H-REV107-1 and PR65-V5 had almost identical distribution patterns in COS-7 cells. Co-localisation of these proteins was obtained in the perinuclear region (Fig. 19, bottom panel – overlap of the H-REV107-1 and PR65 localisation is shown in yellow).

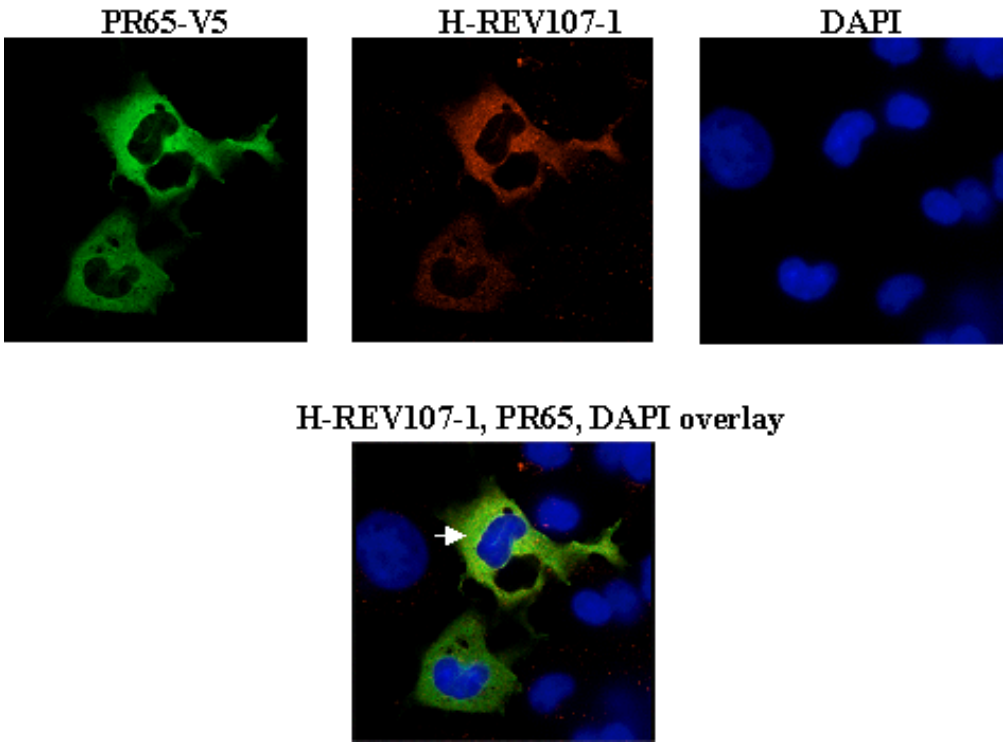

Fig. 19 The H-REV107-1 and PR65-V5 proteins ectopically expressed in COS-7 cells are co-localised in the cytoplasm

COS-7 cells were transiently transfected with PR65-V5 and H-REV107-1 expression vectors. Forty eight hours post transfection cells were fixed and incubated with a monoclonal anti-V5 antibody and polyclonal anti-H-REV107-1 antibody, and with the secondary anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 594 antibodies, respectively. Nuclei were stained with DAPI.

Upper panel: PR65 – green, H-REV107-1 - red, Nuclei - blue. Bottom panel – overlay of all three staining. The perinuclear region where H-REV107-1 and PR65 proteins are co-localised had a yellow-green colour (arrow).

3.3.3 H-REV107-1 Interacts with PR65 in a Cell-Free System

The GST pull-down assay is a well established method to verify the specificity of interaction between two proteins under cell-free conditions. This method allows excluding of an influence of other proteins on the interacting partners.

For this experiment the Glutathione S-transferase (GST) Gene Fusion System (Pharmacia Biotech) was used. GST-fusion proteins can be expressed in E.coli, and purified by affinity chromatography using Glutathione Sepharose. The ΔCH-REV107-1 protein fused at its N-terminus with Glutathione S-transferase (107-GST) was purified from *E.coli*, and incubated with the clarified cell lysate prepared from COS-7 cells over-expressing PR65-V5. As a control the COS-7 protein extract was incubated with the purified Glutathione S-transferase (GST) alone. After washing, proteins bound to the Glutathione Sepharose were separated by SDS-PAGE and subjected to Western blotting using the anti-V5 antibody. This analysis revealed PR65-V5 in a complex with the 107-GST fusion protein (Fig. 20, upper panel, lane 4). Control incubation with an anti-GST antibody revealed a specific 107-GST and GST bands of the predicted size in the E.coli cell lysate (Fig. 20, lane 2 and 1, respectively), and in the Sepharose-bound protein complexes (Fig. 20, lane 4 and 6, respectively).

These experiments demonstrated that the PR65 and H-REV107-1 proteins interacted in COS-7 cells as well as in cell-free conditions. Further experiments aimed at identifying the H-REV107-1 protein domains responsible for the interaction, and understanding the functional role of the H-REV107-1 – PR65 interaction.

3.3.4 Homodimer Formation of H-REV107-1

Western blot analysis of the COS-7 cells over-expressing the ΔCH-REV107-1HA truncated protein revealed two bands of different sizes: one band of 16 kDa, corresponding to the ΔCH-REV107-1HA protein, and the second band of approximately 32 kDa (Fig. 21, middle panel, lane 2). Co-transfection of COS-7 cells with the ΔCH-REV107-1HA and H-REV107-1V5 expression vectors, and subsequent Western blot analysis with an anti-HA antibody resulted also in the observation of two bands of different sizes. This suggested that the H-REV107-1 protein formed homodimers in COS-7 cells.

To prove this hypothesis, COS-7 cells were transiently transfected with the ΔCH-REV107-1HA and H-REV107-1V5 expression vectors, and with the ΔCH-REV107-1HA and PR65-V5 expression vectors as a positive control. Immunoprecipitation was performed with an anti-V5 antibody. Immunoprecipitated protein complexes were analysed by Western blotting with an anti-HA antibody. The ΔCH-REV107-1HA protein was detected in a complex with H-REV107-1V5 or PR65-V5 (Fig. 21, bottom panel, lanes 3 and 4, respectively), confirming the ability of H-REV107-1 to form homodimers in COS-7 cells.

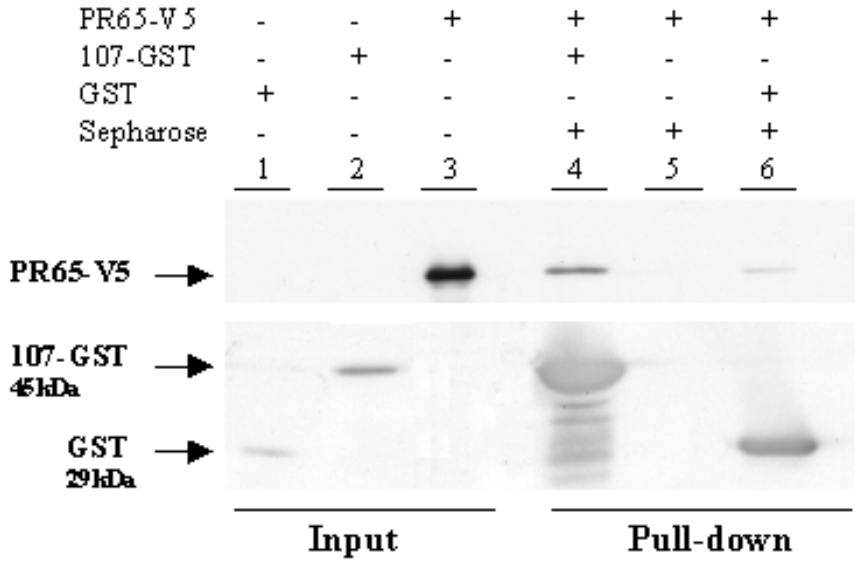

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Fig. 20 H-REV107-1 interacts with PR65 in a cell-free conditions

The H-REV107-1 protein fused at its N-terminus with a GST-epitope (107-GST), and the GST-epitope alone were over-expressed in E.coli B21, and purified with GST-Sepharose. Purified proteins were incubated with a lysate from COS-7 cells over-expressing PR65-V5. 10 μg of total protein extract, and protein complexes bound to the Glutathione Sepharose were subjected to SDS-PAGE and analysed using Western blotting.

Upper panel: Western Blot analysis was done with an anti-V5 antibody. Bottom panel: Western Blot analysis using an anti-GST antibody. Lane 1 – E.coli B21/GST protein extract after sonication; lane 2 – E.coli B21/107-GST sonicate; lane 3 - 10μg of the protein extract from COS-7 cells over-expressing PR65-V5; lane 4 – GST pull-down of PR65-V5 with the recombinant 107-GST protein; lane 5 – GST pull-down of PR65-V5 with the Glutathione-Sepharose; lane 6 – GST pull-down of PR65-V5 with the GST-protein.

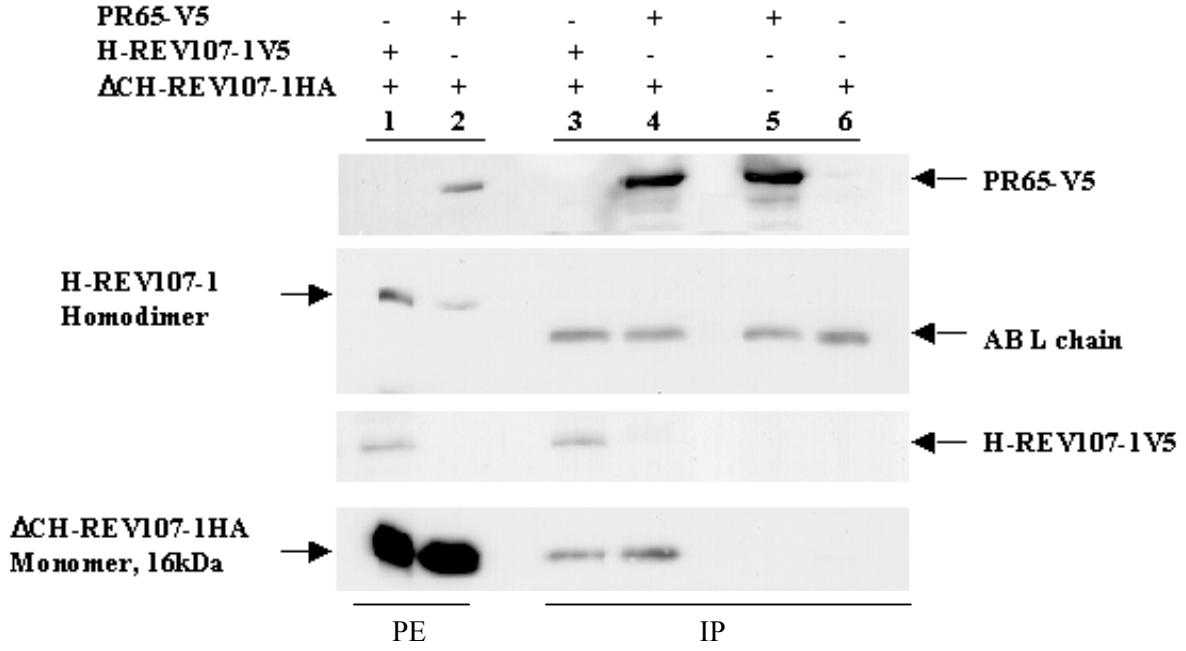


Fig. 21 The H-REV107-1 protein forms a homodimer in COS-7 cells

COS-7 cells were transiently transfected with the ΔCH-REV107-1HA and H-REV107-1V5 expression plasmids, with ΔCH-REV107-1HA and PR65-V5, and with every plasmid alone as a negative control. Forty eight hours after transfection immunoprecipitation with an anti-V5 antibody and Protein G agarose was performed. The co-immunoprecipitated ΔCH-REV107-1HA protein was detected by Western blotting against anti-HA antibody depicted on the bottom panel.

Lane 1 – cell lysate of COS-7 cells transfected with the H-REV107-1V5 and ΔCH-REV107-1HA expression plasmids; lane 2 – cell lysate of COS-7 cells transfected with the PR65-V5 and ΔCH-REV107-1HA expression plasmids; lane 3 – co-immunoprecipitation of ΔCH-REV107-1HA with H-REV107-1V5; lane 4 – co-immunoprecipitation of ΔCH-REV107-1HA with PR65-V5; lane 5, 6 – negative controls.

3.3.5 Determination of the H-REV107-1 Domains Responsible for Interaction with PR65 and Homodimer Formation

3.3.5.1 Generation of the H-REV107-1 Mutant Proteins

To map the region of H-REV107-1 responsible for protein binding, three mutants were generated and tested by co-immunoprecipitation. Selection of the domains to be mutated was based on the analysis of the protein sequence described in the Introduction. In brief, members of the H-REV107 protein family share a highly conserved domain (H-box), the function of which is unknown (Hughes and Stanway, 2000). The ΔC107-HWAY mutant was generated with a point mutation in the H-box leading to the substituting His by Ala at the position 23. The NCE domain was shown to play an important role for the activity of the lecithin retinol acyltransferase (LRAT), a protein which belongs also to the H-REV107 family (Mondal et al., 2000). The ΔC107-NCE mutant comprised the amino acid exchange Cys-112 to Ser-112 in this region. The third ΔC107-ΔN mutant carried a deletion of 20 amino acids at the proline-rich N-terminus (Fig. 22). Such proline-rich domains were shown to be important for protein binding (Kay et al., 2000). All mutants were fused at the C-terminus with the HA-epitope.

3.3.5.2 The N-terminal Domain of the H-REV107-1 Protein is Required for the Interaction with PR65 and for Homodimer Formation

The ability of the ΔC107- NCE, ΔC107-HWAY and ΔC107-ΔN mutants to associate with PR65-V5 was tested using co-immunoprecipitation in COS-7 cells. Proteins were precipitated with the HA-conjugated Sepharose, and examined using Western blot analysis with an anti-V5 antibody. The PR65-V5 protein was detected in a complex with the ΔCH-REV107-1HA protein used as a positive control (Fig. 24 A, upper panel, lane 1), and with the ΔC107-NCE, and ΔC107-HWAY mutant proteins (Fig. 24 A, upper panel, lanes 2, 3 accordingly). The ΔC107-ΔN mutant was unable to bind PR65-V5 (Fig. 24 A, upper panel, lane 4). Upon co-immunoprecipitation with an anti-V5 antibody similar results were obtained. The ΔC107-NCE, and ΔC107-HWAY mutants were shown to associate with PR65-V5 (Fig. 24 B, bottom panel, lane 2, 3). The ΔN107 mutant was not detected in a complex with PR65-V5 (Fig. 24 B, bottom panel, lane 4). This demonstrated that the N-terminal proline-rich domain of the H-REV107-1 protein is required for the interaction with PR65.

To define the protein domain responsible for a homodimer formation, COS-7 cells were transiently transfected with the H-REV107-1V5 expression vector, and a vector expressing each of the mutants, respectively. Immunoprecipitation was done with the anti-V5 antibody. The ΔNH-REV107-1 mutant was also deficient in homodimer formation (Fig. 23, bottom panel, lane 5).

H-REV107-1 protein

1 20 23 27 112 162

MRAPIPEPKPGDLIEIFRPF---**HWAIY**---**NCE**----LIGVMFSRNKRQKQ

ΔC107-ΔN	H-REV107-1 mutant lacking 10 N-terminal aminoacids
ΔC107-HWAY	Mutant carrying a point mutation in HWAY box resulting in His/Ala-23 exchange: HWAIY – AWAIY
ΔC107-NCE	Mutant carrying a point mutation in the NCE domain resulting in Cys/Ser-112 exchange: NCE - NSE

Fig. 22 H-REV107-1 mutants generated for search of the domains responsible for interaction

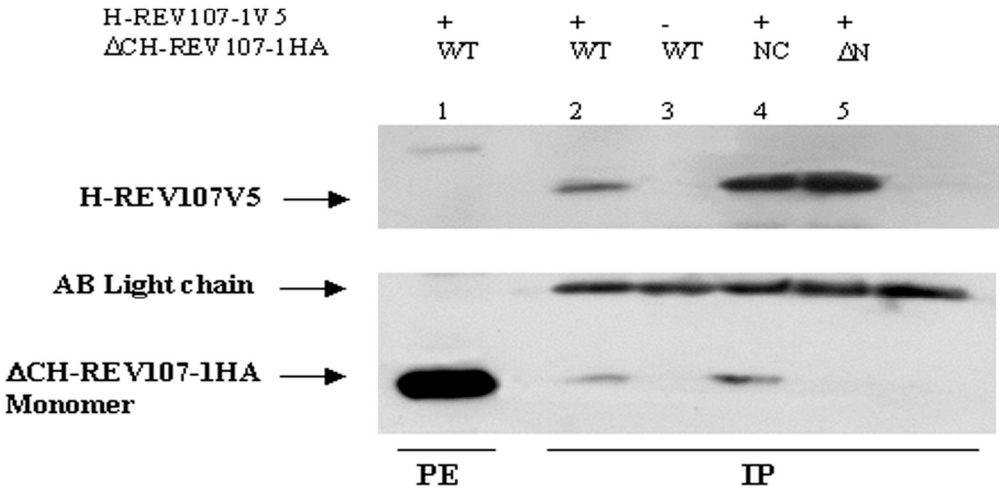


Fig. 23 The ΔC107-ΔN mutant does not form homodimers

COS-7 cells were simultaneously transfected with expression vectors: H-REV107-1V5 and one of the mutants. Protein complexes were precipitated with an anti-V5 antibody. Upper panel – Western blot analysis of the precipitated proteins with an anti-V5 antibody. Bottom panel - Western blotting against anti-HA antibody. Lane 1 – 10μg of protein extract from COS-7 cells transfected with the ΔCH-REV107-1HA expression vector. Lane 2 – immunoprecipitation of ΔCH-REV107HA with H-REV107-1V5 used as a positive control. Lane 3 - negative control. Lane 4 – immunoprecipitation of ΔC107-NCE with H-REV107-1V5. Lane 5 – immunoprecipitation of the ΔC107-ΔN mutant with H-REV107-1V5. Lane 6 – negative control.

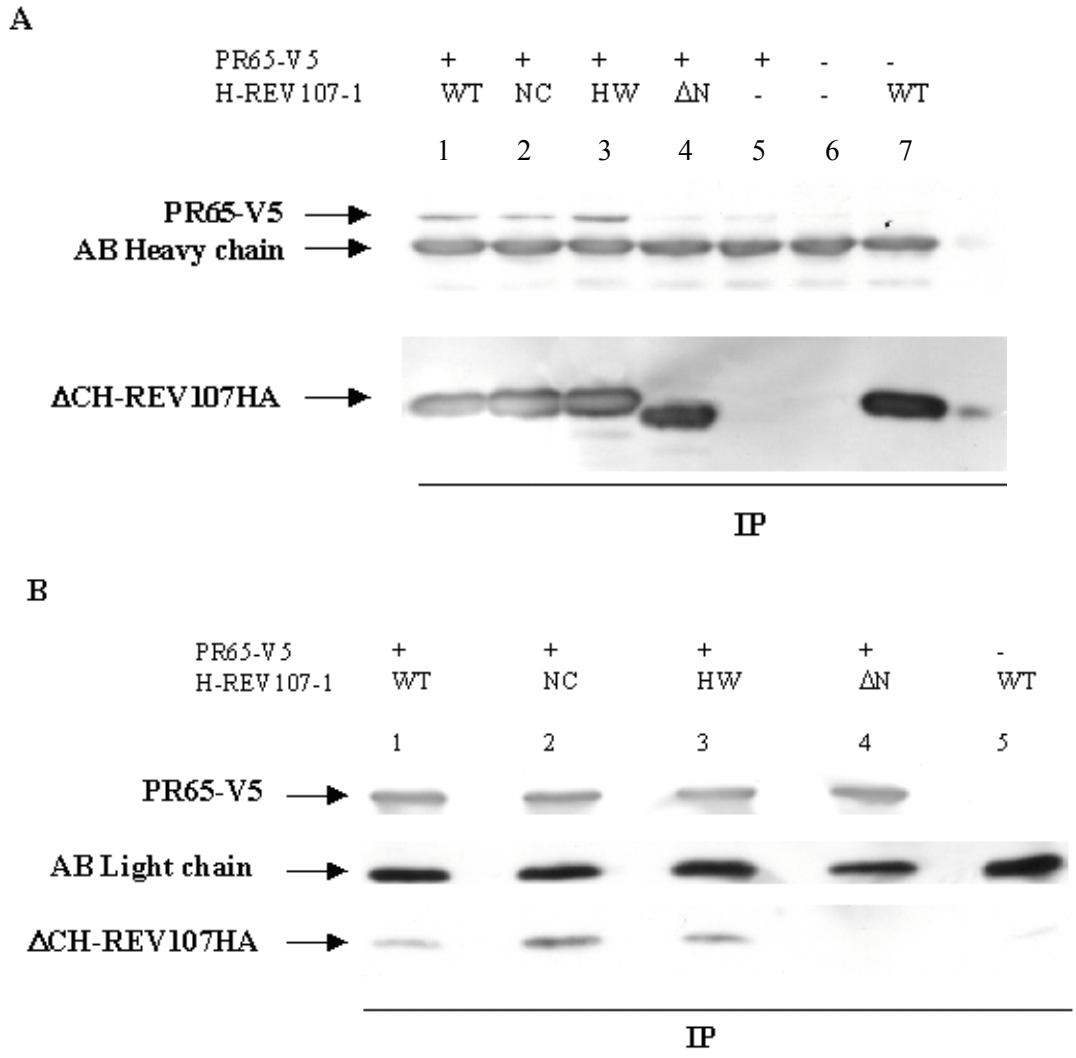


Fig. 24 The ΔN107 mutant fails to interact with PR65

COS-7 cells were co-transfected with the following expression vectors: PR65-V5 and ΔCH-REV107-1 (WT), PR65-V5 and ΔC107-NCE mutant (NC), PR65-V5 and ΔC107-HWAY mutant (HW), and PR65-V5 and ΔC107-ΔN mutant (ΔN). For negative controls plasmids containing epitopes only were transfected (-).

A: immunoprecipitation with HA-conjugated Sepharose. Upper panel – precipitated protein complexes were analysed with an anti-V5 antibody for the PR65-V5 detection. Lane 1 – PR65-V5 was obtained in a complex with ΔCH-REV107-1HA, lane 2 – with the ΔC107-NCE mutant, lane 3 – with the ΔC107-HWAY mutant. Lane 4 – the PR65-V5 protein does not associated with the ΔC107ΔN mutant. Bottom panel – control Western blotting against anti-HA antibody.

B: reciprocal co-immunoprecipitation with an anti-V5 antibody. H-REV107-1 associating with PR65-V5 was revealed by Western blotting against anti-HA antibody. Lane 1 – H-REV107-1 was obtained in the complex with PR65-V5, lane 2 – ΔC107-NCE mutant, lane 3 – ΔC107-HWAY mutant. Lane 4 – the ΔC107-ΔN mutant did not associate with PR65-V5. Upper panel – incubation with an anti-V5 antibody revealed precipitated PR65-V5.

Thus, the N-terminal domain of the H-REV107-1 protein was shown to be responsible for both association with the PR65 protein, and for the homodimer formation. Preliminary experiments were performed to test a potential competition between H-REV107-1 homodimer formation and PR65 binding. In this experiment the PR65 binding eliminated homodimer formation (data not shown).

3.4 Investigation of a Role of the H-REV107-1 - PR65 Interaction in Apoptosis

3.4.1 H-REV107-1 Does not Induces Apoptosis in Rat Fibroblasts FE-8

The aim of further investigations was to explore the mechanism of H-REV107-1 mediated cell death and to define a role of the PR65 – H-REV107-1 interaction in this process. Examination of the role of H-REV107-1 in cell cycle progression by flow cytometric analysis required establishment of stable transfectants over-expressing H-REV107-1. All attempts to generate stable clones in human ovarian carcinoma cell lines OVCAR-3 and A27/80 were unsuccessful, and resulted in the loss of cells over-expressing H-REV107-1. However, an H-rev107 – tetracycline-inducible system in rat fibroblasts FE-8 was available (Sers et al., 1997). Therefore, a potential implication of the H-REV107-1 protein in cell cycle control was tested in this system. Two independent FE-8 clones stably transfected with tet-inducible H-rev107, and one clone transfected with the empty vector as a control (pUHD) were tested in this experiment. Cells were cultured for 24, 48, 72, 96, and 120 hours with or without doxycycline then stained with propidium-iodide (PI) followed flow cytometric analysis. These experiments were performed by Cornelia Giseler, Institute of Pathology, Charité, Berlin. H-rev107 expression was controlled by Western blotting against an anti-H-rev107 antibody (Fig. 25).

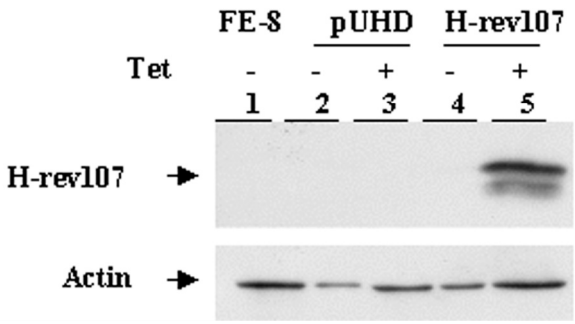


Fig. 25 H-rev107 expression after doxycycline induction in FE-8 cells

Cells were cultured for 48 hours with (+) our without (-) doxycycline, then lysed and subjected to SDS-PAGE. Upper panel – western blot analysis was performed with a polyclonal anti-Hrev107 antibody, bottom panel – anti-actin antibody was used as a loading control. Lane 5 - H-rev107 expression in the FE-8 cells harbouring H-rev107 expression plasmid after induction with doxycycline

Cell lines		G1(%)	G2(%)	S(%)
FE-8 pUHD		38,8	27,8	33,4
FE-8 pUHD + Tet	**48h**	40,2	21,4	38,4
	72h	38,3	25	36,7
	96h	35,6	25,3	39,1
	120h	**37,1**	30,3	32,6
FE-8 H-rev107		42,3	22,9	34,8
FE-8 H-rev107 + Tet	**48h**	51,4	22,9	25,7
	72h	55,9	23,1	21
	96h	59,7	22,6	17,7
	120h	**60**	18,8	21,2

Table 6 Cell cycle analysis of FE-8 cells harbouring tet-inducible H-rev107

FE-8 cells stably transfected with the empty pUHD vector, and cells harbouring H-rev107 under the control of a tetracycline-inducible promoter in pUHD were cultured in Tet-free medium with or without doxycycline. 48, 72, 96 and 120 hours after the induction, cells were stained with propidium iodide and subjected to flow cytometry. Cell cycle progression analysis revealed a tendency of the cells over-expressing H-rev107 to arrest in G1 phase.

Flow cytometry analysis demonstrated that the FE-8 cells over-expressing H-rev107 were arrested in G1, reaching 60% G1 phase after 120 hours. In contrast, only 37% of the pUHD-transfected cells were in the G1 phase at the same time (Table 6). Thus, the H-rev107 protein appeared to induce G1 arrest in rat fibroblasts. At the same time nuclear morphology was analysed using laser confocal microscopy. However no changes were observed after 48, 96, and 120 hours of H-rev107 induction excluding the possibility of apoptosis.

3.4.2 The ΔC107-ΔN Interaction Deficient Mutant Fails to Induce Apoptosis in Human Ovarian Carcinoma Cell Lines A27/80 and OVCAR-3

In contrast to FE-8 cells, forced expression of H-REV107-1 in OVCAR-3 and A27/80 cell lines was already demonstrated to induce apoptosis (Fig. 15). Therefore, we suggested that in different cell types and (or) different species H-REV107-1 exerts different effects on cellular growth. Further investigation of a potential role of the PR65 – H-REV107-1 interaction in the H-REV107-1 – mediated cell death was performed in these cells.

The growth suppressing properties of H-REV107-1 full length, ΔCH-REV107-1, and the interaction deficient mutant (ΔC107-ΔN) were compared using a colony assay. These experiments were kindly performed by Jaqueline Hellwig (Institute of Pathology, Charité, Berlin, Germany). The expression vectors were introduced in A27/80 and OVCAR-3 cells. The number of G418 – resistant colonies was calculated after 2-3 weeks of selection. In OVCAR-3 cells the full length H-REV107-1 protein reduced the number of colonies to 45% of the control. The truncated form (ΔCH-REV107-1) reduced colonies only to 65%, and the mutant (ΔC107-ΔN) was unable to reduce colony formation in OVCAR-3 cells. In A27/80 cells, the colony numbers with full length H-REV107-1 were 78%, the truncated form of the protein inhibited growth up to 55%, and the (ΔC107-ΔN) mutant again failed to reduce number of colonies (Fig. 26).

For further analysis of the H-REV107-1 role in apoptosis induction, the induction of apoptosis by the H-REV107-1 mutants was analysed in OVCAR-3 cells. The cells were transiently transfected with the H-REV107-1, ΔCH-REV107-1, ΔC107-NCE, ΔC107-HWAY, and ΔC107-ΔN expression vectors. Nuclei with apoptotic phenotype were calculated 42 hours after transfection. Expression of the full length H-REV107-1 cDNA resulted in 18,5% apoptotic nuclei, the truncated form lacking the membrane binding domain with or without point mutations led to induction of apoptosis in around 11% of the cells. After transfection of the ΔC107-ΔN deletion mutant only 4,3% of the cells underwent apoptosis. The background was calculated as the number of apoptotic nuclei after transfection with the empty vector, and was found to be 2,6% (Table 7).

These experiments demonstrated that two H-REV107-1 protein domains were important for the growth suppression and induction of apoptosis in OVCAR-3 cells: the C-terminal domain is responsible for the intracellular localisation of H-REV107-1 (Sers et al., 1997), the N-terminal domain mediates the binding of the PR65 protein, and is responsible for homodimer formation.

Transfection	pcDNA3	H-REV107-1	ΔCH-REV107-1	ΔC107-NCE	ΔC107-HWAY	ΔC107-ΔN
Apoptotic cells (%)	2,6	18,5	11,1	12	10,3	4,3

Table 7 H-REV107-1 N-terminal and C-terminal domains play an important role in the induction of apoptosis in OVCAR-3 cells

OVCAR-3 cells were transiently transfected with 6 different expression plasmids. Immunofluorescence analysis was performed 42 hours after transfection. Cells were fixed and stained with DAPI. Apoptotic nuclei were visually determined and calculated using Laser Confocal microscopy. For every transfection more than 300 hundred cells were counted. +

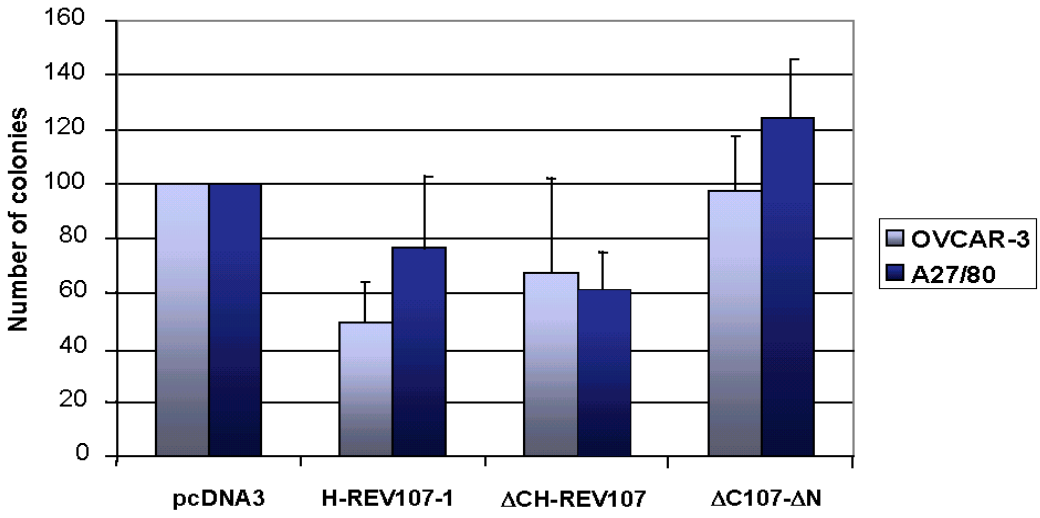


Fig. 26 Reduction of colony formation in OVCAR-3 and A27/80 cells

A27/80 and OVCAR-3 cells were transfected with four different expression plasmids contained: H-REV107-1 full length cDNA, ΔCH-REV107-1, and H-REV107-1 lacking N-terminal and C-terminal domains (ΔC107-ΔN), or pcDNA3 used as a control. G418-resistant colonies were calculated. Number of colonies transfected with pcDNA3 was equated to 100%. The results of four independent experiments were evaluated

3.4.3 Cellular Re-Distribution of the Endogenous PR65 Protein Correlates with the H-REV107-1 Induced Apoptosis in OVCAR-3 Cells

The N-terminal domain of the H-REV107-1 protein was shown to be required for apoptosis in OVCAR-3 and A27/80 cells. This suggested that the interaction of H-REV107-1 with PR65 can be important for this process. Therefore the phenotype of OVCAR-3 cells transiently transfected with the H-REV107-1 and ΔC107-ΔN expression vectors were investigated at a single cell level.

In untransfected cells, PR65 was localised mainly around the nucleoli throughout the interphase, and in the cytoplasm during mitosis (Fig. 27 C, arrow). H-REV107-1 transfection induced a dramatic change in the intracellular distribution of PR65 and in the appearance of the nuclei. In the cells expressing H-REV107-1, PR65 immunofluorescence was seen only in the cytoplasm, that accompanied apoptotic nuclear morphology (Fig. 27 A, yellow arrows). The ΔNΔC107-1 mutant was distributed through the cytoplasm and the nucleus (Fig. 27 B, upper right panel).

The protein was unable either to induce apoptosis, or to change PR65 localisation (Fig. 27 B, yellow arrows).

Colocalisation analysis of H-REV107-1 and PR65 immunostaining revealed that the change in the distribution of the PR65 protein induced by H-REV107-1 correlated with a co-distribution of these two proteins. This can be clearly observed in the overlay images (Fig. 27 A, B, icons “Overlay”) in which yellow colour (a result of the addition of green and red spectra) indicate colocalisation. Yellow coloured areas were seen only for H-REV107-1 and PR65 staining, demonstrating that these proteins are colocalised (Fig. 27 A, icons “Overlay”). For the ΔNΔC107-1 and PR65 proteins colocalisation regions were not obtained. (Fig. 27 B, icon “Overlay”). In order to quantify these observations, the yellow, red and green pixels were counted and depicted as a diagram (Fig. 27 A, B, icons “Colocalisation”; these data were kindly provided by Kerstin Lehmann, MetaGen, Berlin, Germany).

These results demonstrated a direct correlation between interaction of H-REV107-1 with the PR65 protein, and the ability of H-REV107-1 to induce apoptosis in OVCAR-3 cells accompanied by the transport of PR65 from the nucleus to the cytoplasm.

The PP2A protein appears in cells mostly as a dimeric core complex consisting of the 36-kDa catalytic C subunit (PR36, α or β isoforms) and the 65-kDa “scaffolding” regulatory A subunit (PR65, α or β isoforms), or as a trimeric complex containing variable regulatory B subunits bound to the AC dimer (Janssens and Goris, 2001; Millward et al., 1999). The PR65α was demonstrated to interact with H-REV107-1. We asked whether H-REV107-1 – PR65α protein complex might interact with the PP2A catalytic subunits, and if over-expression of the H-REV107-1 protein in OVCAR-3 cells can alter the intracellular distribution of other PP2A subunits. The localisation of the catalytic subunit PR36 was determined in OVCAR-3 cells using immunofluorescence analysis.

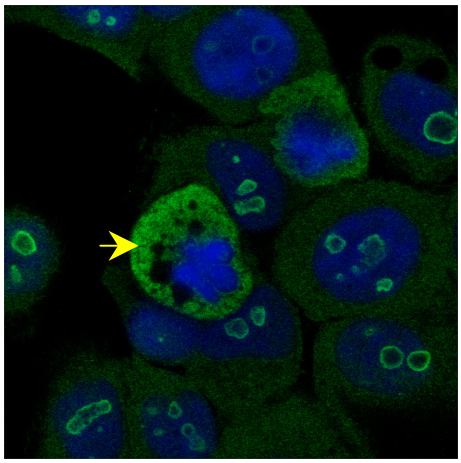
The commercially available antibody used recognised only the PR36 α isoform. Immunofluorescence analysis revealed that the catalytic subunit is distributed invariably through the cytoplasm (Fig. 27 C), and that over-expression of H-REV107-1 did not alter the protein localisation (data not shown). The expression and the intracellular distribution of other PP2A subunits is currently investigated.

3.4.4 H-REV107-1 Inhibits PP2A Activity in vitro

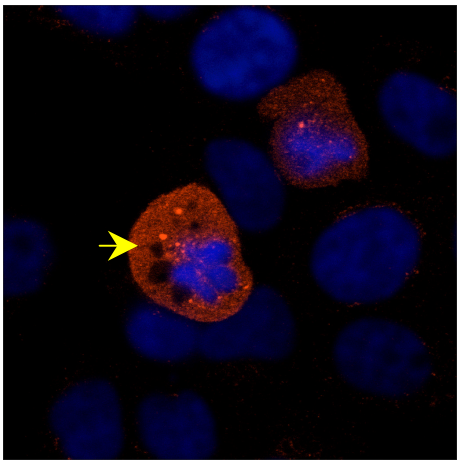
Finally, we asked if the interaction between H-REV107-1 and PR65α might influence the PP2A catalytic activity in OVCAR-3 cells. Therefore, the ability of PP2A, precipitated from OVCAR-3 cells, to dephosphorylate the synthetic substrate p-Nitrophenyl phosphate (pNPP) was measured *in vitro*. OVCAR-3 cells were transfected either with the pcDNA3 vector, or with the H-REV107-1, ΔCH-REV107-1, and ΔC107-ΔN expression vectors. As a control, OVCAR-3 cells were treated during 1 hour with the PP2A inhibitor okadaic acid at 2 nM.

A

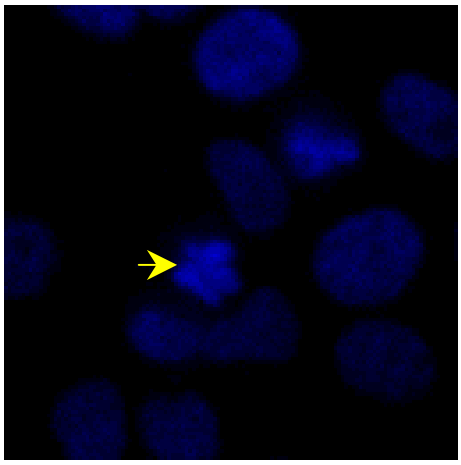
PR65



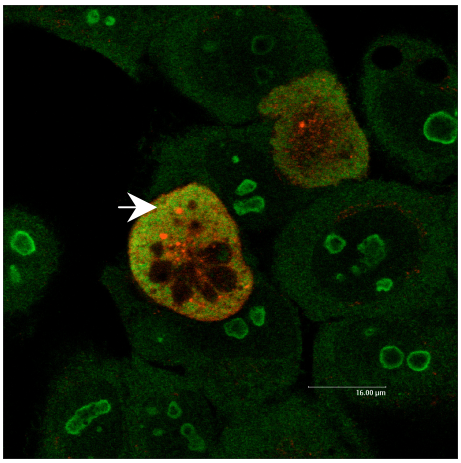
H-REV107-1



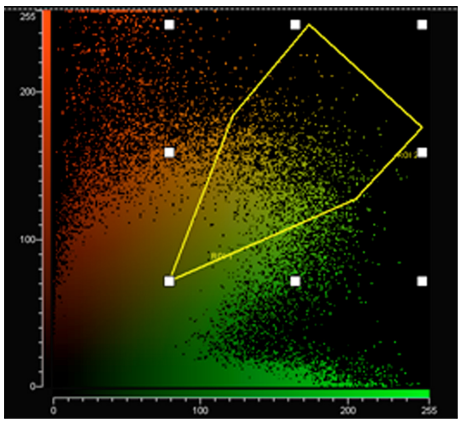
DAPI



Overlay

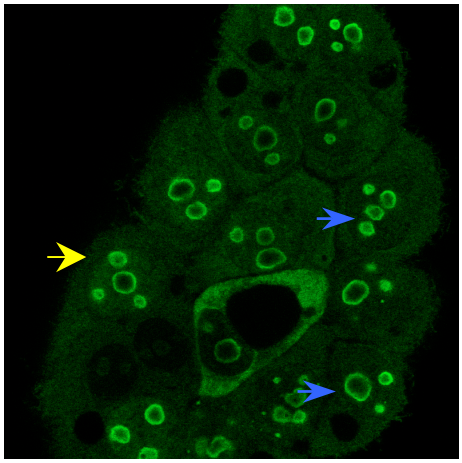


Colocalisation

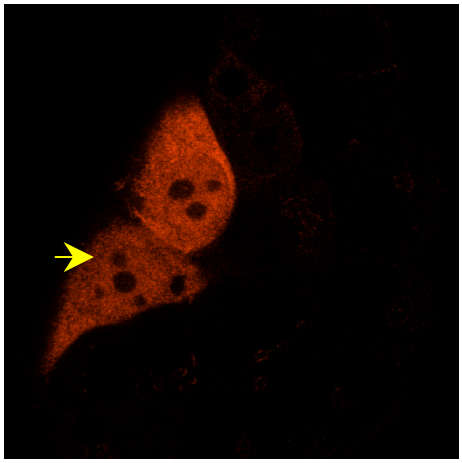


B

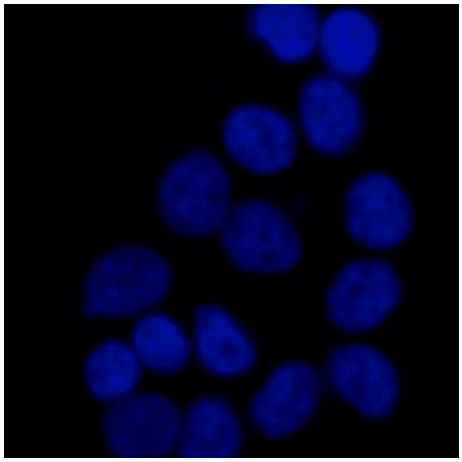
PR65



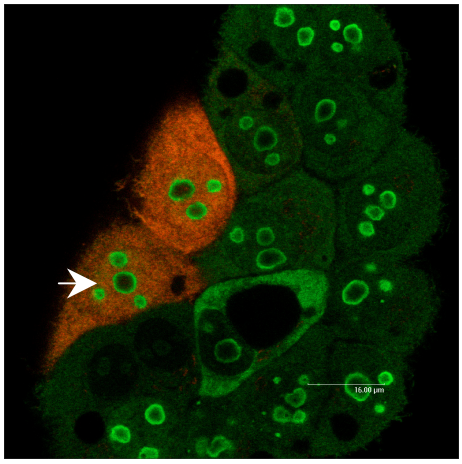
ΔC107-ΔN



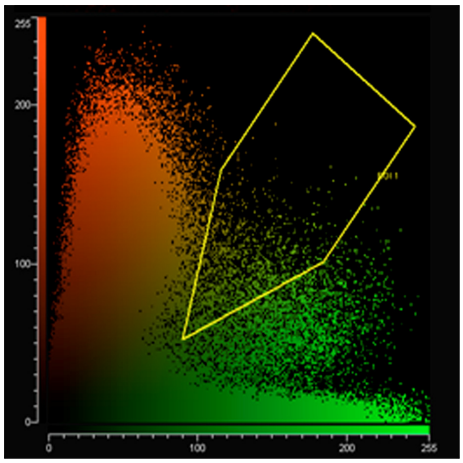
DAPI



Overlay



Colocalisation



C

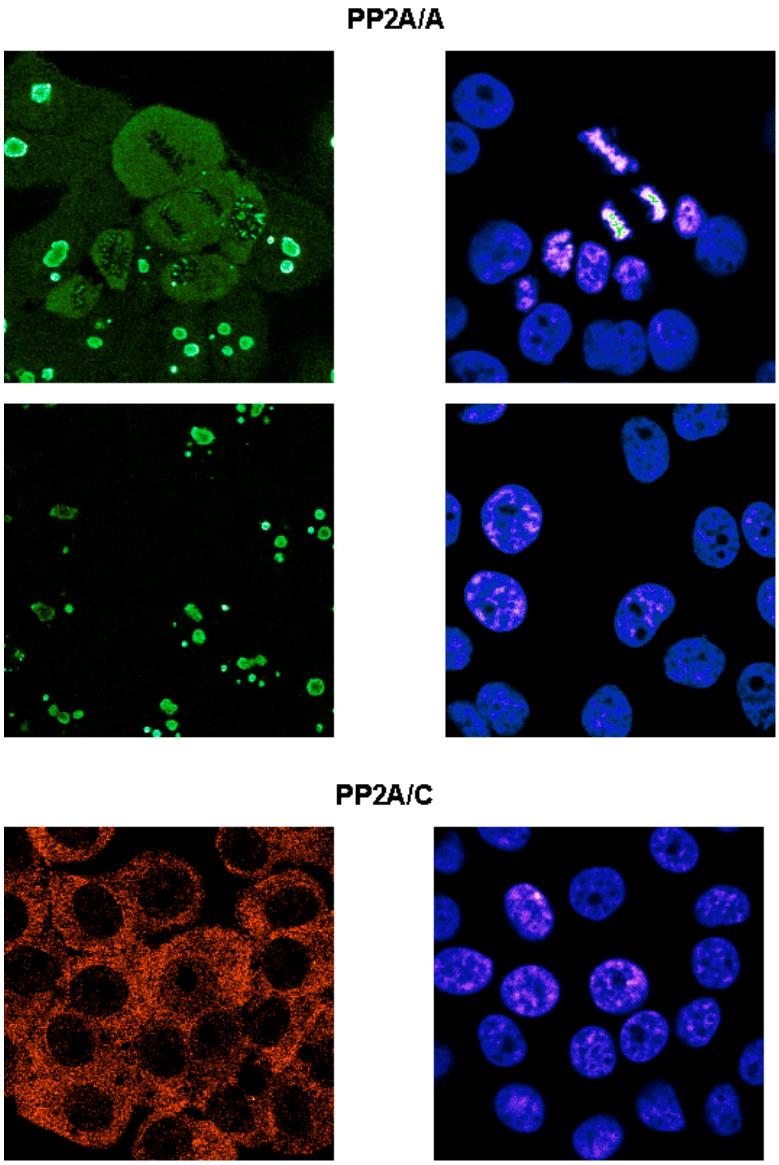


Fig. 27 Induction of apoptosis in OVCAR-3 cells after H-REV107-1 over-expression is correlated with a re-distribution of the PR65 protein.

A, B: OVCAR-3 cells were transiently transfected with the H-RE107-1 (A) and ΔC107-ΔN (B) expression vectors. Forty six hours after transfection immunofluorescence was performed. Cells were stained with monoclonal anti-PR65, and a polyclonal anti-H-REV107-1 primary antibodies, and anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 594 secondary antibodies. Nuclei were stained with DAPI. Immunofluorescence was analysed with the help of laser confocal microscopy. Overlay was performed using LCS Multicolour-Software.

C: Immunofluorescence of untransfected OVCAR-3 cells was performed to investigate the intracellular localisation of the endogenous PP2A catalytic subunit, PR36. Cells were stained with monoclonal anti-PR36 or anti PR65 antibodies, and anti-mouse Alexa-Fluor 488 secondary antibody

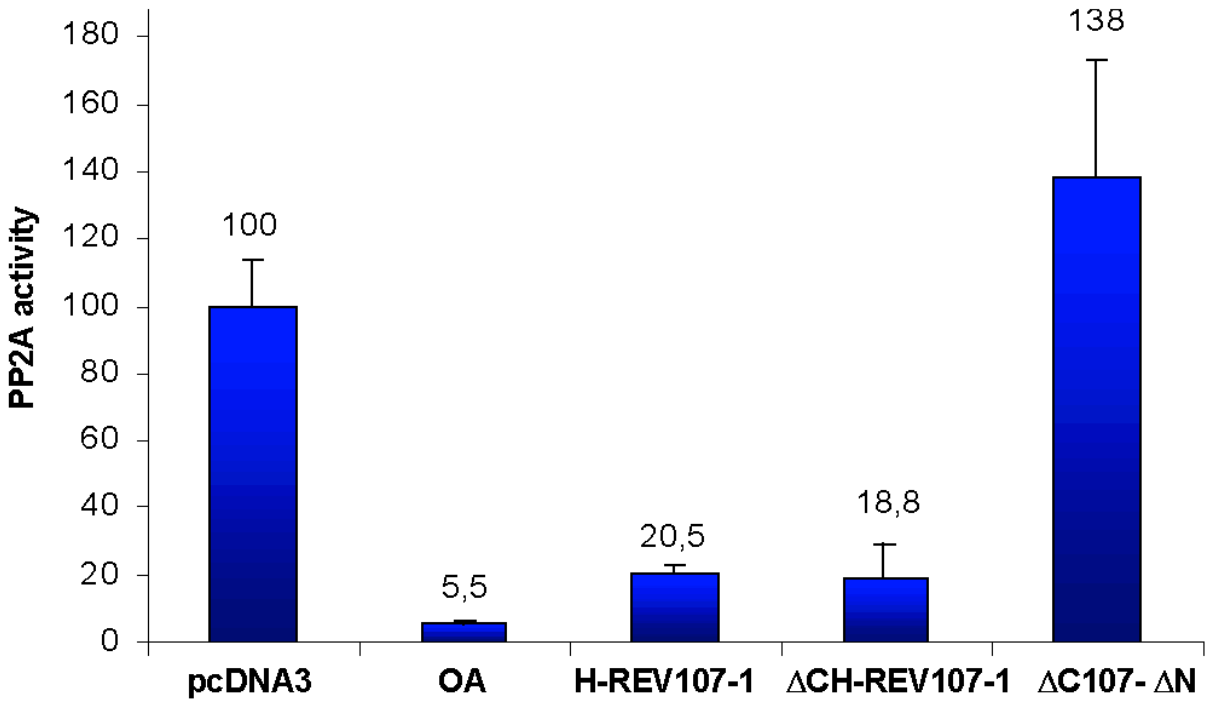


Fig. 28 The H-REV107-1 protein inhibits the catalytic activity of PP2A in vitro

OVCAR-3 cells were transiently transfected with the H-REV107-1, ΔCH-REV107-1, and the ΔC107-ΔN mutant expression vectors. Cells transfected with the pcDNA3 empty vector were used as a positive control. For the negative control untransfected cells were treated for 1 hour with 2 nM okadaic acid. Forty six hours after transfection cells were lysed in phosphatase lysis buffer and immunoprecipitated with an anti-PP2A/A antibody. Protein phosphatase activity of the precipitated protein complexes was measured by pNPP hydrolysis at 405 nm. Absorbance units reflected the phosphatase activity. PP2A activity of cells transfected with a pcDNA3 vector was set 100%.

The amount of precipitated phosphatase, and *H-REV107-1* expression were controlled by Western Blot analysis of the precipitated complexes and protein extracts, respectively (Fig. 29). The PP2A activity measured in the pcDNA3 transfected OVCAR-3 cells was set 100%. Treatment of OVCAR-3 cells with okadaic acid inhibited the phosphatase activity up to 94.5%. Surprisingly, the H-REV107-1 protein also inhibited PP2A activity by almost 80%. The ΔC107-1ΔN mutant was unable to inhibit PP2A (Fig. 28).

Thus, PP2A is active in untransfected OVCAR-3 cells, although regulatory and catalytic subunits are preferentially localised in different cellular compartments. Overexpression of the H-REV107-1 protein leads to the inhibition of the PP2A activity, and the N-terminus of H-REV107-1 appeared to be important for the inhibition, suggesting that the interaction between H-REV107-1 and PR65α is required for this process.

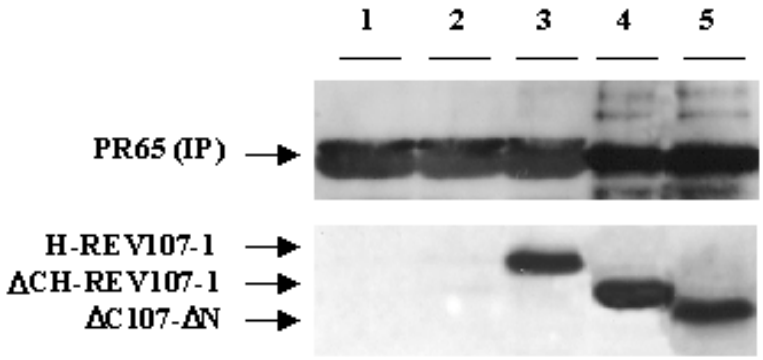


Fig. 29 Control Western blot analysis of the precipitated protein complexes

Upper panel – precipitated proteins were analysed by western blotting with an anti-PR65 antibody. Bottom panel – control western blot analysis of the protein extracts with an anti-H-REV107-1 antibody.

Lane 1 – OVCAR-3 cells transfected with an pcDNA3 plasmid, lane 2 – OVCAR-3 treated for 2 hours with okadaic acid 2 nM (OA), lane 3 – OVCAR-3 cells transfected with the H-REV107-1 expression vector, lane 4 – ΔCH-REV107-1, lane 5 – ΔC107-ΔN protein.

3.4.5 Okadaic Acid Induces Apoptosis in OVCAR-3 Cells

Since the induction of apoptosis correlated with the H-REV107-1 ability to bind PR65 and to inhibit PP2A activity, the natural PP2A inhibitor, okadaic acid was used to study the potential role of the phosphatase in the cell survival. OVCAR-3 cells were treated with 10 nM okadaic acid (OA) for 48 hours and 72 hours, as a control DMSO was added to the medium. The percentages of cells undergoing apoptosis were quantified by dual-coloured flow cytometric analysis after staining with Propidium iodide and Annexin-V. Treatment of OVCAR-3 cells with 10 nM OA for 48 hours resulted in 12,1 % apoptotic cells, after 72 hours 54,8% of cells underwent apoptosis (Fig. 30).

Although in other cell lines PP2A was demonstrated to play a pro-apoptotic role (Klumpp and Krieglstein, 2002; Moon and Learner, 2003), the protein phosphatase 2 A was likely to be important for the survival in OVCAR-3 cells.

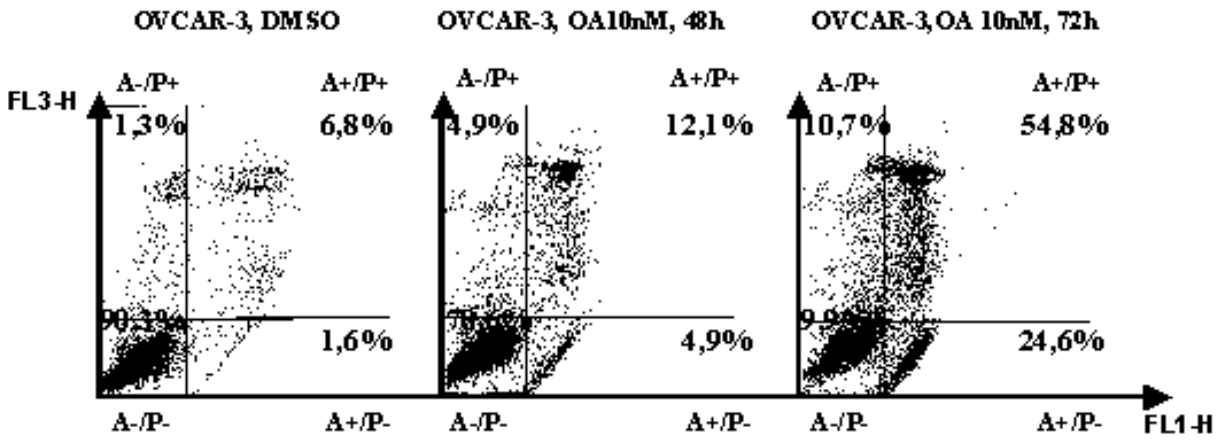


Fig. 30 Induction of apoptosis by OA treatment in OVCAR-3 cells

Subconfluent OVCAR-3 cells were treated either with 10 nM OA for 48 and 72 hours or with its vehicle DMSO for 72 hours. After harvesting the cells were subsequently stained with Propidium iodide (P) and fluorescein isothiocyanate (FITC)-conjugated anti-Annexin-V antibody (A). The cytograms demonstrate Annexin – binding (ordinate) versus Propidium iodide – binding (abscissa) in DMSO and OA – treated cells. Vital cells (A-/P-), pre-apoptotic cells (A+/P-), apoptotic cells (A+/P+), and residual damaged cells (A-/P+) are shown in respective quadrants.

3.4.6 PP2A Inhibition in OVCAR-3 Cells Leads to the Activation of Procaspase-9

Okadaic acid, when applied in a concentration of 10 nM, inhibits the activity of two protein phosphatases: PP2A and PP1 (Ishihara et al., 1989). The PP2A – specific concentration of 0.5 nM was used in further experiment to test if the induction of apoptosis in OVCAR-3 cells required only PP2A inhibition. Two known apoptotic pathways result in the activation of caspases. The first one is receptor-autonomous, depends on the participation of mitochondria and results in the activation of procaspase-9. The second pathway involves the interaction of death receptors with their ligands, and results in procaspase-8 activation (Zimmerman et al., 2001). Induction of these two pathways was investigated in OVCAR-3 cells.

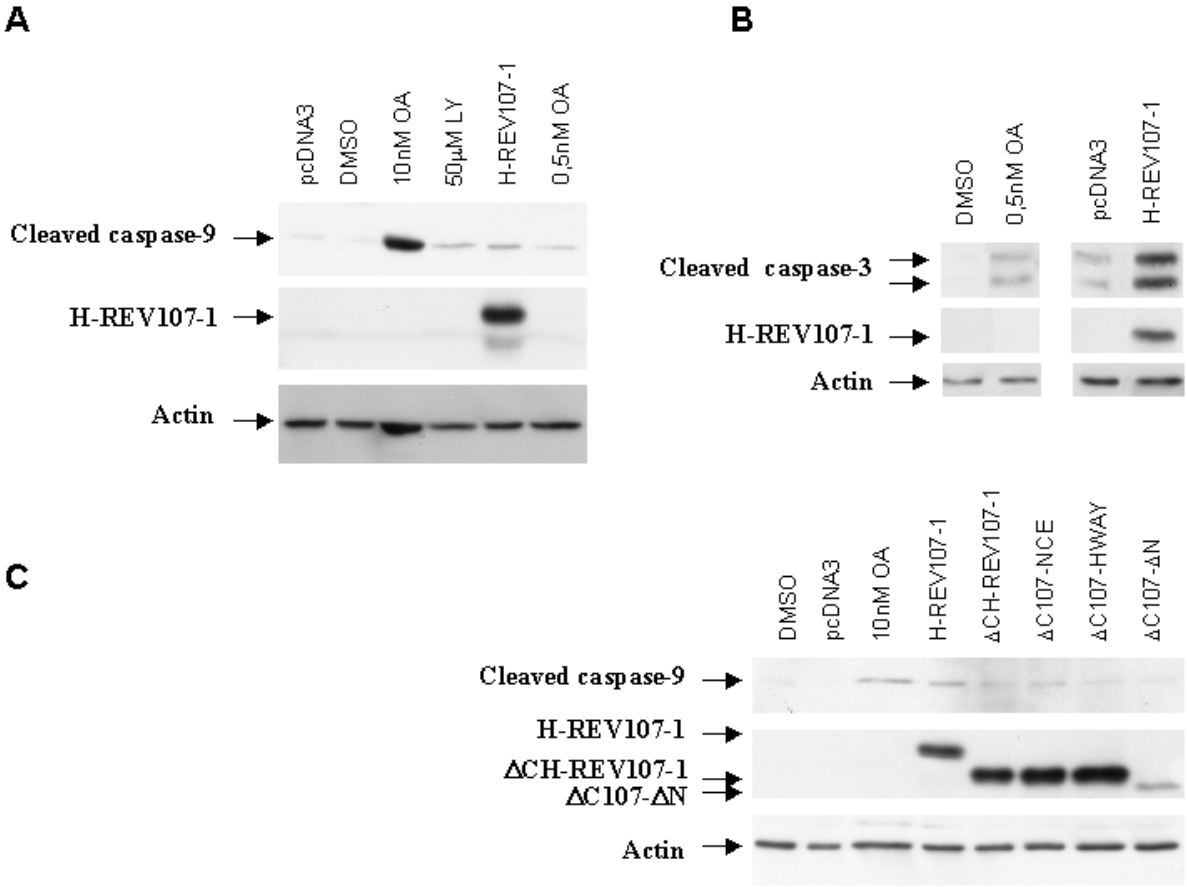


Fig. 31 H-REV107-1 overexpression and OA treatment of OVCAR-3 cells activate procaspases –9 and –3

OVCAR-3 cells were transiently transfected with the H-REV107-1, ΔCH-REV107-1, ΔC107-NCE, ΔC107-HWAY, and ΔC107-ΔN expression vectors, or treated with 0.5 nm and 10 nm OA. Protein extracts were harvested 44 hours after transfection, and subjected to SDS-PAGE followed by Western Blot analysis.

A: Western blotting against cleaved caspase-9 antibody. Activated caspase-9 was revealed in OVCAR-3 cells after OA, or LY294002 treatment, or after H-REV107-1 expression. B: Activated downstream caspase-3 was detected after treatment with OA 0,5 nM and after H-REV107-1 ectopic expression. DMSO and pcDNA3 are negative controls. C: Induction of caspase-9 was tested in OVCAR-3 cells expressing H-REV107-1 wild type or the mutants. ΔC107-ΔN failed to induce cleavage of the caspase-9.

Ectopic expression of the H-REV107-1 protein and okadaic acid treatment resulted in activation of the procaspase-9. Cleaved caspase-8 was not detected (data not shown). Cleaved caspase-3 was also tested as one of the down-stream caspases. The cleaved product was revealed after H-REV107-1 transfection and OA exposition (Fig 31 B). This demonstrated that the H-REV107-1 protein, similar to the PP2A inhibitor okadaic acid, induces apoptosis through the mitochondrial-dependent pathway.

High level of cleaved caspase-9 and -3 were only found after H-REV107-1 overexpression, and after treatment with OA of 10 nM, whereas incubation with 0.5 nM OA resulted in considerably weaker activation of both caspases (Fig. 31 A, B). This suggests that in OVCAR-3 cells 0.5 nM okadaic acid is insufficient for a complete inactivation of the PP2A activity.

The ability of the H-REV107-1 mutants to activate cleavage of procaspase-9 was examined in further experiments. The result confirmed the previous observations. Overexpression of the ΔCH-REV107-1, ΔC107-NCE and ΔC107-HWAY mutants resulted in a weaker activation of procaspase-9 in comparison with the wild type H-REV107-1. The ΔC107-ΔN truncated protein failed to induce procaspase-9 cleavage in OVCAR-3 cells (Fig. 31 C).

3.5 Confirmation of Interaction between H-REV107-1 and RARG, S100A6, ETF1, and P14.5

3.5.1 RARG

The retinoic acid receptor gamma (RARG) belongs to the steroid/thyroid hormone nuclear receptor superfamily (Evan, 1988). This family contains two distinct sub-classes of proteins, the all-trans retinoic acid (ATRA) and the 9-cis retinoic acid receptors RAR and RXR. Each sub-class consists of α, β, and γ isoforms (Gudas, 1992). The RAR and RXR proteins form homodimers or heterodimers, and act as nuclear transcription factors (Zhang et al., 1992). In view of the growth suppressive properties of ATRA, and the participation of RARG in this signaling, we suggested a possible functional co-operation of the H-REV107-1 and RARG proteins in growth control. Therefore, we analysed the interaction between H-REV107-1 and RARG in COS-7 cells.

The mammalian expression vector containing full length of the *RARG* cDNA fused to a V5-epitope (RARG-V5) was purchased from GeneStorm Collection of Invitrogen. For co-immunoprecipitation of ΔCH-REV107-1HA and RARG-V5, COS-7 cells were transiently transfected with the corresponding expression plasmids, however no interaction was detected (data not shown). From the literature it was known that binding with ATRA or its antagonists can alter the binding capacity of RARG (Zhang et al., 1992).

Potential candidates that could improve the interaction between RARG and H-REV107-1 were all-trans retinoic acid (ATRA), an antagonist of ATRA, (E)-4-(2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthelenyl)-propen-1yl benzoic acid) TTNPB, and 27 bp double-strand oligonucleotides containing the retinoic acid responsive element (RARE), DR5. The mutated RARE, DR5M was used as a negative control in co-immunoprecipitation (Fig. 32).

H-REV107-1HA did not interact with RARGV5 without supplementary ligands (Fig. 32 A, lane 4). Addition of either TTNPB, or DR5 weakly enhanced the interaction (Fig. 32 A, lane 5; Fig. 32 B, lanes 3, 5). A combination of TTNPB and DR5 had an additive effect (Fig. 32A, lane 6; Fig. 32 B, lane 2), ATRA did not affect the interaction.

3.5.2 S100A6, ETF1, and P14.5

To verify the interaction between H-REV107-1 and the S100A6, ETF1, and P14.5 proteins by co-immunoprecipitation, we generated several mammalian expression vectors. The open reading frames (ORF) encoding S100A6, and ETF1 proteins fused with an HA-epitope, and P14.5 protein without epitope, were amplified by PCR, and cloned into the pcDNA3.1 mammalian expression vector resulting in the S100A6-HA, ETF1-HA, and P14.5 expression plasmids. Sequencing analysis of the cloned inserts confirmed that S100A6, and p14.5 contained full length cDNAs. The ETF1 insert included 711 bp encoding the C-terminal domain of 237 amino acids of the ETF1 protein, which has a full length of 437 aminoacids.

3.5.2.1 S100A6

The S100A6/Calcyclin protein belongs to the S100 subfamily of Ca(2+)-binding proteins (Ferrari et al., 1987). It was shown to be over-expressed in a variety of tumor cell lines and human tumours (Maelandsmo et al., 2000), to be associated with invasion in colorectal adenocarcinoma and metastasis in liver cancer (Komatsu et al., 2002). The first co-immunoprecipitation experiment under standard conditions revealed a negative result (data not shown). However, the protein-protein interaction between S100A6 and S100A2 proteins required an activation of Calcyclin by Ca^{2+} and Zn^{2+} ions (Filipek et al., 1999). In the following experiments a lysis buffer containing different concentration of Ca^{2+} and Zn^{2+}- ions ranging from 1 nM to 100 nM was tested. A concentration of 2 nM of both ions was found optimal. For the co-immunoprecipitation, COS-7 cells were transiently transfected with the H-REV107-1V5 and S100A6HA expression vectors. The cells were lysed in the lysis buffer containing 2 nM $CaCl_2$ and $ZnCl_2$, and precipitated using the anti-V5 antibody (Fig. 33). Co-immunoprecipitation of the S100A6 protein with H-REV107-1 has been reproduced two times.

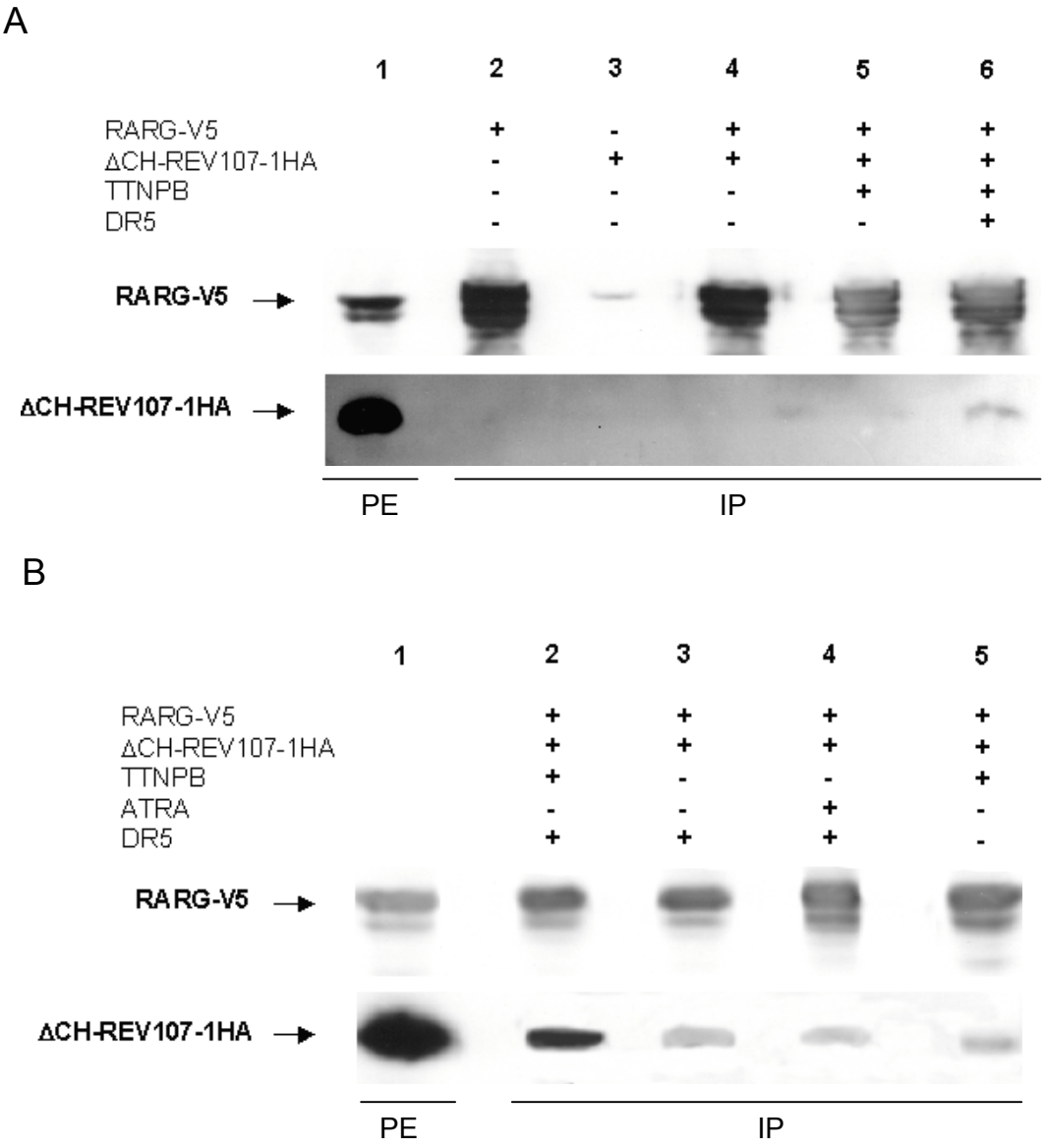


Fig. 32 TTNPB and DR5 are essential for the interaction between RARG and H-REV107-

COS-7 cells were transiently transfected with the H-REV107-1HA and RARGV5 expression vectors, and lysed 48 hours post transfection. As a negative control COS-7 cells were transfected with the epitope – containing plasmids without insert. The whole cell extract was used for immunoprecipitation with an anti-V5 antibody. H-REV107-1 was identified in the immunocomplexes using anti-HA antibody. RARGV5 was identified using an antibody against V5 epitope. A: lane 2, 3 – negative controls, lane 4 – immunoprecipitation in the absence of additional ligands; lane 5 – in presence of TTNPB; lane 6 – with both TTNPB ligand and DR5 RARE. B: Examination of a role of different ligands on the interaction. Lane 2 – DR5 and TTNPB, lane 3 – DR5, lane 4 – DR5 and ATRA, lane 5 – TTNPB.

3.5.2.2 ETF1

Eukaryotic translation termination factor 1 (ETF1) belongs to a family of tightly related proteins catalysing termination of protein synthesis (Guenet et al., 1999). Immunoprecipitation with an anti-V5 antibody was performed in COS-7 cells transiently transfected with the H-REV107-1V5 and ETF1-HA expression vectors. Immunoprecipitated protein complexes were analysed by Western blotting with anti-HA, and anti-V5 antibodies. The interaction between these two proteins was very weak (Fig. 34). This correlated with the results obtained in the mating test where the interaction between H-REV107-1 and ETF1 was also shown to be weak (Fig. 7). A co-immunoprecipitation experiment *vice versa* with an anti-HA antibody revealed a negative result (data not shown). These experiments demonstrated that the interaction between ETF1 and H-REV107-1 in COS-7 cells is very weak, and it was not investigated further.

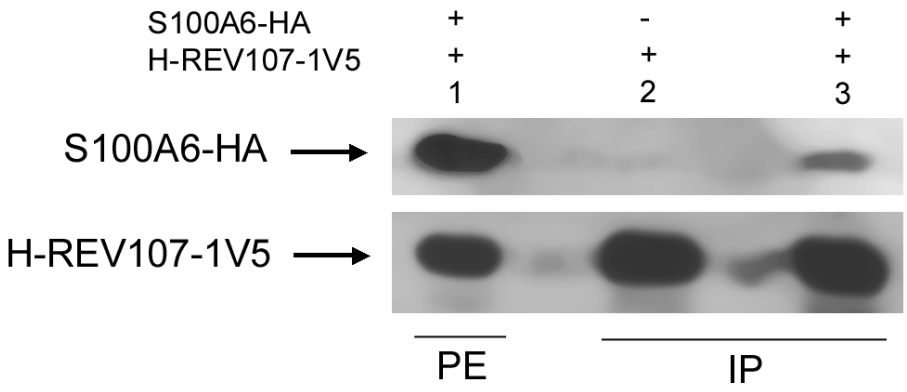


Fig. 33 Calcyclin (S100A6) interacts with H-REV107-1 in COS-7 cells

COS-7 cells were transiently transfected with the H-REV107-1V5 and S100A6HA expression vectors and lysed in the Ca^{2+} and Zn^{2+} containing lysis buffer 48 hours after transfection. As a negative control cells were transfected with epitopes- containing plasmids without inserts. One mg of the protein extract was used for the co-immunoprecipitation with an anti-V5 antibody. Protein complexes were subjected to SDS-PAGE and immunoblotted against anti-V5 and -HA antibodies.

Upper panel – H-REV107-1V5 was immunoprecipitated and detected with an anti-V5 antibody, Lower panel – co-precipitated S100A6-HA was detected with an anti-HA antibody. Lane 1 – 15μg of protein extract used for the co-immunoprecipitation. Lane 2 – negative control. Lane 3 – co-immunoprecipitation of the H-REV107-1V5 and S100A6HA proteins.

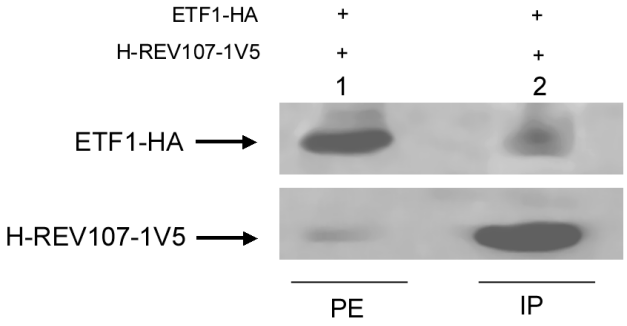


Fig. 34 H-REV107-1 interacts weakly with ETF1 in COS-7 cells

COS-7 cells were transiently transfected with the H-REV107-1V5 and ETF1-HA expression vectors. Co-immunoprecipitation was performed 48 hours after transfection with an anti-V5 antibody.

Upper panel – protein complexes were analysed by Western blotting against an anti-HA antibody, lower panel – against an anti-V5 antibody. Lane "PE" – 10μg of protein extract used for the immunoprecipitation. Lane "IP" – co-immunoprecipitation of the H-REV107-1V5 and ETF1HA proteins.

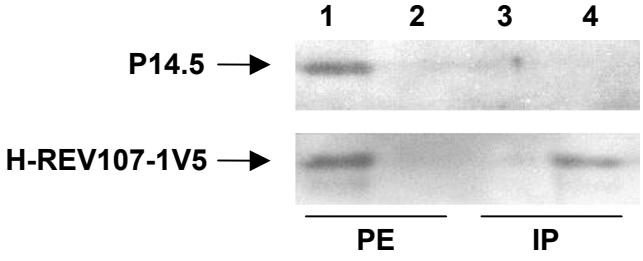


Fig. 35 H-REV107-1 fails to bind P14.5 in COS-7 cells

H-REV107-1-V5 and p14.5 – expression vectors were simultaneously transfected into COS-7 cells. As a negative COS-7 cells were transfected with epitope-containing plasmids without insert. Immunoprecipitation with an anti-V5 antibody was performed 48 hours after transfection.
Upper panel: Western blotting with an anti-p14.5 antibody; lower panel: Western blotting with an anti-V5 antibody.
Lane 1 - protein extracts used for the immunoprecipitation; lane 2, 3 – negative controls; lane 4 – co-immunoprecipitation of the H-REV107-1 and P14.5 proteins.
Abbreviations: PE – protein extract, IP – co-immunoprecipitation.

3.5.2.3 P14.5

The human protein P14.5 was identified in 1996 as the homologue of the rat perchloric acid-soluble protein. Its weak expression in a variety of liver and kidney tumor cells and high expression in fully differentiated cells suggested that the protein is involved in differentiation. The protein was described as an inhibitor of protein synthesis in vitro (Schmiedeknecht et al., 1996). To prove whether H-REV107-1 interacts with P14.5, COS-7 cells were transiently transfected with P14.5 and H-REV107-1V5 expression vectors. The cells were lysed 48 hours post transfection, and H-REV107-1V5 was immunoprecipitated using the anti-V5 antibody. Western Blot analysis of the immunoprecipitated protein complexes revealed H-REV107-1V5 (Fig. 35, lane 4) but not the P14.5 protein, although P14.5 was presented in the protein extract used for co-immunoprecipitation (Fig. 35, lane 4 and 1, respectively). It was concluded that under the conditions used, H-REV107-1 does not interact with the P14.5 protein in COS-7 cells.

4 Discussion

4.1 Yeast Two–Hybrid System

These studies were driven by the desire to understand the mechanism of action of the class II tumor suppressor gene *H-REV107-1*. Since the rat *H-rev107* gene has been cloned, several reports were published about *H-REV107-1* and its related genes in different species (Hajnal et al., 1994; Kuchinke et al., 1995; Sers et al., 1997; Husmann et al., 1998; Di Sepio et al., 1998; Akiyama et al., 1999; Huang et al., 2000; Ito et al., 2001; Sers et al., 2002). The main common features of these genes are that they are down-regulated in tumors and tumor derived cell lines, and possess growth suppressive properties. The primary sequence of the H-REV107-1 product did not provide any clues to the mechanism of its function as a growth suppressor. In order to elucidate the mechanism of human H-REV107-1 mediated growth inhibition, we performed a search for interacting proteins using a yeast two-hybrid assay.

Yeast two hybrid systems were developed in 1990 by Stanley and his colleagues, and provided a novel way of detecting protein-protein interactions *in vivo*. One of the first examples of its application was the identification of the Raf Kinase as a Ras interactor and a downstream effector of Ras function (Vojtek et al., 2003).

We have used a LexA-based yeast two-hybrid system supposed to produce fewer false positives as compared to the GAL4-based system. This proposition is based on the fact that in the LexA system the DNA–binding domain (BD) and the activation domain (AD) are provided entirely by the prokaryotic LexA, and B42 *E. coli* proteins, respectively. The prokaryotic proteins are supposed to interact with very few of yeast or mammalian proteins over-expressed in yeast cells. In contrast, the GAL4 system contains plasmids with eukaryotic GAL4-DNA binding and activation domains which are supposed to be less selective. Nevertheless, some interactions can be recognised only by using a LexA-based yeast two hybrid system, and other interactions can be identified only with the help of the GAL4-based system (Golemis et al., 1994). The reason for this observation is unknown.

Prior to a large scale transformation for a library screening, the human H-REV107-1 protein was kindly tested by E. Cuppen (Department of Cell Biology, Institute of Cellular Signalling, University Nijmegen, The Netherlands) in an established yeast two hybrid approach. This test demonstrated that for a successful application of the approach, the truncated form of the H-REV107-1 protein without the membrane binding domain has to be used. Expression of the full length protein in yeast did not resulted in colony growth. We supposed that the membrane binding domain hampered a transport of the protein into the yeast nucleus where the interactions with library proteins take place. Therefore, we performed the entire screen using a truncated form of the H-REV107-1 protein, lacking the 27 C-terminal aminoacids.

The use of a truncated protein bears the risk of loosing some interactors, which bind to the deleted region. However, it was rather doubtful that a transmembrane domain will participate in protein-protein binding. Most investigations of protein-protein interactions demonstrated that membrane bound domains do not participate in interaction with cytoplasmic proteins. There are only a few examples demonstrating that such domains can be involved in interaction with other proteins, or in the formation of homodimers within a membrane (Langosch et al., 2002). The bacterial protein glycophorin is known to interact with other proteins via its transmembrane domain, although the mechanism of this protein-protein recognition is unclear (Gerber and Shai, 2002). In eukaryotes, large transmembrane proteins participating in transmembrane channel organisation were demonstrated to form homo- and heterodimers via the transmembrane regions (Nakayama et al, 2002).

Although the LexA-and GAL4-based yeast two hybrid systems are powerful tools for detecting protein-protein interactions, they have several disadvantages. They produce false positives, when library proteins intrinsically modulate transcription, for example when they function as transcriptional activators or repressors. In addition, the interaction between bait and pray takes place in the yeast nucleus, where proteins do not undergo posttranslational modifications like terminal glycosylation, sulfation, phosphorylation or methylation. Interactions that require protein posttranslational modifications in the cytoplasm can not be detected by these systems, as well as interactions which require modulating factors present in the cytoplasm or the cell membrane. These aspects were very important for our assay, because we supposed that the human H-REV107-1 protein is subject to posttranslational modification. Five potential tyrosine phosphorylation sites and one serine site were identified with a score more than 0,6 in the H-REV107-1 protein sequence (Fig. 31) using the NetPhos 2.0 program developed by the Technical University of Denmark (http://www.cbs.dtu.dk/services/NetPhos/). An additional search for potential myristoylation, glycosylation or tyrosine sulfation sites of the H-REV107-1 protein revealed negative results. Furthermore, a Western blot analysis of COS-7 cells transiently transfected with an H-REV107-1 expression vector revealed two bands of a slightly different mobility in SDS-PAGE (Fig. 12), which also suggests some kind of posttranslational modification.

Thus, the Lex-A yeast two hybrid assay identified only putative interactors of H-REV107-1 which can bind to the non-modified protein.

H-REV107-1

MRAPIPEPKPGDLIEIFRPFYRHWAIYVGDG**Y**VVHLAPPSE 41

VAGAGAASVMSALTDKAIVKKELL**Y**DVAG**S**DK**Y**QVNNKHD 80

DK**Y**SPLPCTKIIQRAEELVGQEVLYKLTSENCEHFVNELR**Y** 122

GVARSDQVRDVIIAASVAGMGLAAMSLIGVMFSRNKRQKQ 162

Fig. 36 Prediction of protein phosphorylation sites of the H-REV107-1 protein

Five tyrosine and one serine phosphorylation sites were identified using NetPhos program. These residues are shown in red.

Unfortunately, after we had established the LexA yeast two-hybrid system, several new two-hybrid approaches were developed, allowing an improved search for interacting proteins. One example is the CytoTrap two-hybrid system, which enables the discovery of protein-protein interactions in the cytoplasm of yeast cells. This system is based on the activation of Ras signaling pathways in a temperature-sensitive yeast cell line via the recruitment to the cellular membrane of human Sos protein (hSos) fused to the bait. The target proteins are anchored to the membrane. Therefore, interaction between bait and target leads to a close proximity of the hSos and Ras proteins. The hSos protein activates Ras by GDP/GTP exchange, and allows the yeast cells to grow at 37°C. The system was developed for the identification of interacting partners of the Jun protein (Aronheim et al., 1997). It is very well adapted for the identification of protein interactions which take place in the cytoplasm. An interesting modification of this system is, the so called “reverse Ras recruitment system”, in which the bait is anchored to the cellular membrane, and the protein partner (the prey) is fused to a cytoplasmic Ras mutant. Protein–protein interaction between the prey and the bait results in Ras membrane translocation and activation of a viability pathway in yeast (Hubsman et al., 2001).

One of the possibilities to identify interactions requiring tyrosine phosphorylation is the so called “TK (tyrosine kinase) two-hybrid system”. This system is very similar to the standard LexA-based approach. The difference is that an additional tyrosine kinase domain is ligated into the LexA-vector, containing the bait. Expression of this vector in yeast leads to the expression of a bait, and a tyrosine kinase which then phosphorylates the bait protein. Thus, using this system, a specific search of interacting partners of the tyrosine phosphorylated protein can be performed (Vojtek and Hollenberg, 1995).

We screened a human kidney cDNA library with a truncated form of the H-REV107-1 protein. After rescue of the yeast plasmids from positive colonies, and the following sequencing analysis, we searched for homology of the isolated cDNA inserts with known genes in the NCBI database to chose targets for further investigation. This step of analysis is the most critical in the two-hybrid assay. Among the isolated 168 clones, only a few sequences were present more than one time. We excluded typical predicted false positives from further analysis, although it might lead to the loss of putative true positives. For example, heat shock proteins were described to bind many targets unspecifically in yeast (Golemis et al., 1994). Therefore, we excluded the Hsp90 heat shock protein from further analysis. However in many publications it was demonstrated that Hsp90 is a molecular chaperone whose association is required for the stability and function of multiple mutated, and over-expressed signaling proteins that promote cancer growth and cell survival. Hsp90 client proteins include mutated p53, Bcr-Abl, Raf-1, c-Src, Akt, HER2 (Neckers, 2003). It participates in the prevention of tumor cells from apoptosis (Rashmi et al., 2003). Inhibition of Hsp90 results in induction of apoptosis through the activation of caspases–9 and -3 in AML HL-60 cells because of the attenuation of Hsp90-interacting proteins, including Akt, c-Raf-1, and c-Src protein kinases (Nimmanapalli et al., 2003). We demonstrated that ectopic expression and induction by IFNγ of H-REV107-1 in ovarian carcinoma cells leads to the induction of apoptosis through the activation of caspase-9 (Fig. 36). Therefore, in view of these findings, an interaction between Hsp90 and H-REV107-1 might also be plausible and should not entirely be excluded.

We also found a number of unknown genes, which we did not prove in a mating assay, and which were not further analysed in mammalian cells. Putative H-REV107-1 interacting partners might be among them. Therefore we recently repeated the BLAST search of sequences found three years ago. For most inserts still no homologies were found. However, the analysis revealed one novel gene, *TSGA10* isolated in 2001 (Madarressi et al., 2001) without any known function. In addition two cDNA sequences encode proteins which were characterised in the meantime. One of them is the acid cluster protein ACP33, isolated as a novel intracellular binding partner of CD4, proposed to modulate a stimulatory activity of CD4 in T cells (Zeitlmann et al., 2001). The second cDNA insert encodes 100 amino acids at the C-terminus of the novel human protein Scribble, a homologue of the Drosophila protein scrib. Scrib dysfunction results in the false distribution of apical proteins (Bilder and Perrimon, 2001). It has been demonstrated that scrib acts as a tumor suppressor and participates together with two other tumor suppressors, lethal giant larvae (lgl) and discs-large (dlg) in the regulation of cell polarity and growth control (Bilder et al., 2000). The human homologue, hScrib, was shown to be a target of the papillomavirus (HPV) E6 protein, which stimulates its ubiquitination and degradation (Nakagawa and Huibregtse, 2000).

Based on the performed BLAST search analysis, and available literature about found potential interacting partners of H-REV107-1, we chose 11 clones for a mating test (Table 5). The mating assay is a powerful supply for the verification of false positives from the yeast two-hybrid system. Using this assay, we tested whether induction of the reporter genes is a specific response based on the interaction between H-REV107-1 and the corresponding target protein. The assay resulted in the exclusion of 5 proteins from further analysis, because their ability to activate reporter genes was independent of the H-REV107-1 expression (Fig. 7).

For further conformation in the mammalian system, we chose 6 proteins, namely PC4, PR65, RARG, S100A6, ETF1, and P14.5. The aim of the investigation was to prove if the interactions identified in yeast also occur in mammalian cells, and to define a role of the confirmed protein-protein bindings in the H-REV107-1 - mediated growth suppression, and induction of cell death. We verified that H-REV107-1 protein interacts with PC4, PR65, RARG, S100A6, and ETF1 proteins, but not with the P14.5 protein in transfected COS-7 cells.

4.2 H-REV107-1 is a Target of IRF-1 and Modulates IFNγ - Dependent Inhibition of Cellular Growth by Different Mechanisms

Recovery of *H-REV107-1* expression upon IFNγ-treatment was demonstrated in human ovarian carcinoma cell lines. Further experiments revealed that H-REV107-1 is a target of the interferon regulatory factor 1, IRF-1 (Sers et al., 2002). Therefore we asked how H-REV107-1 is involved in the known IFNγ-dependent pathways leading to growth suppression and apoptosis.

Interferon gamma (IFNγ) is a cytokine which was originally identified as the protein responsible for the induction of cellular resistance to viral infection. Subsequently, much evidence has been accumulated with regard to its role in cell growth and differentiation (Pestka et al., 1987). Later, the IFNγ - response has been also postulated to be part of an endogenous tumor surveillance system (Coughlin et al., 1998). It exerts inhibitory effects on tumor cell growth, and recently an improved survival of ovarian carcinoma patients after therapy with IFNγ was described (Windbichler et al., 2000). The biological effect of IFNγ is mediated through a heterodimeric transmembrane receptor which activates a Janus kinase (JAK) – STAT pathway. JAK activates signal transducer and activator of transcription (STAT1) through tyrosine phosphorylation at the cell membrane, followed by dimer formation and migration of the STAT1 homodimers to the nucleus (Stark et al., 1998). Phosphorylated STAT1 enhances the recruitment of transcriptional coactivators, such as P300/CBP, to the promoters of the IFNγ target genes inducing their transcription (Paulson et al., 1999).

Important mediators of the IFNγ response are the STAT1 – target genes, encoding the interferon regulatory factors (IRFs). To date, nine members of the IRF family have been determined, IRF-1 – IRF-9 (Harada et al., 1998). IRF-1 and IRF-2 were identified originally as a transcriptional activator and its antagonistic repressor, respectively, mediating IFN-α, and -β signaling (Harada et al., 1989). Further investigations showed that IRF-1 and –2, both play a key role in cellular growth control, susceptibility to tumorigenic transformation, and induction of apoptosis. Consequently they were suggested to function as a tumor suppressor (IRF1) and oncogene (IRF2) (Harada et al., 1993; Sato et al., 2001).

The tumor suppressor activity of the *IRF-1* gene is further supported by its localisation at chromosome 5q31.1, a region frequently deleted in human leukemias (Willman et al., 1993). The loss of one IRF-1 allele has also been reported in oesophageal and gastric cancer (Nozawa et al., 1998). Other possible mechanisms of IRF-1 inactivation may be alternative splicing of the *IRF-1* mRNA, producing aberrant *IRF-1* in human myelodysplasias and leukemias (Harada et al., 1994). In breast and ovarian carcinomas the *IRF-1* gene exhibits features of a class II tumor suppressor. Down-regulation of *IRF-1*, similar to *H-REV107-1*, has been demonstrated in high grade human ductal carcinomas and in invasive breast cancers (Doherty et al., 2001; Sers et al., 2002). Significant reduction of the *IRF-1,* and *H-REV107-1* mRNA level was revealed in the ovarian carcinoma cell lines OVCAR-3, A27/80, and PA-1 compared to the non-tumorigenic ovarian epithelial cells HOSE (Sers et al., 2002).

Abrogation of the anti-oncogenic IRF-1 activity can also be achieved by inhibiting of its DNA binding ability via direct interaction with a putative ribosome assembly factor, nucleophosmin (NPM)/B23/numatrin, over-expressed leukemias human leukaemia cell lines (Konde et al., 1997). Summarising these data, IRF-1 is a critical tumor suppressor gene, whose inactivation through various mechanisms contributes to the promotion of several human cancers.

The precise nature of the IRF-1 – dependent tumor suppression is not very clear, it is supposed that IRF-1 acts through the up-regulation of a set of genes whose products function as negative regulators of cellular growth (Harada et al., 1993). A number of IFN-stimulated genes which are involved in negative regulation of cell proliferation, have been shown to be IRF-1 targets. Among them are 2-5A synthetase, cyclin-dependent kinase inhibitor $p21^{WAF1}$ (Coccia et al., 1999), lysyl oxidase (Tan et al., 1996), double-stranded RNA-dependent protein kinase, PKR (Beretta et al., 1996), and H-REV107-1 (Sers et al., 2002).

We demonstrated that H-REV107-1 is a direct target of IRF-1 in NIH3T3 cells harbouring estrogen-inducible IRF-1, and in a subset of ovarian cancer cell lines (Sers et al., 2002). Investigation of the *H-REV107-1* and *IRF-1* expression demonstrated their low level in A27/80 and OVCAR-3 cell lines, which was enhanced after IFNγ-exposition.

However, IFNγ suppresses growth of only A27/80 cells, OVCAR-3 cells were demonstrated to be resistant to the treatment (Sers et al., 2002). Analysis of the phenotype of the treated A27/80 and OVCAR-3 cells revealed no changes in A27/80 cells after 48 hours of incubation with IFNγ (data not shown). In contrast approximately 5% of the OVCAR-3 cells revealed a strong up-regulation of the H-REV107-1 expression, which correlated with an apoptotic morphology of nuclei (Fig. 15). This supposed that H-REV107-1 is directly involved in IFNγ - mediated apoptosis in OVCAR-3 cells. However, due to the low number of cells up-regulating H-REV107-1 after IFNγ - treatment this effect had remained undetected in cell growth assay performed earlier.

To investigate the mechanism of H-REV107-1 – mediated growth suppression, A27/80 and OVCAR-3 cells were transiently transfected with H-REV107-1 expression vector. In both cell lines apoptotic nuclear morphology was observed only in cells expressing H-REV107-1 protein (Shayesteh al., 1999; Sers et al., 2002). Thus, forced H-REV107-1 expression leads to a cell death in both cell lines, whereas IFNγ is likely to suppress growth of these cells by different mechanisms. We observed increase of IRF-1, IRF-2 and H-REV107-1 protein levels upon IFNγ - induction only in OVCAR-3 cells (Fig. 14). In contrast in A27/80 cells expression of these proteins was below detection limits (data not shown). To define further the IFNγ - dependent signaling in A27/80 and OVCAR-3 cells, we analysed the expression of the STAT1 protein, which, as was previously described, mediates most of the IFNγ-responses (Stark et al., 1998). In addition we analysed expression of the $p21^{WAF1}$ cyclin dependent kinase, a central mediator of growth arrest and senescence in mammalian cells (Waldman et al., 1995). Increased $p21^{WAF1}$ expression leads to cell growth arrest which occur in both G1 and G2 phases of cell cycle (Niculescu et al., 1998), and is accompanied by the development of morphologic and phenotypic markers of senescence (McConnell et al., 1998). $p21^{WAF1}$ is regulated in response to DNA damage in a p53-dependent manner, but also via IRF-1 (Tanaka et al., 1996). The p53-independent induction $p21^{WAF1}$ expression in response to IFNγ is mediated by STAT1, through direct binding of IRF-1 to the p21 promoter (Coccia et al., 1999). We have asked whether $p21^{WAF1}$ participates in the IFNγ-response in OVCAR-3 and A27/80 ovarian carcinoma cells. We observed up-regulation of STAT-1 expression in both cell lines 24 hours after IFNγ treatment, and even enhanced level after 48 hours. Notably, up-regulation of $p21^{WAF1}$ was observed only in A27/80 cells (Fig. 16). This result supported our hypothesis of different mechanisms of IFNγ-growth inhibition in OVCAR-3 and A27/80 cells.

We proposed the following model of IFNγ-response in OVCAR-3 cells: cytokine induction leads to the activation of STAT1, which stimulates IRF-1 expression. IRF-1 induces H-REV107-1, and IRF-2 transcription.

IRF-2 activates a negative feed-back loop, probably suppressing expression of H-REV107-1 and other IRF-1 targets. Expression of IRF-2 protein was detected already 24 hours after IFNγ treatment, suggesting rapid reverse of the IRF-1 effect (Fig. 14). It is still an open question why a minority of cells express high level of H-REV107-1 upon IFNγ-induction, and undergo apoptosis, whereas other cells are resistant to the IFNγ-treatment.

Although we observed up-regulation of mRNA *H-REV107-1* and *IRF-1* genes in A27/80 cells after IFNγ-induction, the amount of synthesised proteins was below the sensitivity of the method. Alternatively, the proteins were rapidly degraded, before we could perform Western blot analysis. The ubiquitin-proteasome pathway has been reported to play a key role in the down-regulation of the mouse IRF-1 protein (Nakagawa and Yokosawa, 2000). To prove if this pathway mediates degradation of the human H-REV107-1 and IRF-1 proteins, we treated several ovarian tumor cell lines with the MG115 and MG132 protease inhibitors after induction with IFNγ. We observed a stabilisation of the proteins only in human teratocarcinoma cells PA-1 but not in OVCAR-3 and A27/80 cell lines (data not shown). Thus, a protein destabilisation in PA-1 cell line is proteasome dependent, whereas in OVCAR-3 and A27/80 cell lines other mechanisms are responsible for the destabilisation of the H-REV107-1 and IRF-1 proteins.

Analysis of IRF-1, H-REV107-1, and STAT1 expression in A27/80 cells rather suggested an IRF-1 independent growth suppression in A27/80 cells after IFNγ - treatment. Observed up-regulation of $p21^{WAF1}$ suggested that this protein might participate in the IFNγ-response. In addition to p53 and IRF1, the alternative regulator of the $p21^{WAF1}$ expression was demonstrated to be the breast cancer susceptibility gene 1, BRCA1 (Ouchi et al., 1998).

The tumor suppressor BRCA1 has been reported to be implicated in the DNA-repair process (Sculli et al., 1997), and in growth control specifically in breast and ovarian cancer cell lines, but not in colon and lung cancer cells or fibroblasts (Holt et al., 1996). Direct interaction of BRCA1 with the p300/CBP coactivator and with RNA polymerase II holoenzyme suggested that BRCA1 also plays a role in transcriptional regulation (Pao et al., 2000). Later findings have also implicated BRCA1 as a transcriptional regulator of the $P21^{WAF1}$ and *MDM2* genes harbouring a p53-responsive element in their promoter regions. This suggested that BRCA1 can enhance p53-dependent gene regulation (Ouchi et al, 1998). Moreover, $p21^{WAF1}$ was reported to be required for the BRCA1-mediated growth suppression (Somasundaram et al., 1997). It has been demonstrated that activation via IFNγ leads to the interaction between STAT1 and BRCA1 proteins, which stimulate the $P21^{WAF1}$ gene transcription independent of IRF-1 (Ouchi et al., 2001). Thus, BRCA1 is a critical component of the IFNγ - regulated anti-tumor response, and a possible regulator of the $P21^{WAF1}$ expression in A27/80 cells.

The status of the *BRCA1* gene in OVCAR-3 and A27/80 cells is unknown, but regarding the failure of the $p21^{WAF1}$ up-regulation in OVCAR-3 cells after IFNγ-induction, it is likely that *BRCA1* is mutated in this cell line. In A27/80 cells BRCA1 might participate in $p21^{WAF1}$ induction.

Transcriptional activity of BRCA1 has been demonstrated to be maximal in the presence of PC4, although direct interaction between PC4 and BRCA1 was not shown (Haile and Parvin, 1999). We hypothesised that H-REV107-1 might serve as a PC4, and BRCA1, STAT1 binding protein. Such a prediction arose from the fact that we found the PC4 transcriptional coactivator in yeast two-hybrid system as a true H-REV107-1 interacting partner. PC4 serves as a potent coactivator of a diverse group of transcriptional activators in standard *in vitro* transcription systems (Ge et al., 1994). It interacts both with a variety of activation domains and with members of the RNA II polymerase transcriptional machinery, such as the TFIIA general transcriptional factor (Kaiser et al., 1995). We asked whether H-REV107-1 might potentially co-operate with STAT1 and BRCA1 in the IFNγ-response through a formation of a multiprotein complex including STAT1, BRCA1, PC4, and H-REV107-1.

We precipitated a multiprotein complex, consisting of H-REV107-1, PC4, and STAT1 proteins from COS-7 cells transiently transfected with the appropriate plasmids. However, the majority of the PC4 and STAT1 proteins remained in the protein extract, and only a minor fraction was bound to the H-REV107-1 (Fig. 17). We failed to express BRCA1 protein in COS-7 cells. Therefore potential binding of BRCA1 to the protein complex consisting of STAT1, PC4, H-REV107-1 proteins is unclear.

Summarising our investigation of IFNγ-signaling in the two human ovarian carcinoma cell lines OVCAR-3 and A27/80, we conclude that there are two different mechanisms of IFNγ - mediated growth suppression. In OVCAR-3 cells IFNγ leads to cell death trough the activation of STAT1, IRF1, H-REV107-1 signaling (Fig. 37). In A27/80 cells IFNγ leads to a cell cycle inhibition through STAT1, $p21^{WAF1}$, and, hypothetically, BRCA1 activation. Possibly, in this pathway a STAT1, PC4, H-REV107-1 protein complex with or without BRCA1 protein is involved.

Hypothetical scheme of IFNγ-dependent growth suppression in OVCAR-3 and A27/80 cells is depicted in the Figure 37. There are several questions we wish to answer in our future investigations. Does H-REV107-1 interact with PC4 and other proteins the nucleus, and when does a transfer of the H-REV107-1 protein from the cytoplasm to the nucleus take place?. What is the functional role of the PC4, H-REV107-1, STAT1, and, supposedly, BRCA1 complex in IFNγ - signaling? Does H-REV107-1 influence STAT1 and PC4 activity as transcriptional activators in these cells, and is it involved in the regulation of expression of IFNγ - target genes.

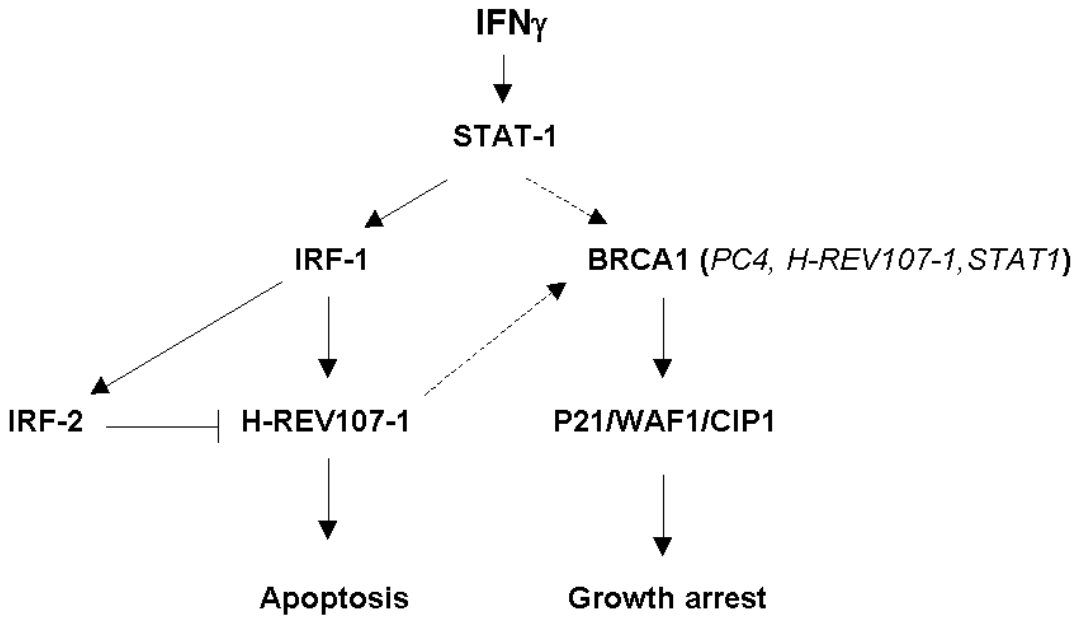


Fig. 37 Hypothetical scheme of H-REV107-1 participation in the IFNγ-signaling in OVCAR-3 and A27/80 cells

4.3 H-REV107-1 Participates in the Cross-Talk between Retinoic Acid and IFNγ-Dependent Pathways

Retinoids (vitamin A and its metabolites) modulate cell growth, differentiation, proliferation, and can act as chemopreventive and chemotherapeutic agents for several types of cancer (Hong and Sporn, 1997). Notably, one of the H-REV107 – related proteins, lecithin retinol acyltransferase (LRAT), plays an important role in retinoid metabolism. It catalyses the esterification of retinol, thereby synthesising retinyl ester, which is supposed to act as a storage form for retinol in epithelial cells in skin, and breast (Chen et al., 1997). Investigation of the retinol metabolism in normal and cancer cells revealed that cancer cells have a greatly reduced ability to metabolise retinol into retinyl ester, This correlated with a significant reduction of LRAT expression (Guo and Gudas, 1998), suggesting that lack of retinyl esters in carcinoma cells may contribute to their tumorigenic phenotype (Guo et al., 2000).

It is known that the biologic activity of retinol and retinoic acid metabolism is mediated by retinoid binding proteins (CRBPs) and diverse retinoid nuclear receptors. There are two families of receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Each family consists of three receptor types, alpha, beta, and gamma (Zhang et al., 2000).

They modulate gene expression by binding to specific DNA sequences (retinoid responsive elements, RARE) in the promoter regions of retinoid-target genes (Naar et al., 1991).

We identified the retinoic acid receptor gamma (RARG) in the yeast two hybrid system as a potential interacting partner of H-REV107-1. Interaction between RARG and H-REV107-1 proteins was a rather surprising finding, because this was a first evidence indicating a potential participation of the H-REV107-1 protein in retinoid signalling. Investigation of the interaction in COS-7 cells demonstrated that the H-REV107-1 protein does interact with RARG only in the presence of supplementary ligands.

To stimulate this interaction we used all-trans retinoic acid (ATRA), a synthetic ATRA antagonist (TTNPB), and a doublestranded sequence of the retinoic acid response element, DR5, which is known to be a target sequence of the RARs in promoter regions of the retinoid target genes (Idres et al., 2002). Although ATRA did not influence the interaction between RARG and H-REV107-1, TTNPB and DR5 weakly enhanced the protein binding. The specificity of binding was tested using a negative control with the mutated DR5 sequence. No enhancement of the interaction was detected (Fig. 32). Nevertheless, this interaction remained very weak, and only a small part of the intracellular H-REV107-1 was identified in the complex with RARG, a major part of the protein did not bind to the retinoid receptor (Fig. 32). Combining these data we suggest that under *in vivo* conditions the interaction might take place in the nucleus, where RARG bound to the promoter of target genes interacts with the H-REV107-1 protein. The weak interaction is likely to be due to the fact that only a small fraction of the H-REV107-1 protein exhibits a nuclear localisation.

It is still an open question if the H-REV107-1 – RARG interaction contributes to retinoid signaling. Recent investigations of RAR – binding proteins shed a new light at the known interaction partners of the H-REV107-1 protein. The STAT5 protein was identified as a critical regulator of enhanced RAR transcriptional activity (Si and Collins, 2002). Most interestingly, the authors described a direct overlap of STAT1 and STAT5-binding sites with RAR elements within the promoters of several genes, for example, RARβ and RARα (Langston et al., 1997). Such colocalisation of regulatory elements suggests a previously unexplored cross-talk between STAT and RAR families of transcriptional factors, following co-regulation of the IFN– and retinoid– dependent genes. Since the H-REV107-1 protein is a mediator of IFNγ-signaling (Sers et al., 2002), it will be definitely interesting to investigate the activity of the RARG in IFNγ-treated cells, and to define a potential role of IFNγ in the modulation of the RARG – H-REV107-1 interaction in more detail.

Another interesting aspect of the interaction between H-REV107-1 and RARG is the enhanced co-immunoprecipitation of the RARG and H-REV107-1 proteins in presence of the TTNPB ligand (Fig. 32). Binding to diverse ligands is supposed to change the conformation of the RARG molecule. Current models of the RAR activation suggest that binding of ligands results in a distinct conformational change, and leads to the release of co-repressors and the recruitment of transcriptional activators (Zhang et al., 2000), such as CBP, or p300. These factors are able to trigger RNA polymerase II to transcribe target genes (Kamei et al., 1996). Interestingly, another coactivator of the RNA polymerase II, the PC4 protein, was also found in the yeast two hybrid assay, as one of the H-REV107-1 binding proteins. Although a role of PC4 in the activation of RARs has not yet been described, its participation in this process cannot be excluded.

In summary, the functional relevance of the H-REV107-1, PC4 and STAT1 proteins on the RARG activity is still speculative, but provides an attractive hypothesis on the role of H-REV107-1 in both the IFNγ and retinoic acid signaling.

Importantly, IFN-γ was found to act synergistically with retinoids, and to enhance the growth inhibitory effect of retinoids in cultured breast and ovarian cancer cells (Hu et al., 2002). Analysis of the *H-REV107-1* expression using cancer profiling arrays demonstrated down-regulation of the gene in breast, ovarian and lung tumors. IFNγ-treatment recovered *H-REV107-1* expression in ovarian carcinoma cell lines OVCAR-3 and A27/80 (Sers et al., 2002). Investigation of the additive affect of IFNγ and ATRA revealed that IFNγ-treatment increased the expression level of RAR-alpha and RARG (Hara et al., 2001). Therefore it will be of interest to investigate if IFNγ-treatment of OVCAR-3 and A27/80 cells will lead to the up-regulation of RARG expression. Retinoids were demonstrated also to contribute to the cross talk between IFN- and ATRA – signaling pathways. In various cell lines, retinoic acid induces directly the expression of the transcription factors STAT1 and IRF-1, which play central roles in IFNγ-signal transduction (Chelbi-Alix and Pelicano, 1999). The *H-REV107-1* gene was reported to be a target of IRF-1 (Sers et al, 2002). Possibly, H-REV107-1 participates in cross-talk between retinoid and IFNγ-signaling in ovarian tumors, although regulation of the gene occurs rather through a retinoid – independent pathway.

In contrast, the H-REV107-1 related protein, the H-REV107-2/TIG3/RIG1, is directly implicated in retinoid acid cellular response (Huang et al., 2000). The *H-REV107-2/TIG3/RIG1* was cloned as an ATRA responsive gene in keratinocytes and cervical and gastric cancer cells, where it acts as a tumor suppressor (Di Sepio et al., 1998; Hung et al., 2000; Deucher et al., 2000).

Further investigation lead to the finding that in gastric carcinoma cells the H-REV107-2/TIG3/RIG1 protein induces apoptosis trough negative regulation of extracellular signal-regulated kinase, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated kinase (Huang et al., 2002). Interestingly, other groups showed that treatment of these cells with ATRA leads to the inhibition of AP1 activity a subunit of which is the JNK-target protein, c-Jun. Investigation of retinoid receptors responsible for the ATRA-mediated growth inhibition of gastric cancer cells revealed that the retinoic acid receptor beta (RARbeta), but not RARG, is required for the AP-1 inhibition, and contributes to growth suppression (Huang et al., 2002). Taking these data together, we suppose that H-REV107-2/TIG3/RIG1 and RARbeta are involved in retinoic acid dependent growth arrest in gastric cancer cells, but not H-REV107-1 and RARG

4.4 H-REV107-1 – Mediated Cell Death through Inhibition of PP2A Activity

Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase in eukaryotic cells. PP2A can be considered a family of phosphatases, minimally containing a highly conserved catalytic subunit C (PR36) α and β isoforms; and a “scaffolding protein”, the regulatory subunit A (PR65), α and β isoforms. The isoforms of the PR65 subunit are 86% identical, and those of the PR36 subunit are 92% identical (Hemmings et al., 1990). The PR65 and PR36 proteins are tightly associated with each other, comprising the so called “core domain”. (Ruediger et al., 1994; Millward et al., 1998). The activity of the “core domain” is regulated by regulatory subunits B, bound to PR65. Four protein families B (PR55), B´ (PR61), B´´ (PR72, PR130, PR49, PR48), and B´´´ (PR93, PR110) are known as regulatory subunits B of PP2A (Table 8; Janssens and Gors, 2001).

Biological and genetic studies have revealed that PP2A enzymes are abundant, ubiquitous, and very conserved during evolution. PP2A dephosphorylates a large number of substrates *in vitro*, and is involved in the regulation of nearly all cellular activities, such as metabolism, transcription, cell cycle, signal transduction, and oncogenic transformation (Sontag, 2000). The PR65β encoding gene, *PPP2R1B* was characterised as a putative tumor suppressor down-regulated or mutated in lung, breast, and colon human tumors (Wang et al., 1998; Janssens and Goris, 2001). PR65α plays a critical role in cell morphogenesis of yeast, probably through regulation of the cytoskeletal network and cell wall synthesis. Deletion of the PR65α encoding gene, *PPP2R1A* is lethal in yeast (Kinoshita et al., 1996). Investigation of PR65α in human tumors demonstrated that *PPP2R1A* gene, is mutated in lung and breast human carcinomas and melanomas (Calin et al., 2000), strengthening a potential role of PP2A in human tumorigenesis.

PP2A		
Catalytic subunit C	**Regulatory subunit A**	**Regulatory subunit B**
PP2A/C (α, β)	PP2A/A (α, β)	PP2A/B
PR36 (α, β)	PR65 (α, β)	PR55, PR61, PR72, PR130, PR49, PR48, PR93, PR110

Table 8 Isoforms of the Protein Phosphatase 2A (PP2A) and their alternative designations.

The homozygous PR36α null mice are embryonically lethal, demonstrating that the PR36α subunit gene is essential for viability, and cannot be replaced by PR36β (Gotz et al., 1998). Recent data suggest that embryonic lethality results from defects in cell adhesion caused by insufficient levels of membrane-associated E-cadherin and β-catenin, suggesting a role of PP2A in Wnt/β-catenin – signaling (Seeling et al., 1999; Patturajan et al., 2002). The importance of PP2A in human cells is indicated by the fact that various pathogenic viruses, namely human immunodeficiency type 1 virus, HIV-1, (Tung et al., 1997), papova viruses (Pallas et al., 1990) or adenoviruses (Kleinberger and Shenk, 1993) express PP2A interacting proteins, which modify the phosphatase activity. Human cytomegalovirus (CMV), a herpesvirus, carries host-derived PP2A, associated with the nucleocapsid fraction (Michelson et al., 1996).

We demonstrated that the H-REV107-1 protein interacts with the regulatory subunit A α of the protein phosphatase 2A (PR65α) in COS-7 cells (Fig. 18), and under cell free conditions (Fig. 20). The interaction of the bacterially expressed H-REV107-1 with PR65α indicated that H-REV107-1 directly binds this protein. Co-immunoprecipitation assays in COS-7 cells with H-REV107-1 mutants delineated a region of 10 aminoacid residues on the very N-terminal end of the protein necessary for the interaction (Fig. 24).

Thus we have presented data demonstrating that the H-REV107-1 mediated apoptosis in OVCAR-3 cells is due to the inhibition of the PP2A activity through physical interaction between H-REV107-1 and the PR65 regulatory subunit. A challenge of our investigation was to define PP2A target proteins whose dephosphorylation plays such important role in cell survival.

Ectopically expressed H-REV107-1 protein forms homodimers in COS-7 cells. We tested the H-REV107-1 mutant proteins for a homodimer formation and observed that the same region responsible for the interaction with PR65α is also required for a homodimer formation. This suggests a competitive character of these interactions. Preliminary experiments demonstrated that binding to the PR65α protein excludes generation of homodimers (data not shown).

We wished to investigate a molecular mechanism of the correlation between H-REV107-1 mediated cell death and the interaction with PR65α. We observed that H-REV107-1 overexpression in human ovarian carcinoma cells OVCAR-3 results not only in the induction of apoptosis, but leads also to the transport of PR65α protein from nucleus into the cytoplasm (Fig. 27). During overexpression of the ΔC107-ΔN interaction deficient mutant neither apoptotic morphology of nuclei, nor transportation of PR65α were obtained. This suggested that H-REV107-1 – mediated apoptosis correlates with its ability to interact with the PR65α protein.

Little known about nuclear PR65α and its interacting partners in ovarian carcinoma cells. In most tissues and cell lines a cytoplasmic localisation of this protein was reported (Thou et al., 2003). We asked where the catalytic subunit PR36 is localised in OVCAR-3 cells, and if other PP2A subunits might interact with the nuclear PR65α protein. For example, the PP2A B56 regulatory subunit γ and δ isoforms were also shown to be concentrated in nucleus (McCright et al., 1996), suggesting that B56 isoforms might somehow be involved in this process. Preliminary analysis of expression of the genes encoding the B56 α, β, γ - proteins via RT-PCR revealed that all isoforms are expressed in OVCAR-3 cells (data not shown).

First, we investigated if H-REV107-1 can influence the intracellular localisation only of PR65α, or of the “core domain” consisting of PR65 and PR36 subunits. Immunofluorescence analysis of the intracellular distribution of PR36α in OVCAR-3 cells revealed an exclusively cytoplasmic localisation. Expression of the H-REV107-1 protein did not have any influence on the localisation of the PR36α protein (data not shown). PR36 is usually bound to the PR65 protein, and does not exist in a cell in free state. Therefore we supposed that the second isoform of the PR65 protein, PR65β, might be expressed in OVCAR-3 cells.

We performed a subcellular fractionation of OVCAR-3 cells, and tested the different fractions with an antibody recognising both, the PR65α and PR65β proteins. Western blot analysis revealed a strong specific signal in the cytoplasmic and in the nuclear fractions (data not shown). Therefore, we suppose that the PR65β is expressed in OVCAR-3 cells, and preferably localised in the cytoplasm, whereas the PR65α is localised in the nucleus, as we observed using immunofluorescence analysis.

Thus, it is likely that in the cytoplasm the PR36α - PR65β protein complex might exist. Taking into consideration that PR65α and PR65β are 86% homologue, we would like to test in our further experiment if H-REV107-1 might interact with PR65β in the cytoplasm, and if the PR36α, PR65β, H-REV107-1 complex exists in OVCAR-3 cells.

We determined a basal level of the protein phosphatase 2A enzymatic activity in untransfected OVCAR-3 cells, and in the cells over-expressing H-REV107-1. As a negative control we used a phosphatase inhibitor, okadaic acid, in the PP2A specific concentration of 2 nM (Holmes et al., 1990). H-REV107-1 overexpression had a strong inhibitory effect on PP2A activity, whereas the interaction deficient ΔC107-ΔN mutant was unable to inhibit the phosphatase (Fig. 30). This suggests that the interaction between H-REV107-1 and PR65α leads to the inactivation of the PP2A enzymatic activity.

The finding that PP2A inhibition leads to apoptosis is rather surprising, because it is in contrast with many other reports, suggesting that the protein phosphatase 2A is a pro-apoptotic phosphatase (Klumpp and Krieglstein, 2002). Additionally, OA has been described as a tumor promoting agent (Afshari et al., 1993; Fujiki and Suganuma, 1993).

A dual role of the protein phosphatase 2A in tumor progression has been already mentioned by other authors. Although most PP2A inhibitors are implicated in tumor promotion, the inhibitor fostriecin displayed a significant anti-tumor activity in Chinese hamster ovary cells inducing a dose-dependent arrest of cell growth during the G2-M phase of the cell cycle (Cheng et al., 1998). Recent research in *Drosophila melanogaster* demonstrated that a trimeric complex PP2A/C, PR65, and B56-regulatory subunit is required for survival and protect cells from apoptosis (Li et al., 2002). Investigation of the sensitivity of different cell lines to OA-induced apoptosis revealed an unexpected result. It was shown that the cell lines harbouring ras mutations are more sensitive than the cell lines with wild-type ras (Rajesh et al., 1999). Using dual-coloured flow cytometry, we demonstrated that treatment of OVCAR-3 cells with 10 nM OA results in cell death due to apoptosis (Fig. 30). Although OVCAR-3 cells do not carry Ras mutations (Patton et al., 1998), activation of one of the Ras-dependent down-stream pathways, the PI3-kinase pathway, has been reported in these cells (Shayesteh et al., 1999). PI3-kinase plays an important role in a cell survival. Amplification of its downstream kinases Akt1, and Akt2 was demonstrated in human ovarian cancer, and inhibition of this pathway led to the cell death (Yuan et al., 2000).

Therefore we included the PI3-kinase inhibitor, LY294002 in further analysis, to test if LY294002 and OA treatment have a similar effect on the OVCAR-3 cells, like H-REV107-1 ectopic expression. Investigation of the apoptotic pathway after H-REV107-1 overexpression, LY294002, and OA treatments revealed that a mitochondrion-dependent, but not a death receptor – dependent pathway is activated in all three cases.

Cleavage of the caspase-9, and its down-stream caspases-3 and –7 was detected (Fig. 31; and data not shown). In contrast, a marker for the death-receptor dependent apoptosis, cleaved caspase-8 was revealed only after treatment with 10 nM OA (data not shown), suggesting that the cellular response to higher concentrations of OA includes activation of additional targets, such as PP1, which activates a death receptor pathway, that differs from the response to the H-REV107-1 protein. Apoptosis induction after treatment with 10 nM OA, and after H-REV107-1 overexpression was more pronounced as after treatment with 0.5 nM OA (Fig. 31, B), probably due to the insufficient inhibition of PP2A activity by low concentration of okadaic acid. Although PP2A is inhibited by OA with K_i 0.2 nM (Shima et al., 1994), various cell lines differ in their membrane's penetrance. Therefore it is difficult to control intracellular OA concentration, and some unspecific effects can occur.

We have analysed PP2A substrate proteins, such as members of the BCL-2 family, directly participating in mitochondrion-dependent apoptosis, and proteins involved in RAS.-dependent signaling. One of the proapoptotic members of this protein family, is the BAD protein function of which is regulated by reversible phosphorylation. Three phosphatases, PP1 (Ayllon et al., 2000), PP2A (Chiang et al., 2001), and PP2B (Wang et al., 2000) have been demonstrated to be implicated in the dephosphorylation of the Ser/Thr residues within BAD. Inhibition of phosphatase activity by treatment of cells with okadaic acid of different concentrations demonstrated that in OVCAR-3 cells BAD is rather a target of PP1 phosphatase but not of PP2A (data not shown). Analysis of other members of the BCL-2 family, Bcl-2 and Bxl revealed negative results as well.

The RAS-downstream signaling pathways were of our particular interest. Two pathway down-stream of Ras, the RAF-MEK-ERK and the PI3K-Akt pathway have been described to be regulated via PP2A (Millward et al., 1999). PP2A modulates the activity of several members of the RAF-MEK-ERK pathway, and has both positive and negative effects (Frost et al., 1994; Alessi et al., 1995; Zhou et al., 2002).

PP2A dephosphorylates an inhibitory site within Raf, Ser-259, the dephosphorylation of which was shown to be necessary for full activation of this kinase (Abraham et al., 2000). We analysed activation-phosphorylation of ERK, MEK, and RAF in OVCAR-3 cells treated with 1 nM and 10 nM OA, with LY294002, and transfected with an H-REV107-1 expression vector. We did not obtained any effect on the phosphorylation of RAF1. Treatment with LY294002 and 1 nM OA led to the reduction of the phosphorylated RAF-down-stream kinase MEK (Nazarenko, unpublished data), suggesting a cross-talk between PI3-kinase and RAF-MEK-ERK kinase pathways. One of the PI3-kinase down-stream effectors, the PKC kinase, has been reported as an activator of MEK (Kolch, 2000).

A large number of studies demonstrated a PP2A inhibitory effect on the PI3-kinase – dependent pathways (Sontag, 2000; Yellaturu et al., 2002). Akt, and PKC were described as substrates of PP2A (Yellaturu et al., 2002; Sontag et al., 1997; Standaert et al., 1999). Interestingly, that PKC revealed an inhibitory effect on Akt - activity (Wen et al., 2002), indicating a negative feedback loop within a PI3-kinase pathway.

Thus, PP2A might activate the PI3-kinase pathway, through inhibition of the PKC activity, and inhibit the PI3-kinase pathway through inhibition of Akt activity. It is possible that different regulatory subunits of PP2A might recruit the enzyme to either PKC, or Akt kinase, and modulate therewith a cell survival or cell death, respectively.

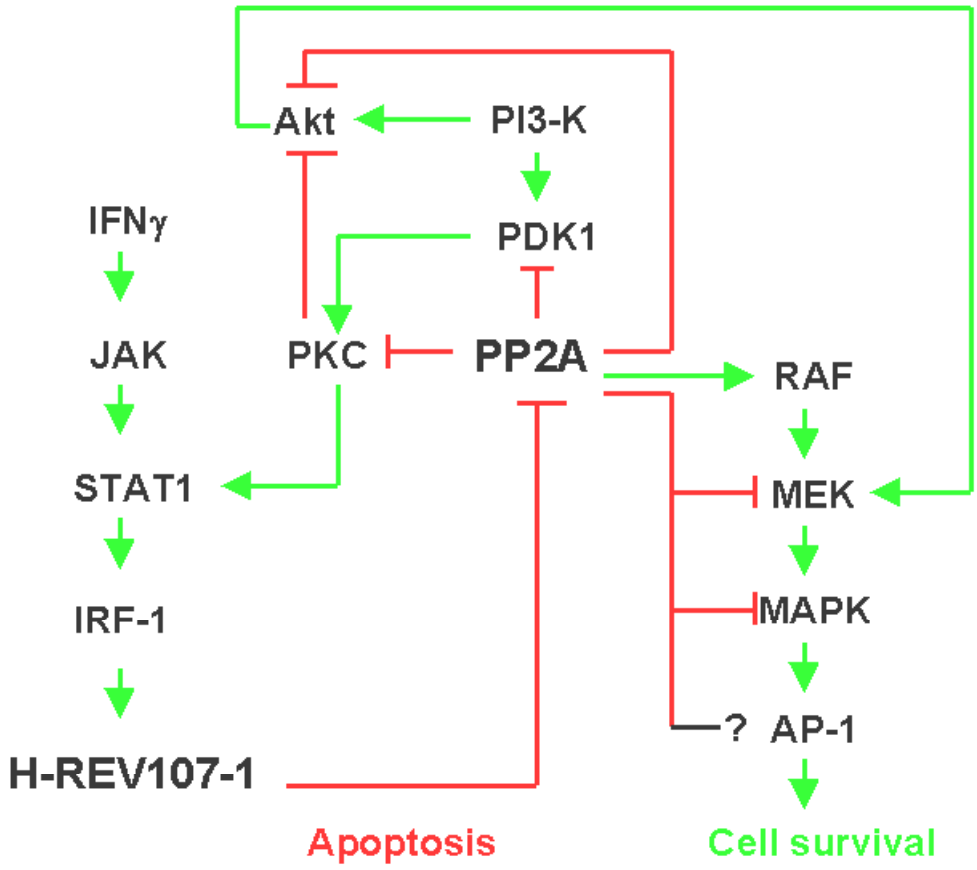


Fig. 38 Schematic presentation of the mechanism of H-REV107-1 – mediated cell death

Inhibition of a pathway is shown with red lines, activation of a pathway is shown with green lines.

It is a desire of our further experiments to elucidate if the PKC kinase is a primary target of PP2A in OVCAR-3 cells. This might answer the question which activity of PP2A, essential for cell survival, inhibits H-REV107-1. A recent publication, demonstrating that PKCε is necessary for the IFNγ-response, supports our hypothesis that PKC might be a target of PP2A in OVCAR-3 cells (Ivaska et al., 2003). The authors demonstrate that the PKCε kinase phosphorylates, and thereby activates the STAT1 protein. PP2A is responsible for a deactivation of PKCε via dephosphorylation. This results in attenuation of the IFNγ-induced phosphorylation of STAT1, and, therefore, inhibition of IFNγ-signaling. It was demonstrated that that IFNγ - function might be recovered by inhibition of PP2A.

We did not yet prove if OA treatment will recover an IFNγ-response in OVCAR-3 cells, but the fact that only the cells over-expressing the H-REV107-1 protein, inhibiting PP2A activity, underwent apoptosis after IFNγ-treatment, substantiates this possibility. A hypothetical scheme of the PP2A signaling pathway in OVCAR-3 cells is depicted in the Figure 38.

4.5 Possible Participation of H-REV107-1 in Calcium Metabolism

One of the H-REV107-1 interacting partners that was confirmed in yeast and mammalian cells is the Ca^{2+} - binding protein calcyclin. Calcyclin belongs to the S100 family of calcium binding proteins, and was characterised as a marker for the malignant phenotype in human malignant melanomas (Maelandsmo et al., 1997), squamous cell carcinomas (Berta et al., 1997), breast (Pedrocchi et al., 1994), and colon cancers (Stulik et al., 2000). There is ample evidence that calcyclin and other members of this family play an important role in neoplastic progression (Weterman et al., 1992). Recent research supports these earlier findings, demonstrating that up-regulation of calcyclin in colorectal adenocarcinomas is directly correlated with Dukes's tumor stage. This suggests that calcyclin may be involved in the progression and invasive process of human colorectal adenocarcinomas (Komatsu et al., 2000; Bronckart et al., 2001).

The interaction between H-REV107-1 and calcyclin is dependent on the presence of Ca^{2+} and Zn^{2+} ions in the lysis buffer (Fig. 33). These ions were demonstrated to bind different sites of calcyclin, independent of each other, changing the protein conformation, and probably the binding capacity of calcyclin (Kordowska et al., 1998). Therefore, it will be of a particular interest to investigate separately a role of these ions on the binding of calcyclin to the H-REV107-1 protein.

Until now we have no further evidences for a functional role of the H-REV107-1 protein in Ca^{2+} signaling. Interestingly, one of the regulatory subunits of PP2A, another H-REV107-1 interacting protein, has been described as a calcium binding protein, and a role of PP2A in calcium metabolism was suggested (Janssens et al., 2003).

The authors evaluated the effects of Ca^{2+} on subunit composition, subcellular targeting, and catalytic activity of a PR72-containing PP2A trimer. Binding of calcium required nuclear localisation of PR72, and mediated PP2A activity in vitro. H-REV107-1 was demonstrated as a negative regulator of PP2A activity in vitro, and its forced expression lead to the transport of the PR65 subunit form nucleus into the cytoplasm.

These data support the notion that the H-REV107-1 protein is directly involved in the calcium metabolism. Therefore it will be of a particular interest to investigate whether calcyclin is able to mediate the PP2A activity in OVCAR-3 cells in presence and absence of the H-REV107-1 protein, and to investigate a potential influence of the Ca^{2+} or Zn^{2+} ions on the H-REV107-1 – mediated inhibition of PP2A activity.

In summary, the application of the yeast two hybrid approach revealed multiple interacting partners of the class II tumor suppressor H-REV107-1, suggesting its participation in various cellular processes. We demonstrated that the H-REV107-1 protein is an important mediator of IFNγ signaling, and induces either growth suppression, or apoptosis through different mechanisms. The STAT1, PC4, H-REV107-1 protein complex is likely to be involved in IFNγ - mediated growth suppression in ovarian carcinoma cells.

Further studies identified a novel oncogenic role of the protein phosphatase 2 A (PP2A) in ovarian carcinoma cells. PP2A is a central serine/threonine phosphatase which is involved in nearly all cellular processes. We demonstrated that for the H-REV107-1 - mediated cell death, the interaction with the regulatory subunit A (PR65) of PP2A is required. The H-REV107-1 - PR65 protein binding results in the inhibition of PP2A activity, which is followed by the induction of apoptotic signaling via a caspase-9 – dependent pathway. The challenge of our future investigation will be to define the direct target of PP2A which is responsible for tumour cell survival, and to elucidate the mechanisms of H-REV107-1 mediated growth inhibition or cell death in more detail.

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Danksagung

In den vergangenen Jahren habe ich unzählige Gespräche mit einer Vielzahl von Personen geführt, die durch ihre Ideen und Anregungen sicherlich auch mit zum Gelingen dieser Arbeit beigetragen haben. Diesen danke ich hiermit ganz herzlich.

Einigen Personen jedoch möchte ich an dieser Stelle einen ganz besonderen Dank aussprechen:

Frau Dr. Christine Sers danke ich für ihre brillanten Ideen, für das sichere Geleit, und ihre Geduld. Ebenso für die Abnahme vieler bürokratischer Angelegenheiten in den letzten viereinhalb Jahren und die besonders herzliche Unterstützung in der Anfangszeit.

Herrn Prof. Dr. Reinhold Schäfer danke ich für sein kritisches Gehör, seine voranbringenden Anregungen und seine Ideen sowohl zu den Versuchen als auch zur Arbeit selbst.

Frau Cornelia Giseler danke ich für die Mitarbeit an zahlreichen Versuchen, das Etablieren des FACS Analyse Verfahrens, dem humorvollen Verständnis bei Mehrfachwiederholung und der Mithilfe beim Ordnen meiner Gedanken.

Der Arbeitsgruppe „Molekulare Tumorpathologie“, in der ich diese Arbeit angefertigt habe, danke ich für das angenehme Arbeitsklima. Insbesondere dem gesamten MTA-Team unserer Arbeitsgruppe für ihre freundliche und kompetente Hilfsbereitschaft.

Frau Dr. Kerstin Lehmann danke ich für die Hilfe bei der Konfokalmiskroskopie.

Selbständigkeitserklärung

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbständig verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Berlin, den 11. Juni 2003

Irina Nazarenko