Adenovirus and VSV: investigations on virus-host-interactions to improve safety and efficacy of oncolytic viruses

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1. Abstract

As conventional anti-cancer regimens like radiation or chemotherapy often fail to cure human cancers new treatment strategies are required. The application of replication-competent viruses as anti-tumor agents – termed virotherapy – represents a novel and promising approach to selectively eradicate cancerous cells while concomitantly sparing normal tissue from destruction. Thereby the therapeutic vector propagated and infected cells are lysed. To restrict viral replication to tumor tissue it is important to understand the molecular mechanisms that govern cancer development, and the interactions of therapeutic viruses with their target cells. This thesis was aimed to explore the interaction between two different therapeutic viral agents – a conditionally replicating Adenovirus (crAd) and the natural tumor virus Vesicular Stomatitis Virus (VSV) – and human cancer cells.

First, the altering transcriptional status of p53 in normal and transformed cells was utilized for the regulation of adenoviral replication applying a novel regulation mechanism that leads to the destruction of the vector genome in normal tissue. This mechanism is based on the p53-dependent expression of the rare-cutting DNA endonuclease I-*Sce* I from yeast. In cells with active p53, I-*Sce*-I specifically cleaves the viral backbone as determined by PCR. Consequently, replication of I-*Sce* I-encoding viruses is impaired in contrast to EGFP-expressing control vectors in p53-positive cell, whereas no difference in cells with non-functional p53 could be observed. Furthermore, this concept can be combined with an additional transcriptional repressor Gal4-KRAB. In summary, tightly regulated, conditionally replicating adenoviruses have been established that combine transcriptional regulation as well as vector destruction mechanisms for improved safety and efficacy of virotherapeutic treatment of solid tumors.

Second, the molecular mechanisms involved in VSV-induced apoptosis were investigated focusing on proteins of the B-cell lymphoma 2 (Bcl-2)-family. VSV was demonstrated to rapidly decrease myeloid cell leukemia 1 (Mcl-1) protein levels. Mcl-1 elimination depends on the combination of VSV-mediated block of cellular protein biosynthesis and continued proteasome-dependent degradation of Mcl-1. Rescue of Mcl-1 inhibited apoptosis confirming that Mcl-1 down-regulation contributes to VSV-induced apoptosis. *In vitro* and *in vivo*, VSV virotherapy in combination with chemotherapy revealed an enhanced therapeutic effect compared to single treatments. In summary, these data suggest that Mcl-1 is a key component of intracellular defense mechanisms against VSV infection. Additionally, strong evidence is provided that this anti-viral mechanism can be successfully exploited by oncolytic VSV to enhance anti-tumor therapy in combination with conventional chemotherapy *in vitro* and *in vivo*.

Keywords: oncolytic virus, apoptosis, Mcl-1

2. Zusammenfassung

Konventionelle Tumorbehandlungen wie Strahlen- oder Chemotherapie führen oftmals nicht zum Heilungserfolg, weshalb neuartige Therapieansätze nötig sind. Der Einsatz von replikativen Viren als therapeutische Agentien – bezeichnet als Virotherapie – stellt einen vielversprechenden und innovativen Ansatz zur selektiven Zerstörung von Krebszellen dar. Dabei wird ausgenutzt, dass virale Vektoren infizierte Zellen lysieren und es gleichzeitig zur Amplifikation der therapeutischen Viren kommt. Um die virale Replikation auf Tumorzellen zu beschränken, ist es von großer Bedeutung die molekularen Mechanismen der Krebsentwicklung und der Virus-Wirtsinteraktion zu verstehen. In dieser Arbeit wurden daher die Interaktionen zwischen therapeutisch relevanten Viren – einem konditionell-replizierenden Adenovirus und dem natürlichen Tumorvirus Vesikuläres Stomatitis Virus (VSV) – und menschlichen Krebszelllinien analysiert.

Der unterschiedliche transkriptionelle Status von p53 in normalen und transformierten Zellen wurde ausgenutzt, um die Replikation eines adenoviralen Vektors durch einen neuartigen, auf der Hefe-DNA-Endonuklease I-*Sce* I basierenden Regulationsmechanismus auf Krebszellen zu begrenzen. Virus-kodiertes I-*Sce* I wurde p53-abhängig exprimiert und spaltete in p53-positiven Zellen das adenovirale Genom an bestimmten Stellen hochspezifisch, während das Gen in Tumorzellen nicht transkribiert wurde. Resultierende Schnittprodukte konnten in wt-p53-Zellen aber nicht in p53-negativen Zelllinien nachgewiesen werden. Die Replikation I-*Sce* I-kodierender Viren wurde durch diesen Schalter im Gegensatz zu entsprechenden EGFP-Kontrollviren ausschließlich in p53-postiven aber nicht in p53-negativen Zelllinien gehemmt. Außerdem war der I-*Sce* I-Schalter mit einem weiteren transkriptionellen Repressor-mechanismus (Gal4-KRAB) kombinierbar. Als Ergebnis wurden stark regulierte, konditionell replizierende Adenoviren entwickelt, die zwei neuartige Regulationsmechanismen kombinieren, um die Sicherheit der onkolytischen Therapie solider Tumoren zu erhöhen.

Darüber hinaus wurde die Apoptose-Induktion durch VSV mit speziellem Fokus auf Proteine der Bcl-2-Proteinfamilie untersucht. Das Proteinniveau von Mcl-1 (*myeloid cell leukemia 1*) sank in VSV-infizierten Zellen schnell und stark ab, was auf der VSV-induzierten Hemmung der zellulären Proteinbiosynthese bei gleichzeitig fortgesetzter proteasomaler Degradation von Mcl-1 basiert. Die Expression von stabilisiertem Mcl-1-Protein führte zur Hemmung der Apoptose-Induktion. Die VSV-induzierte Eliminierung von Mcl-1 konnte *in vitro* und *in vivo* ausgenutzt werden, um die Wirkung einer chemotherapeutischen Therapie zu verstärken. Daraus ergibt sich, das Mcl-1 einen anti-viralen Schalter darstellt, der zudem für eine verstärkte Tumortherapie *in vitro* und *in vivo* ausgenutzt werden kann.

Stichworte: Onkolytische Viren, Apoptose, Mcl-1

3. Introduction

The development of biological science during the last decades allows the complementation of traditional treatment regimens by more targeted therapies. Virotherapy represents an innovative approach to treat human malignancies by viral means. Both a thorough understanding of the disease itself and the virus-host-interactions can lead to concepts where certain viruses are engineered to permit cure of patients at high safety standards and increased success rates compared to standard therapies. In the work presented here, questions regarding both basic research on virus-host-interactions and applied virotherapy were addressed.

3.1 Cancer and tumor development

3.1.1 Cancer

Cancer can be described as a group of diseases underlying one basic phenomenon: uncontrolled cell growth. In contrast to normal, differentiated cells that have lost their replicative capacity, cancer cells have regained the potential for unlimited cell division. Although many differences in genotype and phenotype of different cancers have been observed, there is ample evidence that the emergence of all cancers can be explained by a common set of only a few molecular alterations [1]. It was noted therefore, that neoplasms generally develop in the same way and show the same general behavioral characteristics [2].

Solid tumors are cell masses that lack liquid areas and can be non-cancerous (benign) or cancerous (malignant). Often, a complex, integrated organ-like structure can be observed that comprises interstitial connective tissue, blood vessels and extra-cellular matrix [3;4]. This appearance has several implications for the treatment of solid tumors since accessibility for anti-cancer therapeutics is highly limited by encapsulation and fragmentation of the tumor by stromal components – a fact called physiological resistance [5;6]. Thus, therapeutic success in the treatment of malignant solid tumors especially in comparison to non-solid cancers needs to be improved. As conventional therapies only showed limited success, new strategies taking into account current knowledge of cancer development might pose promising alternatives.

3.1.2 Model of tumor development

In multi-cellular organisms the organization of cells within tissues and organs is strictly regulated. As all mammalian cells carry similar molecular programs regulating their proliferation, differentiation and death, dysregulation of these molecular circuits might lead to the transformation of normal into malignant cells. Based on the observations of human cancers and animal models it was proposed that four to seven rate-limiting, stochastic events [7] suffice for the development of tumors. On the basis of genetic instability, tumor cells acquire up to six alterations that collectively dictate malignant growth: (I) self-sufficiency in growth signals, (II) insensitivity to growth-inhibiting signals, (III) limitless replicative potential, (IV) evasion of programmed cell death (apoptosis), (V) neoangiogenesis, and (VI) tissue invasion and metastasis (reviewed in [1]). Noteworthy, ancillary cells like fibroblast and endothelial cells as well as extracellular matrix components present in a tumor play a key role in driving tumor development by cell-to-cell signaling [1]. Furthermore, cancer phenotype is very much dependent on maintenance of the established modifications, a phenomenon termed “oncogene addiction” [8].

Several barriers restricting tumor transformation have been observed in mammalian cells. Replicative lifespan of somatic cells usually is limited by lack of telomerase activity [9]. Telomeres are repetitive DNA sequences at the end of eukaryotic chromosomes with protective character to prevent end-to-end fusion with other chromosomes. During cell division telomeres are shortened and after continuous erosion the cell responds by entering the state of senescence. If those senescent cells harbor inactive retinoblastoma protein (RB) and tumor suppressor protein p53 pathways they regain ability to multiply until facing a state of massive cell death and karyotypic disarray termed crisis. Only a small number of cells is able to overcome the crisis, thereby acquiring unlimited replicative potential and reach the state of immortalization [10].

Genetic modifications (mutations, chromosomal aberrations) and epigenetic abnormalities promote cancer development in two ways: by inactivating genes that act as tumor suppressors like retinoblastoma protein (RB) and adenomatous polyposis coli (APC), and by transforming proto-oncogenes lsuch as Ras or certain receptor tyrosine kinases. While latter affect the proliferative and/or differentiation state of cells, tumor suppressor proteins represent cellular checkpoints that drive cells into apoptosis upon detection of abnormal intracellular conditions (e. g.

oncogene activation or DNA damage). The most prominent tumor suppressor protein is p53. This protein is involved in various processes that maintain genomic stability (suggesting a role as "guardian of the genome" [11]), induction of temporary or irreversible growth arrest and cell death [12;13]. Additionally, p53 was demonstrated to influence innate immunity [14;15] and angiogenesis [16;17]. p53 unfolds its actions by protein-protein interactions and especially by its ability to act as transcription factor [12;13]. p53 responsive genes possess pro-apoptotic (Bax, Fas), cell cycle control ($p21^{WAF1/CIP1}$, PCNA) and DNA repair activity (GADD45). This paramount position of p53 makes it a preferential target for functional inactivation necessary for tumor development. As a result, genetic modifications (homozygous deletion, mutation) of the p53 gene and altered p53-regulating pathways can be observed in the majority of human tumors [18]. Notably, as p53 simultaneously is involved in anti-tumor and anti-viral defense, many viruses (e.g. Adenovirus) inactivate p53 to prevent premature induction of apoptosis to allow for productive viral amplification.

The connection between anti-viral and anti-neoplastic pathways can be highlighted by the interferon (IFN) system. Interferons are multifunctional cytokines that are involved in cell growth, apoptosis, and anti-viral pathways. During tumor development, the selection pressure for relentless growth and insensitivity toward apoptosis might favour cells that inactivate the interferon system resulting in loss of expression of key interferon genes [19]. In contrast, tumors that disrupt the IFN system might be more susceptible to viral threats. Surprisingly, it has recently been reported that interaction of α/β-interferons and p53 cooperate in fighting viral infections [14;15].

A hallmark of tumor development is the resistance toward cell death signals as almost all cancers acquire this property during the transformation process. Apoptosis – termed programmed cell death I – appears in all metazoans. It is essential to maintain tissue homeostasis and ensure successful organogenesis. Following a precisely choreographed series of steps the morphological manifestations of apoptosis will be apparent: disruption of cellular membranes, break-down of cytoskeleton, extrusion of the cytoplasm, degradation of chromosomes by endonucleolytic cleavage of DNA and condensation of the nuclear compartment [20]. The components of the apoptotic machinery can roughly be divided into sensors and effectors. Sensors control the extra- and

intracellular environments for cell death promoting signals and subsequently regulate components functioning as effectors of apoptosis. While one axis of apoptosis – the so called extrinsic pathway – processes extracellular signals (e.g. FAS ligand, TNFα) eliciting cell death in a sometimes mitochondria-independent manner, the intracellular arm – termed intrinsic pathway – senses and transmits signals from within the cell. Finally, the majority of signals converge on the mitochondria that respond by releasing cytochrome C, a potent catalyst of apoptosis [21]. This process is tightly regulated by members of the Bcl-2 family of proteins. Consisting of three subgroups – classified by structural charateristics – its members possess apoptosis-promoting (Bim, Puma, Noxa, Bax, Bak) or anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1) functions. The cooperation of these members is crucial for balancing survival and cell death but is not yet fully understood [22]. Certain intracellular proteases (caspases) ultimately act as executors of apoptosis. Hierarchically arranged in the process of apoptosis execution, initiator caspases activate effector caspases that selectively destroy cellular structures, the organelles and the genome of the cell [23].

3.1.3 Therapeutic treatment strategies

Conventional therapeutic strategies like radiation or anti-neoplastic chemotherapy primarily affect tumor tissue but their rather unselective action and leads to harsh adverse effects to neighboring tissue or even the whole patient. This brute-force approach was refined over years and significant progress could be achieved especially for non-solid tumors. Chemotherapies today target the high growth rate of cancer cells. Nonetheless, even the emergence of modern biologicals like therapeutic antibodies eventually could not prove to cure cancer patients but rather provides a limited survival advantage at higher quality of life.

Taken together, great advances in cancer therapy have been achieved over the last 60 years since Farber's introduction of aminopterin as one of the first chemotherapeutics [24]. Unfortunately for certain cancers – especially solid tumors – the outlook often remains fatal as conventional therapies are not capable to eradicate the entire tumor. At this point, the concept of oncolytic virotherapy addresses the major drawback of these therapies: conventional therapies lack the capacity to extinguish the whole tumor entity. Therefore, oncolytic viruses are tailored, replication-competent viruses aimed to specifically kill tumor cells by

concurrently propagating to amplify the anti-tumor agent itself. Selectivity of these oncolytic viruses is achieved by exploiting oncogenic features or pathways of cancerous cells. Ideally, the viral propagation stops and virus is cleared from the body when the last susceptible tumor cells are killed. An idealized concept of virotherapy is schematically depicted in figure 1 and explained in detail in the following chapters.

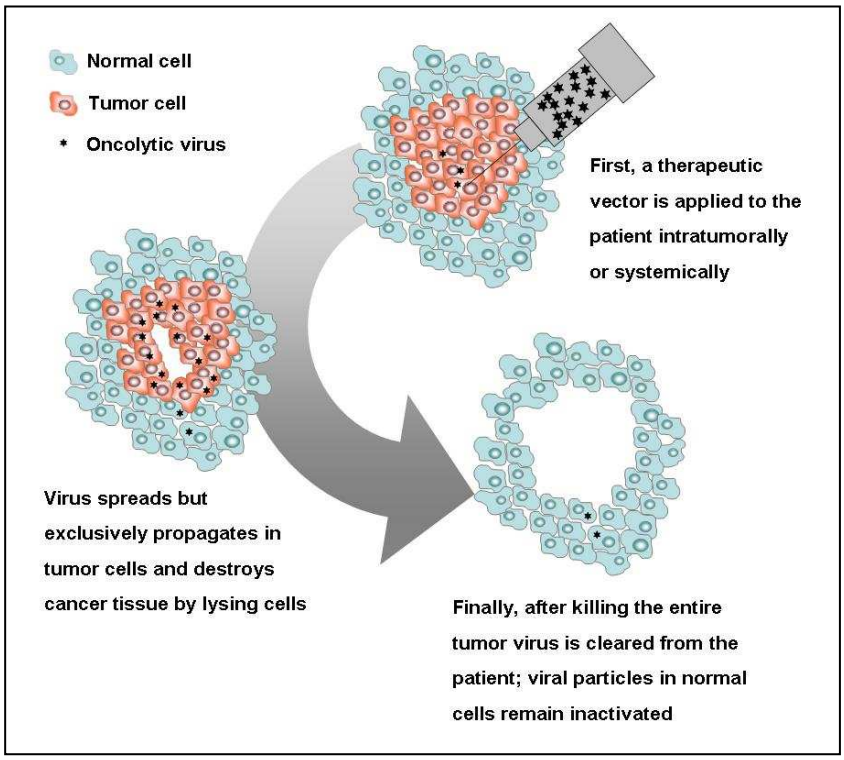

Figure 1. Theoretical concept of virotherapy. The therapeutic virus is injected into the patient and primarily targets cancer cells. Destruction of the infected cell and release of viral progeny allow infection malignant tumor tissue. As therapeutic viruses should be highly selective for cancer cells, healthy tissue is spared from cell lyses. When destruction of the tumor is completed, viral propagation comes to an end and remaining viral particles are cleared from the patient by immune cells. Particles that infected normal tissue will remain idle without causing viral replication and cell lysis.

3.2 Virotherapy

3.2.1 Development of cancer therapies by viral means

With more than 2,400 known species viruses represent a vast biological resource. Usually known to cause severe illnesses ranging from infectious diseases to cancer, perception is not only recently emerging that these ‘miniature biological machines’ or ‘nanoparticles’ can be exploited to serve in the fight against several sicknesses [25;26]. First case reports documenting tumor regression coinciding

with naturally acquired viral infections go back to the mid-1880s [27;28]. Suggestions aiming at exploiting viruses for therapeutic use arose at the beginning of the 19th century, but only in the late 1940s first clinical experiments were performed [29]. A hallmark of virotherapy is represented by the studies of Moore who used newly developed rodent cancer models to show that a virus (Russian Far East encephalitis virus) can selectively kill cancer cells *in vivo* [30;31]. Among Hepatitis viruses, Epstein-Barr virus, flaviviruses and others, Adenoviruses were identified as oncolytic agents in the 1950s (then known as adenoidal-pharyngeal-conjuctival virus) [32]. To control virulence and escape rapid clearance of the oncolytic agent by the patient's immune system because of earlier encounters much attention was drawn on animal viruses resulting in the discovery of several so called naturally tumor viruses. Nonetheless, only Newcastle Disease Virus (NDV) [33] and Vesicular Stomatitis Virus (VSV) [34] have been pursued as natural oncolytics today. With the advent of reverse genetics, direct manipulation of viruses became feasible to target viruses more specifically to cancerous cells. Subsequent to the generation of non-replicative viruses the potential of replication-competent agents was recognized. Eventually, this resulted in the first market approval for H101, a genetically modified human Adenovirus type 5, in 2005 for combination treatment with chemotherapy of patients suffering from head and neck cancer [35].

In the following chapter an introduction to therapeutically exploited viruses is provided that focuses on the molecular background of replicative oncolytic viruses and strategies to increase their tumor selectivity in general (3.2.2). Subsequently, special attention is paid to human Adenoviruses (3.3) and Vesicular Stomatitis Virus (3.4) applied as virotherapeutic agents.

3.2.2 Strategies for the exploitation of viruses as oncolytic agents

The application of viruses for the treatment of human patients requires a high level of safety. Therefore, an oncolytic virus should display two properties: selective propagation in and destruction of tumor tissue while sparing normal cells. This not only includes a high specificity for cancer cells, but also the evolution of a therapeutic virus into a pathogen within the patient has to be prohibited.

While some viruses display a natural specificity for cancer cells, others have to be engineered to increase therapeutic efficacy. The underlying targeting principles –

irrespective of the oncolytic virus applied – can be reduced to four groups: transductional, transcriptional, translational and apoptosis targeting (reviewed in [26]).

Transductional targeting includes different strategies to restrict entry of oncolytic viruses to cancer tissue. Other concepts allow infection of non-cancerous cells but aim to prevent viral replication by regulating transcription of viral key proteins (transcriptional targeting). Cells have developed different mechanisms to react on viral infections. During tumor development, cancer cells inactivate some of these anti-viral pathways what has been reported for the interferon system for example [36]. Thus, certain viruses preferentially replicate in cancer tissue exploiting the loss of anti-viral barriers. In contrast, normal cells block translation of viral mRNAs (translational targeting). Another concept is the modification of viral genes whose products are essential for virus propagation in normal cells but are dispensable in tumor cells (apoptosis targeting) [37].

3.3 Adenoviruses as oncolytic agents

The family of *Adenoviridae* comprises non-enveloped viruses with a linear, non-segmented, double-stranded DNA (30 – 38 kb) as genome. Until today, more than 40 species of Adenoviruses are known to infect animals and men. Currently, there are over 50 serotypes known that infect humans primarily being responsible for mild diseases of the upper respiratory system but also for conjunctivitis and gastroenteritis. On the other hand, these viruses pose a major threat to immune-compromised patients causing severe problems with high rates of mortality. First described in 1953 [32], Adenoviruses are well studied today. Adenoviral DNA is not integrated into the host genome, consequently risk of virus-induced mutagenesis is low, and particles can be produced of high titers and purity. Thus, certain adenoviral subtypes have been developed as anti-cancer therapeutics – so called oncolytic Adenoviruses [38-42]. So far, the adenoviral vectors called Gendicine (2003) – a non-replicative virus containing the tumor suppressor gene p53 – and H101/Oncorine (2005) – a modified replication-competent vector for oncolytic therapy – are the only viruses that have been approved for treating human cancers [35;43].

3.3.1 The human Adenoviruses type 5

To infect target cells human Adenovirus type 5 relies on the presentation of the coxsackievirus adenovirus receptor (CAR) on the surface of cells. CAR is bound by the knob domain of the adenoviral fiber protein. Additional binding to the co-receptor αV integrin (CD51) results in endocytosis of the virus particle via clathrin-coated pits. The virion is released to the cytoplasm by acidification of endosomes and subsequent structural changes of the capsid. Exploiting components of the cytoskeleton, the virion is transferred to nuclear pores where the viral DNA is released into the nucleus.

The replication cycle of Adenoviruses consists of an early and a late phase in which distinct gene products are expressed. Early genes (E1 – E4) possess regulatory functions: first, host cell gene expression is altered to allow for viral DNA synthesis; second, premature cell death of an infected cell by innate or adaptive immune defense mechanisms is prevented; and third, other viral genes are activated [44]. Genes from the E1 locus are separated into E1A and E1B genes that both express various splice variants. These two early gene families are most prominent in modifying the infected cell to promote viral replication. E1A activates adenoviral transcription and targets RB family members to drive the host cell into S phase of the cell cycle. As the actions of E1A induce pro-apoptotic signals, E1B variants, like E1B-19k (a viral Mcl-1 mimic) and E1B-55k, inhibit apoptosis by sequestering p53 in the cytoplasm [45], impairing p53 function as transcriptional activator (E1B-55k) [46;47] and by inactivating pro-apoptotic Bax and Bak proteins (E1B-19k) [48;49]. In conjunction, these proteins are able to immortalize and transform primary cells *in vitro* [50]. Replication of the viral genome occurs with the terminal protein acting as primer by covalently binding to the 5'-ends of the genome. For adenoviral replication, entry of the host cell into S phase is a prerequisite [44]. In the late phase structural proteins for packaging the viral genome are produced. Assembly of viral particles occurs in the nucleus of the infected cell that is finally lysed to release infectious virions.

3.3.2 Adenoviruses as oncolytic vector

The fact that Adenoviruses are DNA viruses with a relatively small genome facilitated its application as genetically modified therapeutic agent. There have

been several developmental waves of recombinant Adenoviruses generated for the treatment of human diseases. First-generation vectors were rendered replication-deficient by deleting E1 or both E1 and E3 region. Usually, these vectors were used to express therapeutic genes [51;52]. To minimize immunogenicity and to increase cloning capacity, second-generation vectors were constructed that additionally lack E2 and E4 genes. This trend finally led to third-generation adenoviral vectors – also termed gutless vectors – that completely lack the viral genomic information except of the inverted terminal repeat sequences (ITR) and the viral packaging signal [53].

Since these vectors did not lead to satisfyingly therapeutic efficacy, replication-competent Adenoviruses were constructed. Replicative vectors can elicit cell death in human cancer cells by different mechanisms: cytotoxicity mediated through viral proteins, induction of antiviral immunity, sensitization to chemotherapeutics or expression of heterologous therapeutic genes [38]. Limitation of side-effects while retaining oncolytic potential and the capacity to replicate efficiently can be achieved by targeting recombinant Adenoviruses to cancer cells by certain means. Adenoviruses can be transductionally targeted by replacing attachment proteins responsible for the native viral tropism. This can be necessary not only to gain selectivity but also because cancer cells often reduce or completely shut down expression of the native Adenovirus surface receptor CAR [54]. Additionally, tumor-selectively replicating adenoviral vectors were generated by modification of viral genes [40;55], repression of cellular genes that have essential character for virus replication [56] or even tumor-specific genome rearrangements of viruses [57]. Transcriptional control of adenoviral genes by tumor- or tissue-specific promoters (such as MUC1, AFP, PSA, kallikrein-2, pS2, alpha-lactalbumin, CXCR4, hTERT or Flk-1/endoglin) is another powerful tool to restrict replication to cancer cells [58-66]. Unfortunately, application of these promoters strongly narrows the virus to a certain cancer or tissue. However, the exploitation of a more general tumor feature would greatly broaden the field of application for transcriptionally targeted viruses.

3.3.3 p53-dependent adenoviral vectors

Since the tumor suppressor protein p53 is impaired in the majority of human tumors by genetic alterations, decreased protein stability or other mechanisms [67-

71] its transcriptional status (active or inactive) could be applied to differentiate between cancerous and normal cells and, thus, increase tumor-selectivity while potentially treating a wide range of tumors. In pioneering works it was hypothesized that deleting the p53-binding protein E1B-55k would restrict viral replication to cells with genetic defects in the p53 pathway [55]. However, p53-dependency of the resulting adenoviral vector Ad*dl*1520 (Onyx-015) was questioned. Rather, differential late viral mRNA export seems to be determining selectivity of Onyx-015 [72].

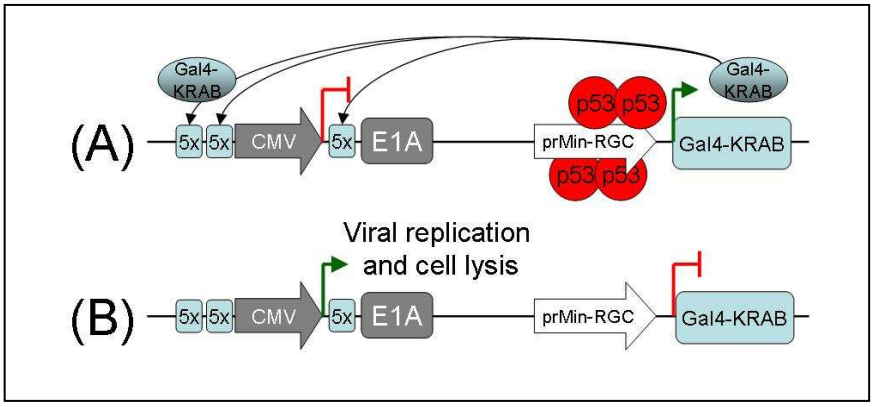

Figure 2. Schematic representation of the p53-dependent regulator system as part of the adenoviral genome. **(A)** Within an adenoviral backbone the p53-dependent promoter prMin-RGC drives the expression of the regulator gene Gal4-KRAB in presence of p53. The Gal4-Domain of the fusion protein binds to specific DNA-binding sequences in the artificial CMV promoter that controls expression of the E1A gene. Following, the KRAB-domain acts as a transcriptional repressor and E1A is not expressed. **(B)** In contrast, when p53 is absent or inactivated (like in the majority of human tumors) prMin-RGC is inactive and, thus, E1A is transcribed and subsequently can start viral replication finally leading to viral offspring and lysis of the infected cell.

In an alternative concept (figure 2), an artificial p53 dependent promoter called prMin-RGC has been constructed that allows for selective expression of genes in p53 wild type cells [73;74]. This promoter contains thirteen p53-binding sites derived from the ribosomal gene cluster (RGC), in combination with a minimal CMV-promoter providing a TATA-box motive. In a replication-competent Adenovirus, prMin-RGC controlled the expression of the transcriptional repressor fusion protein Gal4-KRAB (Gal4-DNA binding domain fused to the KRAB repressor). Gal4-KRAB in turn repressed E1A expression in p53 positive cells by binding to an artificial Gal4-KRAB-dependent CMV promoter that controls the expression of E1A. In absence of functional p53, Gal4-KRAB is not expressed, thus, allowing expression of E1A that promotes viral replication [74]. Supporting this strategy, in cells expressing functional p53 the protein level can be further

elevated by chemotherapy, e.g. Doxorubicin treatment. Furthermore, Adenoviruses itself stabilize p53 [75], thus enhancing prMin-RGC-controlled expression. To facilitate comprehension, the concept is depicted in figure 2.

3.3.4 The endonucleolytic enzyme I-*Sce* I

A replication deficient adenoviral vector was developed that expresses the rare-cutting endonuclease I-*Sce* I [76]. In contrast to virotherapeutic approaches this concept focuses on DNA repair and gene targeting by generating specific DNA double strand breaks. Nonetheless, this enzyme might be suitable to increase selectivity of conditionally replicating Adenoviruses when applied in the right setup.

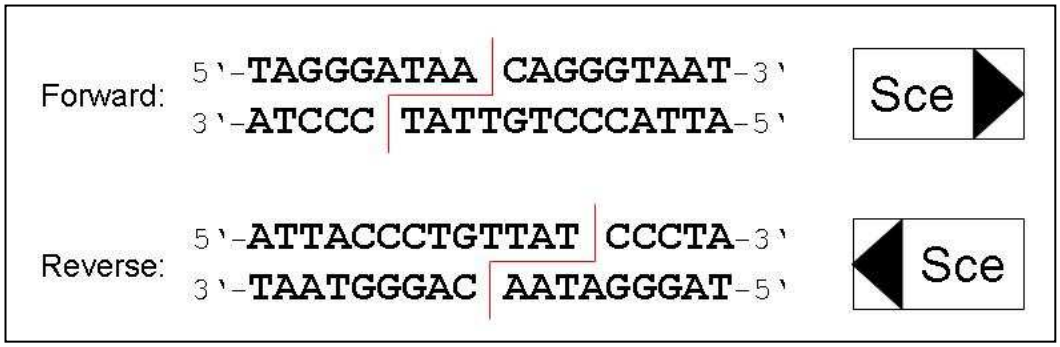

Figure 3. I-*Sce* I recognition sequence. The 18 base pair long recognition sequence of I-*Sce* I is non-palindromic and therefore can be described in forward and reverse direction. Cleavage (red lines) results in two parts that differ in length. The icons on the right side will be employed in following figure.

The meganuclease I-*Sce* I is a 26 kDa site-specific homing-endonuclease encoded by a mitochondrial intron of *Saccharomyces cerevisiae* [77-79]. Homing nucleases recognize long nucleotide sequences (14 – 40 bp) and therefore represent a class of extremely rare cutting enzymes. The core cleavage sequence of I-*Sce* I was revealed as a non-palindromic 18 bp long sequence [78] depicted in figure 3. On a random basis, this 18 bp sequence can be found as one in 70 billion base pairs (4^{18} bp), thus, allowing the introduction of a single or several cleavage sites into complex genomes, e.g. oncolytic DNA viruses. This capability makes the enzyme not only a powerful tool for the study of DNA repair processes [80;81] but also provides a smart instrument for targeted genome engineering in nearly all organisms like for example recombinant adenoviral vectors. Most likely, acceptor organisms are not affected by I-*Sce* I expression [82].

3.4 Vesicular Stomatitis Virus (VSV)

Vesicular Stomatitis Virus (VSV) is a bullet-shaped member of the Rhabdovirus family that causes a contagious disease among a broad range of host species including horses, cattle, and pigs [83], whereas the infection in humans is usually asymptomatic. In contrast, non-lethal infections of animals provoke lesions in the mucosa of nose and mouth and intranasally infected mice even suffer from neuropathy since neurons are highly susceptible for VSV infection. Naturally occurring in Latin America, VSV repeatedly caused epizootic diseases in the USA [84]. Serological analysis revealed the existence of at least two VSV serotypes known as New Jersey (VSV-NJ) and Indiana (VSV-IN) subtype [85].

3.4.1 Structure of Vesicular Stomatitis Virus

VSV is an enveloped virus with a non-segmented 11 kb long negative-stranded RNA genome that encodes five primary gene products, the nucleocapsid (VSV-N), polymerase proteins, (VSV-L) and (VSV-P), surface glycoprotein (VSV-G) and a peripheral matrix protein (VSV-M) [86]. Viral replication proceeds in the cytoplasm of infected cells. VSV does not undergo genetic recombination or reassortment. It neither and possesses known transforming potential nor it does integrate parts of its genome into the host DNA [87]. These properties make VSV attractive for use as therapeutic agent for humans.

VSV-G is the major known antigen and is densely packed on the viral surface. As a consequence, intact virions exhibit only one antigenic site that is a target for neutralizing antibodies *in vivo* [88]. VSV-N encases the viral genome but polymerase proteins L and P are attached to the RNA as well. The multifunctional VSV-M protein binds to viral components like RNA genome/nucleocapsid core (RNP) and VSV-G. On the other hand, the matrix protein is the major component of VSV responsible for interactions with the host cell (as described below).

3.4.1.1 Replication cycle of VSV

The infection cycle of the Vesicular Stomatitis Virus (VSV) starts with the binding of the glycoprotein to a yet unidentified receptor on the surface of the target cell. According to the broad range of infected cell types including mammalian as well as insect cells, the receptor is supposed to be an ubiquitously occurring molecule

that acts as binding partner for VSV-G [89]. So far, it is known that phosphatidyl serine is required for endocytosis but it has been excluded to serve as prime surface receptor of VSV [90]. In the cytoplasm of the infected cell viral polymerase proteins carried within the virus capsid start sequential transcription of the five viral genes resulting in capped and polyadenylated RNAs that are translated by the host cell's translation machinery [91;92]. Interestingly, the proteins generating the subgenomic RNAs are also responsible for the replication of the RNA genome. Newly synthesized N, P and L proteins associate with genomic RNA, thus forming RNP cores. These cores bind to regions of the cell membrane that are enriched with viral G and M proteins. Viral offspring will be released by budding that is followed by cell destruction. In comparison to other viruses VSV generates a tremendous amount of viral progeny within a short period of time.

3.4.1.2 Virus-host-interactions

3.4.1.2.1 VSV replication is highly susceptible to the actions of type I interferons

Host cells facing a viral infection usually limit viral amplification by activating components of the immune system. The adaptive immunity clears virus infected cells and neutralizes free-floating virus. But even on the cellular level an arsenal of anti-viral mechanisms is available that have an adverse effect on virus replication. These mechanisms are part of the innate immune response that when altered renders cells highly susceptible to viral threats and also plays a crucial role in cancer development. A functional adaptive immune response can not compensate an impaired innate immunity since animals lacking innate immunity components die from the cytopathic nature of VSV infection.

VSV is called a natural tumor virus or naturally smart virus as it is lytic to numerous transformed and malignant cell lines and much less so to normal tissue. It could be shown that VSV is extremely sensitive to the anti-viral action of the type I interferons [34;93]. The interferon (IFN) system mediates host defense by utilizing several components that sense a viral infection, promote signal transduction and affect virally transduced and uninfected neighboring cells. Signaling components like the Toll-like receptor pathway, the NF-κB pathway and interferon-regulatory factor 3 (IRF-3) initially sensing the infection by a virus induce transcription and secretion of IFN-β. Interferon α/β receptors (IFNARs)

transmit the signal to the Jak/STAT pathway which finally leads to the transcription of IFN-stimulated genes (ISGs) that provide an antiviral response [34;94]. Not surprisingly, VSV's selectivity to human tumor cells is based on the fact that IFN-responsive pathways are often defective in human cancers [34;93;95;96]. In contrast, functional IFN pathways in normal cells limit VSV amplification by induction of a robust anti-viral response. Namely, type I interferons highly restrict transcription of subgenomic mRNAs [97;98], mRNA cap methylation [99], mRNA translation [100;101] and viral assembly [102]. Research on virus-host interactions revealed several key components of the IFN system to fight VSV which will be briefly described below.

The eIF2α kinase PKR has been considered a crucial checkpoint against viral infections in general as it shuts down protein biosynthesis via phosphorylation of the eukaryotic initiation factor 2 (eIF2α) and elicits an apoptotic response after binding to the extensive secondary structures of double-stranded RNA (dsRNA). The role of PKR within the defense line against VSV is discussed controversially. VSV induces activation of PKR and mice lacking the interferon-inducible double-stranded RNA-dependent protein kinase are reported to be highly susceptible to VSV infection in comparison to wild type mice [103;104]. Other studies support this point of view [105-107] while more recent publications are questioning the role of PKR as a major component of IFN-mediated resistance to VSV infection. Rather, two other members of the eIF2α kinase family, the general control non-derepressible-2 (GCN2) protein and the endoplasmic reticulum (ER) kinase PERK/PEK which is activated by the presence of unfolded protein in the ER, are shown to exclusively contribute to the inhibition of VSV replication [108;109]. Additionally, while VSV can trigger IFN induction by recognition of single-stranded RNA via toll-like receptor 7 (TLR7) [110;111] there was no dsRNA detectable in VSV infected cells – although being expected to emerge within the replication of the genomic RNA – that might have induced activation of PKR suggesting that other viral components are more relevant for the induction of an anti-viral response [112].

Other members of the IFN-mediated anti-viral mechanisms that were reported to play a certain role in fighting VSV infections are the interferons themselves [113;114] and Stat-1 [115] suggesting the IFN system to be a key mechanism for the defense against VSV infections.

Interestingly, ISGs not only act anti-virally but concomitantly play a physiological role in preventing cancer development [36]. For example, induction of the tumor suppressor p53 was reported following interferon treatment [14] suggesting a possible anti-viral activity of p53. Although no VSV-associated mechanism is known that specifically targets p53 to abrogate its function – a common feature among viruses – two recent studies provide evidence of limiting VSV amplification by p53. Upon infection of mouse embryo fibroblasts (MEF) with VSV p53 is phosphorylated in an ataxia telangiectasia mutated (ATM)-dependent manner at Ser18 and subsequently leads to the transcription of p53 target genes [14]. Noteworthy, p53 up-regulation is dependent on the interferon α/β receptor (IFNAR) indicating a strong link between p53 and IFN pathways. Compared to $p53^{-/-}$ MEFs, wild type cells underwent a rapid induction of apoptosis, thus limiting viral production. *In vivo*, deletion of p53 renders mice susceptible to low doses of VSV while wild type mice remain unaffected. Questioning the pleiotropic effects of an absence of p53 on antiviral responses another group observed a limited VSV propagation in mice bearing an additional copy of the p53 gene ("super p53 mice") and did explain it by an enhanced p53-dependent induction of apoptosis [116]. However, posing a significant effect in MEFs *in vitro* (virus yield in wild type mice was 10-fold higher than in super 53 mice and at certainly lower apoptotic levels) the survival rate of infected wild type and super p53 mice did not show statistical differences [116].

3.4.1.2.2 VSV usurps the cellular protein biosynthesis machinery

Although coding for only five proteins, VSV massively modulates protein biosynthesis of the infected cell including transcription, the export of mRNAs and translation. It has been reported that the multi-functional matrix protein of VSV inhibits the cellular RNA-polymerases I-III [117;118] preventing generation of mRNAs. By binding to the mRNA export factor Rae1, transfer of mRNAs to the cytoplasm is efficiently suppressed [119]. Another component of the host cell translation machinery that is primarily targeted to limit protein biosynthesis of the infected cell is a part of the multi-subunit eIF4F complex, termed eIF4E. VSV-mediated dephosphorylation of eIF4E at serine 209 (via dephosphorylation of the eIF4E-binding protein 4E-BP-1) between 3 and 6 h post infection leads to the disassembly from the eIF4F complex resulting in a block of translation of host

mRNAs [120]. Thus, it has been reported that inhibition of interferon genes is achieved by general inhibition of host RNA and protein synthesis [121]. Despite this, the translation of viral 5'-capped mRNAs proceeds since viral mRNAs contain cis-acting structural elements that enhance translation efficacy of viral mRNAs [122]. This grants the virus exclusivity to cellular resources. By phosphorylation of the alpha-subunit of eIF2α infected cells can react on this hijacking of the cellular translation apparatus by blocking global translation. It should be noted, that in contrast to normal cells, the guanine nucleotide exchange factor eIF2B downstream of eIF2α was frequently aberrant in human tumors, reversing eIF2α activity and finally allowing VSV mRNA translation [96], and thus posing another selectivity mechanism for VSV.

3.4.1.2.3 Induction of apoptosis in VSV-infected cells

During evolution, many viruses have acquired mechanisms to interfere with induction of apoptosis, a major reaction of infected cells to limit viral spread. VSV infected cells induce apoptosis in a caspase-3/9-dependent manner [93;95;123-127] wherein the pro-apoptotic Bax protein might represent a central point of convergence finally leading to mitochondrial membrane depolarization [128]. Mechanisms to prevent this induction are not known so far. However, it can rather be speculated whether this rapid induction by a virion constitutive component [125] could be beneficial for the virus. As wild type VSV induces apoptosis via the mitochondrial (intrinsic) pathway, it has been analyzed whether members of the Bcl-2 family influence viral amplification. For the pro-survival members Bcl-2 and Bcl-xL it was shown that over-expression promoted survival of infected cells of neural origin [126]. VSV-mediated apoptosis can even be partially suppressed by over-expression of Bcl-2 [126]. For currently developed VSV-M mutants that show a more promising safety profile than their wild type counterparts, natural Bcl-2 over-expression observed in primary chronic lymphocytic leukemia cells prevents the oncolytic effect of the viral therapeutic [129].

VSV causes a visible cytopathic effect characterized by the typical rounding of infected cells. This is due to the action of the matrix protein that disorganizes actin, microtubles and intermediate filaments in cooperation with the VSV-L protein [130;131]. This action of VSV-M is strongly correlated with its ability to shut down host gene expression [132]. Moreover, VSV disrupts cell-signaling

pathways such as STAT signaling probably attenuating inflammatory responses to VSV infection [133]. Another effect related to innate immunity is the suppression of antigen presentation by VSV-M [134]. Taken together, mainly – but not exclusively – by the multiple abilities of the M-protein, VSV hijacks the infected cell to transform it into a virus production factory. Due to these diversified roles of the matrix protein in VSV replication and pathogenesis it has even been termed the 'brain' of the virus [135].

3.4.2 VSV as oncolytic vector

The rapid replication cycle, the natural selectivity for tumor cells and the ability to infect a wide variety of human cancer cells make VSV an attractive tool for cancer therapy. Wild type VSV was repeatedly reported to cause growth inhibition on human cancer cell lines exploiting defects in the innate immune response. Additionally, the ability of the virus to kill cancer cells *in vivo* even when administered systemically in immunocompetent mice together with the fact that no overt symptoms could be observed in these animals holds great promise for wild type VSV as effective oncolytic agent [34;95;136]. Oberserving that interferon non-responsive tumors can be eradicated by VSV *in vitro* and *in vivo* [34], it became apparent that tumors that bear defects in the frontline anti-viral IFN system are susceptible for oncolytic VSV therapy [93]. Finally, the development of VSV as oncolytic vector was further promoted, first by engineering a DNA-based platform to generate recombinant VSV particles [137] and, second by the discovery of VSV mutants providing an enlarged therapeutic window as they induce a powerful IFN-β response [138].

Certain mutations of the VSV matrix protein neutralize its abilities to impair the IFN response in infected cells. Namely, the resulting attenuated virus (AV) mutants AV1 (M51R), AV2 (V221F and S226R) and AV3 (MΔ51) possess a vastly improved therapeutic index compared to wild type VSV as they potently induce an interferon response in neural tissue [94]. Whereas wild type and mutant viruses induce IFN-β transcription, in cells infected with mutant VSV these IFN-β mRNAs were detectable in the cytoplasm indicating that mutations of the VSV matrix protein disrupt the block of export of mature mRNAs to the cytoplasm [94]. In contrast to wild type VSV, the above described mutants induce apoptosis via the extrinsic pathway by Fas and Daxx [139;140].

A wealth of different concepts regarding engineered VSV particles was established aiming at an increased selectivity of VSV for human cancer cells by recombinant means. Interestingly, recombinant VSV genomes can accommodate large amounts of foreign RNA, a strong prerequisite for successful modification [141]. Additionally, inserted genes are usually reported to be expressed at high levels. Like other viruses VSV has been armed with pro-drug-converting enzymes, such as HSV-thymidine kinase [142] and cytosine-deaminase [143] or immunomodulatory genes like interleukin-4 [142].

Modification of the viral tropism by replacing the glycoprotein by chimeric Sinbis virus surface proteins [144;145] yielded a higher selectivity for certain tumor cells by restricting the host cell range to cells exposing specific surface tumor markers. Furthermore, surface modifications of VSV influence the recognition of host adaptive immunity [146]. The integration of host innate immunity genes (IFN-β, IL-12) into the genome of VSV improved specificity and enhanced anti-tumor efficacy of the resulting vector by combining oncolytic and immunomodulatory strategies [147;148].

In two recent studies the potential of VSV has been explored to augment an enhanced therapeutic effect when combined with conventional chemotherapy [149;150]. Both studies demonstrated *in vitro* and *in vivo* that VSV replication is not impaired by gemcitabine or cisplatin chemotherapy, and enhanced anti-tumor activity by combined virochemotherapy could be observed. However, both studies could not provide a molecular explanation for enhancement of therapy just assuming enhanced apoptosis on a narrow experimental basis.

4. Objectives

The presented work is focused on advancing knowledge in the field of virotherapy of solid human tumors. First, a conditionally replicating adenoviral vector was constructed employing a novel tool to increase selectivity for cancer cells. Second, the virus-host-interaction between Vesicular Stomatitis Virus and different human cancer cell lines regarding induction of apoptosis was explored paying special attention to the members of the Bcl-2 family proteins.

Several approaches to limit replication of oncolytic viruses for the treatment of human cancers have been described. However, silenced viral particles lying dormant in infected cells can be reactivated to start replication by physiological changes within the cell. Thus, destruction of the viral backbone in non-target cells would prevent the rise of break-through mutants after long persistence in normal cells. Hence, based on a p53-responsive system, a recombinant adenoviral vector was constructed to express the yeast endonuclease I-*Sce* I as a self-destruction switch in p53-dependent manner to disrupt the viral backbone and increase selectivity of the oncolytic vector.

In a second project, infection of human cancer cells by wild type VSV rapidly leads to induction of apoptosis, but the underlying mechanisms are not yet fully understood. Previous work on other viruses strongly suggests the implication of proteins of the Bcl-2 family to be involved in sensing and reacting to viral infections. Here, the focus of the investigation was set on the role of the anti-apoptotic proteins of the Bcl-2 family in fighting VSV infection. As these proteins are reported to confer resistance against chemotherapy, a possible sensitization mediated by VSV infection was explored.

5. Materials and methods

All experiments were performed in S1 or S2 laboratories at Hannover Medical School equipped for molecular biological work. Displayed exclusively are substances, devices or methods that might have influenced the outcome or the form of the experiments or exceed the status of standard laboratory equipment.

5.1 Materials

5.1.1 Cell lines

5.1.1.1 Purchased/provided cell lines

Cell line	Origin	Source	Main items
A549	Human, lung	ATCC	Adenocarcinoma, $p53^{+/+}$
HepG2	Human, liver	ATCC	HCC, $p53^{+/+}$
Huh-7	Human, liver	Japanese Collection of Research Bioresources (JCRB)	Hepatoma, mut-p53 (220C, transcriptionally inactive form)
H1299	Human, lung	ATCC	NSCLC, $p53^{-/-}$
Hep3B	Human, liver	ATCC	HCC, $p53^{-/-}$
HEK 293	Human, kidney	ATCC	Expressing adenoviral E1A and E1B genes
BSR T7/5	Hamster, kidney	Matthias Schnell, Max von Pettenkofer Institut, Munich	T7-Polymerase expressing BHK-21-derivative
Phoenix cells	Human, kidney	Lars Zender, HZI Braunschweig	HEK 293 based packaging cell line for generation of retroviral particles

All cell lines were maintained in growth medium (DMEM + Glutamax, Life Technologies) supplemented with 10 % fetal bovine serum (Life Technologies), 100 units/mL penicillin and 100 µg/ml streptomycin (Seromed, Berlin, Germany) at 37°C in 5 % CO_2. BSR T7/5 cells (kind gift of Matthias Schnell, Munich) required growth medium plus 4 % tryptose phosphate (Life Technologies).

5.1.1.2 Stably transfected cell lines

The following transgenic cell lines were generated in the context of this work:

Parental cell line	Inserted plasmid(s)	Inserted promoter-gene combination	Selection marker (concentration)
A549	pS4871	CMV-wt-hMcl-1	Puromycin (800 ng/ml)
	pS4865	CMV-mut-hMcl-1	Puromycin (800 ng/ml)
	pS5060	CMV-wt-hMcl-1	Neomycin (600 µg/ml)

Parental cell line	Inserted plasmid(s)	Inserted promoter-gene combination	Selection marker (concentration)
A549	pS5070	CMV-mut-hMcl-1	Neomycin (600 µg/ml)
	pS5600	CMV-EGFP	Neomycin (600 µg/ml)
	SK301	CMV-YFP-LC3	Neomycin (600 µg/ml)
	SK301 + pS4865	CMV-YFP-LC3 + CMV-mut-hMcl-1	Neomycin (600 µg/ml) + Puromycin (800 ng/ml)
Huh-7	pS5060	CMV-wt-hMcl-1	Neomycin (300 µg/ml)
	pS5070	CMV-mut-hMcl-1	Neomycin (300 µg/ml)
	pS5600	CMV-EGFP	Neomycin (300 µg/ml)
	SK301	CMV-YFP-LC3	Neomycin (300 µg/ml)
	pS5926	Scrambled shRNA	Neomycin (800 µg/ml)
	pS5839	shRNA anti-Bax	Neomycin (800 µg/ml)
	pS5848	shRNA anti-Bak	Neomycin (800 µg/ml)
	pS5862	shRNA anti-Bax/anti-Bak	Neomycin (800 µg/ml)
HepG2	SK301	CMV-YFP-LC3	Neomycin (800 µg/ml)

Transgenic cell lines were generated by lenti- or retroviral gene transfer and propagated in growth medium supplemented with Neomycin (Calbiochem Corp. San Diego, CA) or Puromycin (Sigma-Aldrich, Taufkirchen, Germany).

5.1.2 Bacteria

Strain	Genetic properties, supplier
JM109	Modified *E. coli* safety strain; Gibco; prepared as electro- and chemically competent cells
XL1-blue	Modified *E. coli* safety strain; Stratagene; prepared as electro-competent cells

Bacteria were grown in LB medium containing Ampicillin (100 µg/ml) or Kanamycin (10 µg/ml) over night.

5.1.3 Mice

Strain	Properties
NMRI-nu/nu	Female, pathogen-free nude mice aged 6-8 weeks from the Central Animal Facilities of Hannover Medical School

5.1.4 Plasmids

5.1.4.1 Provided plasmids

Plasmid	Supplier/ Source	Properties
pBlueScript	Stratagene	Cloning vector (to sequence DNA fragments), used as nonsense-DNA for transfections
pQCXIP	Clontech	Retroviral vector for the generation of stably transfected cell lines containing a CMV-gene of choice – IRES – Puromycin unit
pQCXIN	Clontech	Retroviral vector for the generation of stably

Plasmid	Supplier/ Source	Properties
		transfected cell lines containing a CMV-gene of choice – IRES – Puromycin unit
pGL2	Promega	Reporter vector containing the firefly luciferase (luc)
pGL4.12	Promega	Reporter vector containing a modified firefly luciferase (luc2CP) to respond more rapidly and with greater magnitude to changes in transcriptional activity
CMV-lacZ	Florian Kühnel	Vector containing a lacZ gene controlled by a CMV promoter, used to normalize efficiency of transfection
CMV-GFP	Florian Kühnel	Vector containing an EGFP gene controlled by a CMV promoter, used to estimate efficiency of transfection microscopically
Gal4-KRAB (FK2337)	Florian Kühnel	Vector containing the gene of the fusion protein of Gal4-DNA-binding domain and the KRAB (Krüppel associated box) transcription repressor domain
pAdHM4	Mark A. Kay	Plasmid containing the genome of human Adenovirus type 5 lacking the E1 (342-3523 bp) and E3 (28133-30818 bp) regions
K92	Thomas Wirth	Shuttle vector containing the Adenoviral E1 region where E1B transcription is linked to EGFP by an IRES
NW1187	Norman Woller	pBluescript containing a U6-promoter to drive shRNA-expression
SK301	Sarah Knocke	pQCXIN containing the gene for the fusion plasmid YFP-LC3 [151]
pVSV-G	Norman Woller	Plasmid coding for VSV-G controlled by a CMV promoter
NW4739	Norman Woller	pLVTHM w/o *Xba* I site
pMDL g/p RRE	Norman Woller	Helper plasmid for Lentivirus generation
pMD-G	Norman Woller	Helper plasmid for Lentivirus generation
pRSV-Rev	Norman Woller	Helper plasmid for Lentivirus generation
psiCHECK-2	Promega	Vector designed to optimize RNAi target sequences by expressing two different luciferases: *Renilla* luciferase (with a multiple cloning site situated downstream of the stop codon to allow integration of target gene sequences) and firefly luciferase (for normalization of *Renilla* luciferase expression)
pRK5.LHA-Sce1	Toni Cathomen (Charité, Institute of Viorology, Berlin)	Plasmid containing a codon-optimized (humanized) version of the I-*Sce* I gene with N-terminal HA-tag and nuclear localization sequences
FK1212	Florian Kühnel	pBluescript vector containing a CMV_{350}-

Plasmid	Supplier/ Source	Properties
		promoter that controls a luciferase gene
DS122	Florian Kühnel	pBluescript vector containing a CMV_{350}-promoter, an artificial MCS and a poly-adenylation signal (expression plasmid)
FK1738	Florian Kühnel	pGL-2 vector with p53-dependent prMin-RGC-promoter driving the expression of luciferase reporter gene
pIRES2-EGFP	Clontech	Vector to co-express EGFP with a gene of choice linked by a internal ribosomal entry sequence (IRES)
pVSV-XN2	John K. Rose (New Haven, CT, USA)	Recombinant VSV genome (cloning platform)
pVSV-GM-CSF		Recombinant VSV genome including an additional GM-CSF gene
pVSV-EGFP		Recombinant VSV genome including an additional EGFP gene
pBS-N		Expression vector for VSV-N-protein
pBS-P		Expression vector for VSV-P-protein
pBS-L		Expression vector for VSV-L-protein
FK6570	Florian Kühnel	pBluescript vector containing a CMV230 promoter with Gal4-binding sites upstream (15x) and downstream (5x) of the promoter
pSE1A_1	Nina Strüver	N-terminally deleted E1A (66bp)
pSE1A_2	Nina Strüver	Wt-E1A
pSE1A_3	Nina Strüver	N-terminally deleted E1A (66bp), insertion of a single *Bam*HI-site
pSE1A_4	Nina Strüver	N-terminally deleted E1A (254bp)
EG263	Engin Gürlevik	NW1187 vector controlling a shRNA against EGFP under control of a U6-promoter
p3xFLAG-CMV-mut-Mcl-1	Xiaodong Wang, Dallas, TX, USA	Plasmid containing the sequence of human Mcl-1 altered by certain Lys→Ala-changes to prevent ubiquitination of Mcl-1

5.1.4.2 Constructed plasmids

Plasmid	Properties
DS122-prMin-RGC	DS122 with the p53-dependent promoter prMin-RGC replacing the CMV promoter
pS209	Expression plasmid for I-*Sce* I controlled by prMin-RGC-promoter
pS261	Expression plasmid for I-*Sce* I controlled by prMin-RGC-promoter with a Kozak sequence and an C-terminal His_6-tag sequence
pS432	DS122-prMin-RGC controlling the expression of the Gal4-KRAB gene
pS584	K92 with excised IRES-EGFP region
pS575	Expression plasmid for I-*Sce* I controlled by prMin-RGC-

Plasmid	Properties
	promoter with a Kozak sequence and an additional MCS between promoter and I-*Sce* I gene
pS716	pS575 including the IRES from pIRES2-EGFP between promoter and I-*Sce* I gene
pS725	pS716 with the Gal4-KRAB-sequence inserted between prMin-RGC-promoter and IRES
pS1111	DS122-prMin-RGC controlling the expression of EGFP with an 5'-UTR-situated I-*Sce* I-recognition site (fw)
FK6451	pGL2 vector containing the expression cassette: CMV_{350} – Sce_{fw} – EGFP – Sce_{fw} - luciferase
FK6456	pGL2 vector containing the expression cassette: CMV_{350} – Sce_{fw} – EGFP – Sce_{rev} - luciferase
pS2019	pGL2 vector containing the expression cassette: CMV_{350} –Sce_{rev} - luciferase
pS3075	DS122 coding for the human Bcl-x gene
pS3208	pS725 replacing the I-*Sce* I gene by EGFP
pS3251	pBluescript vector containing a Gal4-KRAB sensitive CMV_{230} promoter with an I-*Sce* I-recognition site (fw) at the 3'-end of the promoter fragment
pS3288	Exchange of the IRES-EGFP module in K92 by an artificial sequence containing ATG triplets (one for each reading frame) and an I-*Sce* I-recognition site (fw)
pS3310	Exchange of the IRES-EGFP module in K92 by an artificial sequence containing ATG triplets (one for each reading frame) and an I-*Sce* I-recognition site (rev)
pS3399	pBluescript vector containing a Gal4-KRAB sensitive hTERT promoter with an I-*Sce* I-recognition site (fw) at the 3'-end of the promoter fragment
pS3704	Exchange of the wt-E1A sequence in pS3310 by a N-terminally truncated form (del66) of E1A
pS3786	Exchange of the wt-E1A sequence in pS3288 by a N-terminally truncated form (del66) of E1A
pS4098	Insertion of the I-*Sce* I-sensitive CMV_{230}-promoter (pS3251) into pS3704 upstream of E1A
pS4112	Insertion of the I-*Sce* I-sensitive hTERT-promoter (pS3399) into pS3704 upstream of E1A
pS4116	Insertion of the I-*Sce* I-sensitive CMV_{230}-promoter (pS3251) into pS3786 upstream of E1A
pS4129	Insertion of the I-*Sce* I-sensitive hTERT-promoter (pS3399) into pS3786 upstream of E1A
pS4237	Insertion of prMin-RGC-I-*Sce* I-unit from pS261 into pS4116 downstream of the E1 genes (CMV, Sce_{fw} §)
pS4226	Insertion of prMin-RGC-I-*Sce* I-unit from pS261 into pS4098 downstream of the E1 genes (CMV, Sce_{rev} §)
pS4240	Insertion of prMin-RGC-I-*Sce* I-unit from pS261 into pS4129 downstream of the E1 genes (hTERT, Sce_{fw} §)
pS4233	Insertion of prMin-RGC-I-*Sce* I-unit from pS261 into pS4112 downstream of the E1 genes (hTERT, Sce_{rev} §)
pS4303	Insertion of prMin-RGC-Gal4-KRAB-IRES-I-*Sce* I-unit from

Plasmid	Properties
	pS725 into pS4116 downstream of the E1 genes (CMV, Sce_{fw} §)
pS4315	Insertion of prMin-RGC-Gal4-KRAB-IRES-I-*Sce* I-unit from pS725 into pS4098 downstream of the E1 genes (CMV, Sce_{rev} §)
pS4356	Insertion of prMin-RGC-Gal4-KRAB-IRES-EGFP-unit from pS3208 into pS4129 downstream of the E1 genes (hTERT, Sce_{fw} §)
pS4372	Insertion of prMin-RGC-Gal4-KRAB-IRES-EGFP-unit from pS3208into pS4116 downstream of the E1 genes (hTERT, Sce_{rev} §)
pS4416	Insertion of IRES-EGFP from pIRES2-EGFP into DS122-prMin-RGC
pS4532	Insertion of prMin-RGC-I-*Sce* I-unit from pS4416 into pS4098 downstream of the E1 genes (hTERT, Sce_{rev} §)
pS4565	Insertion of prMin-RGC-I-*Sce* I-unit from pS4416 into pS4112 downstream of the E1 genes (hTERT, Sce_{rev} §)
pS4865	Integration of mut-Mcl-1 in pQCXIP
pS4871	Integration of wt-Mcl-1 in pQCXIP
pS5060	Integration of mut-Mcl-1 in pQCXIN
pS5070	Integration of wt-Mcl-1 in pQCXIN
Ad4226	Integration of E1 and prMin-RGC region of pS4226 in pAdHM4
Ad4532	Integration of E1 and prMin-RGC region of pS4532 in pAdHM4
Ad4233	Integration of E1 and prMin-RGC region of pS4233 in pAdHM4
Ad4565	Integration of E1 and prMin-RGC region of pS4565 in pAdHM4
Ad4303	Integration of E1 and prMin-RGC region of pS4303 in pAdHM4
Ad4356	Integration of E1 and prMin-RGC region of pS4356 in pAdHM4
Ad4315	Integration of E1 and prMin-RGC region of pS4315 in pAdHM4
Ad4372	Integration of E1 and prMin-RGC region of pS4372 in pAdHM4
pS5408	EGFP in pQCXIP
pS5477	shRNA against Bax (shBax_1) in NW1187
pS5483	shRNA against Bax (shBax_2) in NW1187
pS5486	shRNA against Bax (shBax_3) in NW1187
pS5490	shRNA against Bak (shBak_1) in NW1187
pS5493	shRNA against Bak (shBak_2) in NW1187
pS5498	shRNA against Bak (shBak_3) in NW1187
pS5504	Fragment of the human Bak-gene in psiCHECK-2
pS5510	Fragment of the human Bax-gene in psiCHECK-2
pS5600	EGFP in pQCXIN
pS5653	shRNAs against Bax (shBax_1) and against Bak (shBak_2) in NW1187
pS5670	shRNAs against Bak (shBax_2) and against Bax (shBax_2) in NW1187
pS5685	Puromycin resistance gene (PCR product from pQCXIP) in pBluescript
pS5701	Neomycin resistance gene (PCR product from pQCXIN) in pBluescript
pS5757	Puromycin resistance gene from pQCXIP in NW4739
pS5779	Neomycin resistance gene from pQCXIN in NW4739
pS5839	U6-promoter controlling shRNA against Bax (shBax_1) in pS5757

Plasmid	Properties
pS5848	U6-promoter controlling shRNA against Bak (shBax_2) in pS5757
pS5862	U6-promoter controlling shRNA against Bax (shBax_1) and Bak (shBak_2) in pS5757
pS5926	U6-promoter controlling shRNA against EGFP in pS5757

§ Sce_{fw}: TAGGGATAACAGGGTAAT; Sce_{rev}: ATTACCCTGTTATCCCTA

5.1.5 Adenoviruses

5.1.5.1 Provided Adenoviral vectors

Ad-(Plasmid)	Supplier/ Source	Properties
Ad-p53Sensor	Florian Kühnel	Conditionally replicating human Adenovirus type 5 selective for cells with transcriptionally inactive p53 (from plasmid stock)
wt-Ad5	Florian Kühnel	Wild type human Adenovirus type 5 (from infectious particles)
ONYX-015	Frank McCormick	Conditionally replicating human Adenovirus type 5 with deleted E1B region from ONYX Pharmaceuticals, Inc. (from infectious particles)

5.1.5.2 Constructed Adenoviral vectors

Plasmid	Properties
Ad4226	Replicative human Adenovirus type 5 regulated by a prMin-RGC-I-*Sce* I cassette (E1A controlled by CMV_{230}-promoter)
Ad4532	Replicative human Adenovirus type 5 regulated by a prMin-RGC-EGFP cassette (E1A controlled by CMV_{230}-promoter)
Ad4233	Replicative human Adenovirus type 5 regulated by a prMin-RGC-I-*Sce* I cassette (E1A controlled by hTERT-promoter)
Ad4565	Replicative human Adenovirus type 5 regulated by a prMin-RGC-EGFP cassette (E1A controlled by hTERT-promoter)
Ad4303	Replicative human Adenovirus type 5 regulated by a prMin-RGC-Gal4-KRAB-IRES-I-*Sce* I cassette (E1A controlled by CMV_{230}-promoter)
Ad4356	Replicative human Adenovirus type 5 regulated by a prMin-RGC-Gal4-KRAB-IRES-EGFP cassette (E1A controlled by CMV_{230}-promoter)
Ad4315	Replicative human Adenovirus type 5 regulated by a prMin-RGC-Gal4-KRAB-IRES-I-*Sce* I cassette (E1A controlled by hTERT-promoter)
Ad4372	Replicative human Adenovirus type 5 regulated by a prMin-RGC-Gal4-KRAB-IRES-EGFP cassette (E1A controlled by hTERT-promoter)

5.1.6 VSV

VSV-(Plasmid)	Source	Description
wt-VSV Indiana	Mahtab Nourbakhsh, MHH, Hannover	Wild type Vesicular Stomatitis Virus Indiana serotype (from infectious particles)
AV1	Oliver Ebert, TU Munich	Attenuated VSV with mutant matrix protein (M51R)

5.1.7 Oligonucleotides

All oligonucleotides were purchased from Eurofins MWG GmbH.

Name	Sequence in 5'-3'-direction	Description
SceHis_3	AGAAGCTT*GCCACCATGG*GATCAAGATCGCCAAAAAAG	Insertion of a *Hin*dIII-cleavage site and a Kozak-site (italic)
SceHis_4	AAGGATCCTTA*ATGGTGATGGTGATGATG*ACTTCCTTTCAGGAAAGTTTCGGAGGAGATAGTG	Insertion of a *Bam*HI- cleavage site and a His_6-tag (italic)
Sce-Primer_5	ATAGGCCTAAAAACTAGTGCTAGCTATTCCATGGGATCAAGATCGCCAAAAAAGAAGAGAAAGG	Insertion of cleavage sites for *Stu* I, *Spe* I, *Nhe* I and *Nco* I
Sce-Primer_6	CCAAGCTTCTGCAGGTCGACTCTAGAGGATCC	Insertion of a *Bam*HI- cleavage site
SceEGFP_fw	ACAAGCTTGGATCCGATATC*TAGGGATAACAGGGTAATAG**CCACCATGG*TGAGCAAGGGCGAGG	Insertion of cleavage sites for *Hin*dIII, *Bam*HI, *Eco*RV, *I-Sce* I and *Nco* I and a Kozak-site (italic)
SceEGFP_rev	AAAGATCTGTCGACACGCGTCCTCTCTGTCTCGGGTAAGATACATTGATGAGTTTGGACAAACC	Insertion of cleavage sites for *Bgl* II, *Sal* I, and *Mlu* I
bcl-x_fwd	AAGCTAGCAATGTCTCAGAGCAACCGGGAGC	Insertion of a *Nhe* I cleavage site
bcl-x_rev	TTAGATCTGGATGGTCAGTGTCTGGTCATTTCC	Insertion of a *Eco*RI cleavage site
sceE1B_fw1	AAGAATTC*ATG*AAATAAAGCA*ATGAATG*CACACCAACAACATCAT*ATGGTAGGGATAACAGG*	Insertion of a start codon (ATG) in all three reading frames and part of a I-*Sce* I-recognition site
SceE1B_rev1	TTGCGGCCGCAT*TACCCTGTTATCCCTA*CCATATGATGTTGTTGG	Insertion of an I-*Sce* I-recognition site
sceE1B_fw2	AAGAATTCATGAAATAAAGCAATGAATGCACACCAACAACATCATATGGAT*TACCCTGTTATCC*	Insertion of a start codon (ATG) in all three reading frames and part of a I-*Sce* I-recognition site
sceE1B_rev2	TTGCGGCCGC*TAGGGATAACAGGGTAAT*CCATATGATGTTGTTGG	Insertion of an I-*Sce* I-recognition site

Name	Sequence in 5'-3'-direction	Description
sceCMV 230_fw	AAGGATCACAGGGTAATCCATGGTGATGCGGTTTTGGCAGTACATCAATGG	Insertion of a *Nco* I-cleavage site
sceCMV 230_rev	TTGCTAGCTCTAGAAT*TACCCTGTTATCCCTA*TAGCGGATCTGACGGTTCACTAAACCAGC	Insertion of an I-*Sce* I-recognition site
scehTERT_fw	AAGCGCCAGGGTACTCCATGGATGCTGCGCTGTCGGGGCCAGG	Insertion of a *Nhe* I-cleavage site
scehTERT_rev	TTGCTAGCTCTAGA*ATTACCCTGTTATCCCTA*CGAGCCCGCTGCCTGAAACTCGC	Insertion of an I-*Sce* I-recognition site
Mcl_fw	AAAAGCTTAAGGATCCATGTTTGGCCTCAAAAGAAACGCG	Insertion of cleavage sites for *Hin*dIII and *Bam*HI,
Mcl-rev	TTTCTAGACCGAATTCCTATCTTATTAGATATGCCAAACCAGCTCC	Insertion of cleavage sites for *Xba* I and *Eco*RI
E1A_cut control_rev	CGAGGAGGCGGTTTCGCAGATT	Forward primer to generate PCR product specific for I-*Sce* I dependent religation
E1B_cut control_fw	CTAAGATATTGCTTGAGCCCGAGAGC	Reverse primer to generate PCR product specific for I-*Sce* I dependent religation
mir30-Xba	AAATCTAGAGAATTCCGAGGCAGTAGGCA	Primer providing a *Xba* I-site and 5'-flanking sequnence for shRNAs
mir30-Xho	TTCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG	Primer providing a *Xho* I-site and 3'-flanking sequnence for shRNAs

Sequencing primer

Name	Sequence in 5'-3'-direction
Seq-Luc-primer	GGATAGAATGGCGCCGGG
Rvprimer3	CTAGCAAAATAGGCTGTCCC
Rvprimer4	GACGATAGTCATGCCCCGCG
SeqPrim_E1B_end	TGCTGGATGTGACCGAGG
seq_E1Astart_rev	CAAAGCGAACACATAATATCTGGGTCCCCC
E1Bterm_fw	TTAGGAACCAGCCTGTGATGCTGG
ProtIX_rev	TAGTAGAGTTTGCGGGCAGGACG
SeqPrimpQCXI_fw	CCATCCACGCTGTTTTGACC
U6 primer 1.2	GGACTATCATATGCTTACCGTAACTTG

5.1.8 Antibodies

5.1.8.1 Primary antibodies

Antibody	Antigen	Origin	Dilution	Supplier
sc-1615	Actin	Goat, polyclonal	1:1000	Santa Cruz Biotechnology
sc-430	Ad2-E1A	Rabbit, polyclonal	1:1000	Santa Cruz Biotechnology
Y164 (ab32371)	Bak	Rabbit, polyclonal	1:500	Abcam plc
E63 (ab32503)	Bax	Rabbit, polyclonal	1:1000	Abcam plc
N-19 (sc-492)	Bcl-2	Rabbit, polyclonal	1:100	Santa Cruz Biotechnology
L-19 (sc-1041)	Bcl-XS/L	Rabbit, polyclonal	1:1000	Santa Cruz Biotechnology
AF680	Bid	Goat, polyclonal	1:800	R&D Systems
2819	Bim	Rabbit, polyclonal	1:1000	Cell Signaling Technology
BP7052	eIF2alpha (pSer52)	Rabbit, polyclonal	1:1000	Acris Antibodies GmbH
sc-577	Gal4-DNA-binding domain	Rabbit, polyclonal	1:2000	Santa Cruz Biotechnology
12CA5	HA-tag	Mouse, monoclonal	1:1000	Roche Applied Science
A190-108A	HA-tag	Rabbit, polyclonal	1:2000	Bethyl Laboratories, Inc.
AAP-240	Mcl-1	Rabbit, polyclonal	1:7000	Stressgen
sc-372	NFkappaB (p65)	Rabbit, polyclonal	1:1000	Santa Cruz Biotechnology
IMG-349A	NOXA	Mouse, monoclonal	1:500	Imgenex
4976	PUMA	Rabbit, polyclonal	1:1000	Cell Signaling Technology
53015.11	PARP	Rat, polyclonal	1:1500	R&D Systems (Bettina)
12-6766	PARP	Rabbit, polyclonal	1:600	eBioscience
AB-6 OP43	p53	Mouse, monoclonal	1:500	Calbiochem
632375	rGFP	Mouse, monoclonal	1:4000	BD Living Colors
A190-130A	VSV-G	Goat, polyclonal	1:5000 1:100 (IHC)	Bethyl Laboratories Inc.
2042	XIAP	Rabbit, polyclonal	1:1000	Cell Signaling Technology

5.1.8.2 Secondary antibodies

Antibody	Antigen	Origin, properties	Dilution	Supplier
AQ127P	Mouse	goat, HRP-coupled	1:10.000	Chemicon Intl.
7074	Rabbit	goat, HRP-coupled	1:10.000	Cell Signaling Technology
sc-2056	Goat	donkey, HRP-coupled	1:10.000	Santa Cruz Biotechnology
705-066-147	Goat	donkey, biotin-coupled	1:200 (IHC)	The Jackson Laboratory
	Rat	donkey, HRP-coupled	1:10.000	Dianova

5.1.9 Chemicals

All experiments were performed in S1 or S2 laboratories at Hannover Medical School equipped for molecular biological work. Displayed exclusively are chemical substances that might have influenced the outcome or the form of the experiments.

Substance	Supplier
5-Fluorouracil	Hannover Medical School
5x Bradford-solution	Bio-Rad Laboratories GmbH
Ampicillin	Sigma-Aldrich
ATP (for DNA ligation)	Sigma-Aldrich
Carbenicillin	Serva Electrophoresis GmbH
Chloroquine	Sigma-Aldrich
4',6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Sigma-Aldrich
Doxorubicin	Hannover Medical School
Kanamycin	Sigma-Aldrich
Lipofectamin2000	Invitrogen Corp.
MG132	Calbiochem
Mowiol	Roth
Neomycin	Calbiochem
10x PBS	Invitrogen Corp.
Polyethylenimine (25 kD)	Polysciences Europe GmbH
Puromycin	Sigma-Aldrich
To-Pro3	Invitrogen Corp.
zVAD-fmk	Calbiochem

5.1.10 Molecular weight standards

Name	Application	Supplier	Cat. number
1 kb plus DNA ladder	DNA	Invitrogen Corp.	10787-018
peqGOLD Prestained Protein-Marker III	Protein	Peqlab Biotechnolgie GmbH	27-1110

5.1.11 Enzymes

Antarctic phosphatase	New England Biolabs GmbH
DNA Polymerase I, Klenow fragment	New England Biolabs GmbH
Herculase Hotstart DNA Polymerase	Stratagene Europe
Lysozyme	Sigma-Aldrich Chemie GmbH
Proteinase K	Roche Diagnostics GmbH
Restriction endonucleases	New England Biolabs GmbH
Reverse Transcriptase	Qiagen GmbH
RNase	Boehringer
Streptavidine-HRP	Zymed Laboratories
T4-DNA Ligase	New England Biolabs GmbH
T4-Polynucleotide kinase	New England Biolabs GmbH

5.1.12 Kits

Qiagen Endofree Plasmid Midi/Maxi Kit	Qiagen GmbH
QIAquick Nucleotide Removal Kit	Qiagen GmbH
QIAquick Gel Extraction Kit	Qiagen GmbH
QIAquick PCR Purification Kit	Qiagen GmbH
Qiagen RNeasy Kit	Qiagen GmbH
ECL detection Kit	GE Healthcare Europe GmbH
Adeno-X rapid titer Kit	Clontech
Vivapure AdenoPACK 20	Sartorius AG
AEC Histostain Plus Broad Spectrum Kit	Zymed Laboratories,
In Situ Cell Death Detection Kit, POD	Roche Diagnostics GmbH
Caspase-3 Activity Assay Kit	Clontech

5.1.13 Devices

Fluorometer	Biotek Instruments GmbH
Photometer	Biotek Instruments GmbH
Lumat (Luminometer)	Berthold
HM 500 OM Cryostat	Microm
Zeiss LSM 510 Meta scan head on ZEISS Axiovert 200M equipped with an oil immersion objective lense 63 × /1.4, zoom factor 2.0	Carl-Zeiss MicroImaging
Axiovision software	Carl-Zeiss MicroImaging
Zeiss LSM Image Browser 4.2	Carl-Zeiss MicroImaging
QWinV3 software	Leica
GraphPad Prism 3.02	GraphPad Software Inc.

5.1.14 Media and buffers

For cell culture

Storage medium for liquid nitrogen
80 % DMEM + 10 % FCS, 10 % DMSO, 10 % FCS; sterile filtration

For culture of bacteria

LB medium: 10 g bactotryptone, 5 g yeast extract and 10 g NaCl solved in water adjusted 1 l and autoclaved. The medium is supplemented with antibiotics prior usage. Storage at room temperature.

Agar plates: LB medium containing 1.5 % agar select was autoclaved, chilled to ~ 60°C and supplemented with antibiotics. The final mix was applied to agar dishes and chilled until solidified. Storage at 4°C.

SOC medium: 2 g tryptone, 0.5 g yeast extract, 0.05 g NaCl, 1 ml 1 M $MgCl_2$, 1 ml 1 M $MgSO_4$ and 0.4 g glucose are filled up with water up to 100 ml sterilely filtered. Storage at -20°C.

Buffers

1 M TRIS pH 6.8	1 M TRIS-HCl pH 6.8 0.4 % SDS
1.5 M TRIS pH 8.8	1.5 M TRIS-HCl pH 8.8 0.4 % SDS
1x TAE	40 mM TRIS-HCl pH 8.5 1 mM EDTA 40 mM acetic acid
2x BBS pH 6.95	280 mM NaCl 50 mM BES 1.5 mM Na_2HPO_4 x ($2H_2O$) pH 6.95 (optimization by transfection experiments necessary) sterile filtration
2x storage buffer (Adenovirus)	10 mM TRIS-HCl pH 8.0 100 mM NaCl 0.1 % BSA 50 % Glycerol sterile filtration
5x extraction buffer (luciferase)	125 mM TRIS-HCl pH 7.8 (H_3PO_4) 10 mM EDTA 50 % glycerine 5 % Triton-X-100
5x SDS protein load	310 mM TRIS-HCl pH 6.8 250 mM DTT 20 % glycerine 2 % SDS 0.2 % bromophenol blue
10x DNA loading buffer	20 % Ficoll 400 100 mM EDTA pH 8.0 1 % SDS bromophenol blue
10x SDS buffer	250 mM TRIS HCl pH 8.3 1.92 M glycerine 1 % SDS
ATP stock for luciferase assay	100 mM ATP 200 mM TRIS storage at -20°C
β-Galactosidase reaction buffer	60 mM Na_2HPO_4 39 mM NaH_2PO_4 10 mM KCl 1 mM $MgSO_4$ prior use: 2 mM DTT

	1 mg/ml ONPG
Cesium chloride gradient	density 1.2 g/ml (50 ml) 13.25 g CsCl 0.5 ml 1 M TRIS-HCl pH 7.5 density 1.4 g/ml (50 ml) 26.75 g CsCl 0.5 ml 1 M TRIS-HCl pH 7.5
Dialysis buffer	10 mM TRIS-HCl pH 8.0 1 mM $MgCl_2$ 140 mM NaCl
Luciferine	25 mM D-luciferine sodium salt in water storage at -20°C
Luciferase reaction buffer	25 mM glyceryl glycine 15 mM $MgSO_4$ 50 mM ATP prior use
RIPA-buffer	60 ml 10x PBS 6 ml Igepal 3 g deoxycholic acid-sodium salt 0.6 g SDS final volume adjusted 600 ml Before use: 5 µl protease inhibitor mix (Calbiochem) per 1 ml
STET buffer	50 mM TRIS-HCl pH 8.0 50 mM EDTA 8 % sucrose 0.5 % Triton X-100
TAIL buffer	50 mM TRIS-HCl pH 8.0 100 mM EDTA pH 8.0 5100 mM NaCl 1 % SDS 20 µg/ml proteinase K added before use
TE	10 mM TRIS-HCl pH 8.0 1 mM EDTA pH 8.0
TBS-Tween	20 mM Tris-HCl pH 7.6 150 mM NaCl 0.3 % Tween 20
Western blot transfer buffer	40 mM glycine 50 mM TRIS-HCl pH 8.3 1 mM SDS 1 l methanol final volume adjusted 5 l

5.2 Cell biological methods

5.2.1 Cell culture techniques

Cell culture was performed according to modern guidelines for S1 and S2 safety levels. Regularly, contaminations with mycoplasma species were checked by a PCR protocol. 75 cm^2 T-flasks, DMEM supplemented with FCS and an antibiotic Penicillin/Streptomycin were usually used to grow cells in incubators providing 37°C and 5 % CO_2.
To propagate cells, medium was discarded and the cell layer washed with 1x PBS. Trypsinization (0.5 % Trypsin in 1x PBS) was performed to detach cells. Cells were resuspended in fresh medium and grown to 70 – 90 % confluence before next passage.
To determine viable cell number per ml medium hemocytometer was used. Dead cells were excluded by Trypan blue staining.
Cells were stored in liquid nitrogen resuspended in freezing medium consisting of 80 % growth medium, 10 % DMSO and additional 10 % FCS. To prevent contamination with microorganisms the solution was sterilely filtered.
Culturing cells from nitrogen storage started with a quick thaw. Washing with growth medium eliminated residual DMSO traces. Cells were resuspended in fresh medium that was refreshed after cell attachment to remove cellular debris.

5.2.2 Transfection of cell lines

5.2.2.1 Lipofectamin2000

Cells were propageted in a 60 mm plate to achieve a nearly confluent layer (~ 90 %). Immediately before transfection medium was replaced by 2 ml Optimem (serum-free medium). 8 µg DNA were resolved in 500 µl Optimem as was the transfection reagent (16 µl; Lipofectamine2000). Both solution were combined and mixed gently. About 20 min at RT the mix was applied to the cells and incubated at 37°C for 4 h. Transfected cells were treated according to the manufacturer's protocol.

5.2.2.2 Calciumphosphate

About 0.9x 10^6 cells were seeded to 60 mm plates and grown to about 70 % confluence. For transfection 5 µg of DNA were mixed with 150 µl 250 mM $CaCl_2$ and 150 µl 2x BBS and spun down according to Chen and Okayama. After incubation an RT for 15 – 25 min the mixture was applied to cells with 2 ml freshly provided growth medium by slightly shaking the plate. Fresh medium was provided 12 -15 h after start of the transfection. Cells were monitored and treated due to the experimental setup.

5.2.2.3 Polyethylenimine (PEI)

Cells were seeded in 60 mm plates to achieve about 90 % confluence (approximately 1.2x 10^6 cells). Before transfecting target cells medium without FCS had to be applied. 5 µg DNA and 10 µl of a 10 mg/ml PEI solution were separately resolved in 200 µl serum-free medium. Both solutions were mixed and incubated at RT for additional 25 min to form complexes. 400 µl of the

transfection mix were added to the cells and incubated for 4 h. Then, the transfection medium was discarded and replaced by fresh growth medium. Cells were monitored and harvested due to the experimental setup.

5.2.3 Microscopical methods

5.2.3.1 Fluorescence microscopy

TUNEL and nuclear staining with DAPI were analyzed at the fluorescence microscope Leica DM4000B. Merged images of both signals were created by Leica QWinV3 software (blue – DAPI staining; green – TUNEL staining).

5.2.3.2 Confocal Laser Scanning Microscope (CLSM)

The intracellular distribution of the fusion protein YFP-LC3 that was stably expressed in A549, HepG2 and Huh-7 cell lines was analyzed by confocal laser scanning microscopy at the Laser Microscopy Facility at Hannover Medical School with a Zeiss LSM 510 Meta scan head on ZEISS Axiovert 200M equipped with an oil immersion objective lense 63 × /1.4. Generally a zoom factor of 2.0 was applied to record fluorescence images. Later on these images were processed with software from Carl-Zeiss MicroImaging (Axiovision software, Zeiss LSM Image Browser 4.2).

1 – 2x 10^5 cells were grown on cover glasses in 6 well plates over night. For experiments medium was refreshed (1 ml) and 100 µl of virus or chloroquine solution was added. After incubation of indicated times cover glasses were rinsed with 1x PBS and fixed in 4 % paraformaldehyde for 10 min at RT. After an additional washing step cover glasses were tissue dried and cells were stained with about 60 µl of 1 µM To-Pro3 solution (nuclear staining). Glasses were incubated (10 min, RT) in a humid and dark chamber to prevent fading of the dye. After a washing step (1x PBS) cover glasses were dried and put on a glass slides prepared with mounting medium (Mowiol). Incubation at RT (30 min) and 4°C (over night) provided samples appropriate for CLSM analysis.

Before recording microscopic parameters for adequate measurement range were set by positive and negative control samples. This preparation was performed before each experiment but the established configuration was never changed within a single experimental setup. Pictures were recorded as single slices on a certain cellular level and as scanning picture series (up to 15 slices per cell). Resulting files were processed with the above mentioned software to present YFP, To-Pro3 signals and merged images of both signals.

5.2.4 Tissue staining

To test the intratumoral effect of applied therapies, tumors were analyzed by tissue staining. Preparation of tumor slices, Haematoxylin/Eosin (HE) and VSV-G staining were performed by Meriame Nasiri and Gisela Weier at Hannover Medical School.
For this reason A549 tumors were grown on nude mice, treated like indicated and finally explanted. To perform HE staining and staining against VSV-glycoprotein protein tumors were incubated in 4 % paraformaldehyde in PBS over night (4°C)

and embedded in paraffin to prepare 5 µm sections from tumor tissue. Resulting sections were subjected to specific staining that is described in the following two chapters.

5.2.4.1 Haematoxylin/Eosin (HE)

A549 tumors in nude mice were treated for several weeks and subsequently explanted. Paraffin tumor sections were dried, deparaffinized and, rehydrated. Haematoxylin staining was performed for 30 – 90 s. After washing with water samples were Eosin stained and dehydrated. Samples were covered with glasses using Entelan. Samples were analyzed with a bright light microscope.

5.2.4.2 Immune histochemistry

Distribution of VSV within A549 tumors was analyzed by detecting the glycoprotein of VSV (VSV-G) that is located in the cell membrane of infected cells. Tumor sections were stained with goat-anti VSV-G antibody (1:100), biotinylated donkey-anti-goat antibody (1:200) and streptavidine-HRP. Using the AEC Histostain Plus Broad Spectrum Kit (Zymed Laboratories, San Francisco, CA) infected cells were visualized. Samples were monitored with a bright field microscope.

5.2.4.3 TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining

A549 tumors in nude mice were treated for up to 5 days and subsequently explanted. Paraffin tumor sections were dried and deparaffinized. TUNEL assay was performed according to the manufacturer's guidelines (In Situ Cell Death Detection Kit, POD Roche Diagnostics GmbH). Cellular peroxidases were blocked by incubating slides in 3 % H_2O_2 (in methanol) for 10 min. Washing twice in water preceded an incubation in 0.01 M citrate buffer (pH 6.0) for 5 min at 750 W and additional 15 min at 150 W in a microwave. After chilling the slides for 10 min at RT and washing twice in 1x PBS the tissue was permeabilized with proteinase K (20 µg/ml in 1x PBS) for 30 min. Proteinase K was removed by washing twice in 1x PBS. Then, samples were fixed in 4 % paraformaldehyde for 1 h and washed again. Before the final TUNEL reaction samples were incubated in 0.1 % sodium citrate and 0.1 % Triton-X-100 for 30 min and washed twice in 1x PBS. Freshly prepared TUNEL reaction mix to stain DNA double strand breaks was applied to the samples for 1.5 h and removed by washing with 1x PBS. Finally, nuclei were counterstained with DAPI and covered with glasses in mounting medium (Mowiol). Images were recorded on a fluorescence microscope.

5.3 Protein biochemical methods

5.3.1 Preparation of protein extracts from cell culture

Cell layers were controlled by bright field microscopy. After washing the cell layer twice with 1x PBS cells were harvested in RIPA buffer containing a pan-protease inhibitor cocktail and incubated on ice for 30 min. Cell debris was pelleted in a

table top centrifuge (10,000 g, 10 min, 4°C) and supernatants were transferred to fresh tubes.

5.3.2 Determination of protein concentration

Dilutions of protein containing 0 to 6 mg/ml BSA (NEB) were applied as standards (in the same buffer as samples are harvested).
The purchased Bradford solution was diluted 1:4 in water. Fresh tubes were provided with 198 µl water and 2 µl of sample/standard. 800 µl of diluted Bradford solution was added to each protein dilution and mixtures were shaken slightly. Per sample 200 µl of the final mixture were applied to a flat bottomed 96 well plate. Absorbance was measured from duplicates at 595 nm and concentrations were calculated by Gen5 software (Biotek Instruments GmbH).

5.3.3 SDS-PAGE and western blot analysis

Separation of protein solutions was performed by denaturing SDS-polyacrylamid gel electrophoresis. Composition of the resolving gels (10 or 12.5 %) and the stacking gel is shown in the tables below.

Resolving gels					
%	Acrylamid/ Bisacrylamid	1.5 M TRIS pH 8.8 + 0.4 % SDS	H_2O	10 % APS	TEMED
10.0	8 ml	6 ml	9.75 ml	240 µl	10 µl
12.5	10 ml	6 ml	7.75 ml	240 µl	10 µl

Stacking gel					
	Acrylamid/ Bisacrylamid	1 M TRIS pH 6.8 + 0.4 % SDS	H_2O	10 % APS	TEMED
	1.65 ml	2.5 ml	5.85 ml	50 µl	10 µl

10 – 100 µg protein were separated using a Biometra apparatus. Subsequently, proteins were transferred to a HyBond N-membrane (Millipore, Eschborn, Germany) by electroblotting. Membranes were blocked in TBS-Tween containing 5 % milk and subjected to immunostaining. Antibodies were diluted in TBS-Tween containing 5 % milk and applied to the blocked membrane over night (4°C) or for 1 h (RT). Membranes were washed in TBS-Tween six times for 10 min and incubated with the secondary antibody solution (in TBS-Tween) for 1 h. After washing in TBS-Tween (6x 10 min) membrane were developed applying the enhanced chemiluminescence detection system (ECL) according to the manufacturer's protocol (Amersham, GE Healthcare Europe GmbH).

5.3.4 Luciferase assays

5.3.4.1 Firefly

Transfected cells were incubated in growth medium for a certain time, then washed twice with 1x PBS. 5x Extraction buffer was freshly prepared and 350 µl were applied to the rinsed cell layer. After 10 min incubation at RT lysed cells were transferred to a fresh tube and centrifuged (16,000 g, 5 min, 4°C) to separate cell debris. Supernatant was applied to a fresh tube and stored at -20°C.

Measurement of luciferase activity was performed usinga Lumat apparatus. For this purpose 300 µl of freshly prepared reaction buffer were mixed with 50 µl harvested cell lysat in measurement tubes and luciferine solution was applied. Per sample two measurements were carried out.

5.3.4.2 Dual luciferase reporter system

Promega's psiCHECK-2-system allows analysis of shRNA efficiencies and simultaneous normalization of transfection efficiencies by expression of two different luciferases. The 3'-UTR of *Renilla* luciferase contains a MCS for integration of shRNA target gene sequences of choice. Firefly luciferase is expressed constitutively in an unaffected manner to allow for normalization of *Renilla* luciferase expression.

Target gene sequences were generated by PCR from cDNA of A549 and inserted in psiCHECK-2 by *Xho* I and *Not* I cleavage. Plasmids coding for specific shRNA sequences controlled by a U6-promoter and the psiCHECK-2 vectors were co-transfected in HepG2 cells. 48 h after transfection cells were lysed in freshly prepared extraction buffer and measurement of activities of both luciferases were performed according to the manufacturer's protocols. Comparison of the normalized *Renilla* luciferase activities of specific shRNAs against a non-target shRNA revealed the down regulating potential of certain shRNA sequences. Based on these results shRNA sequences were selected for the generation of cell lines constitutively down regulating certain genes.

5.3.5 β-Galactosidase assay

To normalize transfection efficiencies plasmids coding for lacZ gene were co-transfected. Activity of the β-galactosidase enzyme was analyzed by the ONPG assay. 20 mg ONPG (the substrate of β-galactosidase) was mixed with 20 ml β-Galactosidase reaction buffer and 40 µl 1 M DTT. 10 µl cell lysat were applied to a 96 well plate and the final ONPG solution (150 µl) was added. The reaction was carried out at RT (high β-galactosidase activity) or at 37°C (low β-galactosidase activity). After stopping the conversion of ONPG to a yellow product by applying 75 µl of 1 M Na_2CO_3 the absorption was measured at 405 nm in Biotek's photometer and normalization was usually performed by Excel calculation.

5.3.6 Caspase-3-activation assay

Cell death by induction of apoptosis can be monitored by measuring the activity of the enzyme caspase-3 playing a decisive role in this pathway. Clontech's Caspase-3 Activity Assay was applied to all experiments described in this work.
For this reason, cells were seeded in 60 mm plates and treated according to the experimental plan. Cells were harvested by cell scraper in medium, centrifuged (1,000 g, 2 min, 4°C) and washed with 1x PBS. Pelleted cells were lysed in about 200 µl of the provided cell lysis buffer and incubated for 10 min on ice. Samples were shock frozen in liquid nitrogen and stored at -80 C.

Cell debris was removed by centrifugation (20,000 g, 2 min, 4°C). Protein concentrations of the remaining supernatants were determined by Bradford assay. According to the manufacturer's protocol caspase-3 activities of 7 µl of sample

were measured 20 and 80 min after applying an enzyme substrate (Ac-DVED-AFC-substrate, 5 mM in DMSO) or an inhibitor (Ac-DEVD-CHO, 1.5 mM in DMSO). Fluorescence (502 nm) was measured at the fluorometer (Bioteck Instruments GmbH) and fluorescence units per µg protein and hour were calculated.

5.4 Molecular biological methods

5.4.1 DNA amplification and purification

In this work different techniques were applied to purify DNA out according to requirements for amount and/or quality of the purified DNA and will be described in this chapter. The *E. coli* safety strains JM109 and XL1-blue were used to amplify plasmids. While XL1-blue cells were electroporated exclusively JM109 bacteria were accessible as chemically competent preparations as well.

5.4.1.1 Mini format

DNA from agar plate colonies or pAdHM4 plasmid was usually extracted from bacteria by a specific preparation protocol (preventing use of resins to bind DNA). Transformed bacteria were grown 7 – 14 h in LB medium and centrifuged (3,000 g, 2 min, 4 °C). Medium was discarded except residual 100 µl to resuspend cells by vigorous shaking. The suspension was mixed with 300 µl STET buffer + 300 µg/ml lysozyme. To weaken bacterial cell walls by lysozymal digestion cells were incubated 2 min at RT prior to boiling at 95°C for 90 s. The supernatant of the following centrifugation (20,000 g, 20 min, 4°C) was mixed with 400 µl 2-propanol and centrifuged (20,000 g, 15 min, 4°C) again to precipitate DNA. The resulting pellet was washed with 70 % ethanol and resuspended in 15 – 30 µl TE buffer.

5.4.1.2 Midi/Maxi format

For the preparation of highly purified DNA in midi or maxi format appropriate kits (Qiagen Endofree Plasmid Midi/Maxi Kit) from Qiagen GmbH were applied. The purification method is based on a modified alkaline lysis procedure and functions by binding of plasmid DNA to an anion exchange resin.

5.4.1.3 Phenol-Chlorofrom extraction

In certain cases large DNA plasmids (e. g. pAdHM4) were purified by phenol-chloroform extraction as this preparation method minimizes the problem of DNA shearing in contrast to column based methods, e. g. purification kits described above.
The DNA containing solution was adjusted to 100 µl by TE buffer and mixed with 100 µl commercial phenol-chloroform solution from Roth (Roti-Phenol/Chloroform). After vigorous shaking aqueous and phenolic phases were separated by centrifugation (20,000 g, 2 min, 4°C). The upper hydrophilic phase was transferred to a fresh tube and supplemented with 25 µl 10 M ammonium acetate and 400 µl 100 % ethanol. DNA was precipitated by incubation at -20°C

(30 min), adding glycogen and a final centrifugation (20,000 g, 15 min, 4°C). After washing the pellet with 70 % ethanol DNA was resuspended in an appropriated volume of TE buffer or 10 mM TRIS-HCl pH 8.0.

5.4.2 DNA sequencing

For sequencing DNA fragments commercial services of GATC Biotech AG, Konstanz and Eurofins MWG GmbH, Ebersberg were employed.

5.4.3 Transformation

5.4.3.1 Chemical Transformation

To amplify DNA plasmids 80 µl chemically competent JM109 cells were incubated with up to 15 µl DNA solution (e. g. ligation mix) for 30 min on ice. Bacterial solution was heat-shocked at 42°C for 90 s and subsequently cooled on ice for 2 min. After resuspending cells in 100 µl SOC medium and culturing at 37°C for 30 min bacteria were spread on an agar plate and cultured over night. Plasmids from arising colonies were monitored by restriction enzyme analysis.

5.4.3.2 Electroporation

To transform bacteria with large DNA plasmids a method termed electroporation was used. 40 µl of electro-competent JM109 or XL1-blue cells were mixed with up to 1.5 µl of DNA solution and applied to a chilled electroporation cuvette between to electrodes. Electroporation was performed at voltages of 1.7 – 1.8 kV in a specialized apparatus called electroporator (Bio-Rad *E. coli* pulser). Immediately after the voltage pulse up to 1 ml of SOC medium was added to the bacteria and transformed cells were incubated at 37°C for 30 min. After spreading on an agar plate bacteria were cultured over night and resulting colonies were analyzed for plasmid specimen.

5.4.4 DNA recombination techniques

To recombine different DNA fragments DNA was cleaved using restriction enzymes purchased from New England Biolabs, Inc. (NEB). If necessary, sticky DNA ends resulting from enzyme cleavage were blunted using Klenow fragment (NEB). Desired fragments were separated by agarose gel electrophoresis and purified using Qiagen's Gel Extraction Kit. Appropriate DNA fragments were mixed and *in vitro* ligated by T4 DNA ligase (NEB). To prevent religation of DNA 5'-ends cleaved vectors were dephosphorylated by Antarctic phosphatase (NEB) prior ligation. Subsequent transformation of successfully ligated plasmids resulted in transformed bacteria that were applied for DNA amplification.

Constructed, purified or provided plasmids were analyzed by restriction enzyme cleavage. Resulting fragments were separated on agarose gels and compared to 1 kb+ DNA standard to gain knowledge of the quality of analyzed plasmid sample. If the cleavage pattern was not applicable for analysis target DNA regions were sequenced.

5.4.5 PCR

In this work the polymerase chain reaction was used to introduce cleavage sites for restriction enzymes, to extend hybridized oligonucleotides and to detect certain I-*Sce* I-mediated DNA religation products. All three procedures are described below.

5.4.5.1 Insertion of RE sites

To introduce certain recognition sites for restriction enzymes oligonucleotides were designed comprising corresponding sequences. To estimate annealing temperatures (T_M) the following formula was applied for primers > 20 bases:

T_M = 58.9°C + 0.41x (GC) – 600x (length)

GC % of G or C nucleotides in the binding sequence
length number of nucleotides that bind to the template

When RE sites were introduced usually two annealing temperatures had to be calculated (for partial and total annealing of the primers) and the PCR program consisted of two-parted.
For the generation of UTR-modified genes proof-reading HotStart Herculase was used to minimize the rate of mutation. In some cases DMSO (1 – 5 %) and melting substances were necessary to generate the desired products.

5.4.5.2 Generation of miR30 DNA fragments

MiR30 short hairpin sequences (116 bp) were generated within a single polymerase chain reaction using Herculase Hotstart DNA Polymerase. Four primers were used, two constant and two specific for the desired shRNA sequence (figure 4). Depicted below are the composition of a 100 µl PCR mix and a schematic picture of the PCR principle:

	concentration	**volume (µl)**
Herculase reaction buffer	10x	10
DMSO	100 %	2
dNTP	10 mM	4
shPrimer forward	100 µM	1
shPrimer reverse	100 µM	1
mir30-Xba	100 µM	1
mi30-Xho	100 µM	1
Herculase enzyme	5 u/µl	1
water		79

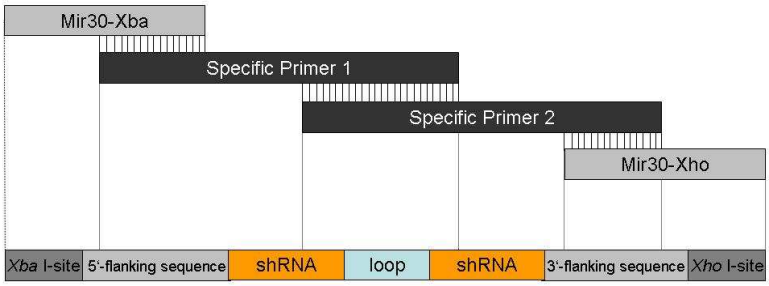

Figure 4. Generation of functional shRNA molecules by PCR. Two primers providing flanking sequences and cleavage sites for cloning and two primers specific for every target RNA region were applied in a single PCR to generate a single shRNA molecule.

The reaction program was three-parted:

Temperature (°C)	Time (s)	Repeats
95	600	
95	30	5x
55	30	
72	30	
95	30	5x
56	30	
72	30	
95	30	30x
60	30	
72	30	
72	600	

5.4.5.3 PCR-assisted detection of I-*Sce* I-cleavage products of the E1 region

The activity of Adenovirus-encoded I-*Sce* I protein could be detected by a simple PCR method that exploits the efficient religation of a certain cleavage product by host cell DNA repair mechanisms. Briefly, a fragment of the Adenoviral genome spanning about 3,000 nucleotides is framed by two I-*Sce* I recognition sites. When cleaved the linear DNA could be religated at a certain position. This two-step-event could be monitored by a PCR method.

Target cells were infected with recombinant Adenoviruses and incubated for an indicated time. Cell layers were washed and lysed by TAIL buffer and DNA was prepared by 2-propanol precipitation and RNA digestion. 300 ng of the purified DNA were added to the PCR mixture with a total volume of 50 µl:

	concentration	volume (µl)
Template (purified DNA)	50 ng/µl	6
Primer E1A_cutcontrol_rev	10 µM	2.5
Primer E1B_cutcontrol_fw	10 µM	2.5
DMSO	100 %	2
Taq-Master (Qiagen)	2x	25
Water		12

The PCR program is depicted below:

Temperature (°C)	Time (s)	Repeats
95	600	
95	30	35x
55	30	
72	45	
72	600	

PCR products were analyzed in agarose gel and by DNA sequencing.

5.4.6 DNA extraction from cell culture

About 1x 10^6 PBS-washed cells were lysed in TAIL buffer and incubated at 56°C over night. Cell debris was removed by centrifugation (10,000 g, 10 min, 4°C). Supernatant was mixed with the same volume 2-propanol and centrifuged (20,000 g, 15 min, 4°C) to precipitate DNA. After washing with 70 % ethanol DNA was resuspended in 25 µl TE buffer and RNA was digested for 1 h at 37 °C. The concentration of DNA was determined by photometric measurement.

5.5 Virological techniques

In the context of this work recombinant Adenoviruses and wild type Vesicular Stomatitis Virus were analyzed regarding their host cell interaction. For this purpose, stably expressing cell lines were created by the help of retro- or lentiviral gene transfer. Methods related to the virus-assisted generation of these cell lines are described in this chapter likewise.

5.5.1 Adenovirus

5.5.1.1 Cloning

Construction of recombinant replicative Adenovirus vectors was based on an *in vitro* ligation method described by Mizuguchi and Kay 1998. To insert foreign DNA cassettes into an adenoviral DNA backbone two unique restriction sites (I-*Ceu* I and PI-*Sce* I) were introduced in a vector – termed pAdHM4 (30.3 kb) – containing a complete E1, E3-deleted human Adenovirus type 5 genome. A pHM3-based shuttle plasmid providing the Adenoviral E1 region was used as construction platform for the development of DNA cassettes coding for regulative elements.
The final recombinant E1-region was inserted in pAdHM4 according to Mizuguchi and Kay. Briefly, 2 µg shuttle plasmid and 3 µg of a DNA minipreparation of pAdHM4 were digested with I-*Ceu* I and PI-*Sce* I. Insert DNA was gel-purified and concentrated by QIAquick Gel Extraction Kit. To prevent shearing of the large pAdHM4 DNA it was exclusively purified by phenol-chloroform extraction. *In vitro* ligation was performed over night at 16°C and 1.5 µl of the ligation mix was used to transform XL1-blue cells by electroporation. After selection of positive clones by restriction analysis DNA was used to transform electro-competent JM109 cells yielding a higher amount of DNA than XL1-blue.

5.5.1.2 Production and amplification

Final pAdHM4 based plasmids coding for recombinant Adenoviral genomes were *Pac* I cleaved to generate linear DNA that could be utilized as transcription and replication template. About 9x 10^5 HEK 293 cells were seeded in a 60 mm plate and transfected with the linearized vector. 5 days after transfection cells were scraped in medium and viral particles were released by three freeze-thaw-cycles (in liquid nitrogen/37°C). Cell debris was removed by centrifugation and the remaining supernatant was added to a fresh layer of HEK 293 cells. About 50 h later cells were harvested again as described above. Successful virus production could be monitored by cytopathic effect and rapid turning of the medium's color to yellow. Scaling up the number of infected cells increased the produced virus amount.
Two different purification methods were applicable for Adenoviral particles: ultracentrifugation and a column-based procedure. The first required about 40 confluent 75 cm^2-flasks of infected HEK 293 cells, the latter 2 flasks.

Adenovirus purification by ultracentrifugation

About 40 75 cm^2-flasks of infected HEK 293 cells were infected with an Adenovirus vector and harvested about 60 h later when a clear CPE occurred. Cells were separated by centrifugation (500 g, 5 min, 4°C) and resuspended in dialysis buffer. To free viral particles from cell nuclei cells were freeze-thawed three times. The lysat was cleared by centrifugation (2,000 g, 5 min, 4°C) and the pelleted cells subjected to an additional freeze-thaw cycle. After centrifugation both supernatants were combined and layered over a discontinuous CsCl-gradient containing 3.5 ml CsCl density 1.2 g/ml and 3.5 ml of CsCl density 1.4 g/ml in a polypropylene tube (Beckman) for SW28i rotor. Particles were sedimented by ultracentrifugation for 4 h at 28,000 rpm (10°C). The visible virus band was harvested by a fine syringe (0.8 mm, Braun) puncturing the centrifugation tube carefully. To increase purity the obtained virus solution was adjusted 7 ml with dialysis buffer and centrifuged again in a CsCl gradient. Harvested virus was dialysed to remove excessive CsCl and stored at -80°C. Viral titers were determined for total and infectious particles. As this purification methods yield high amounts of purified virus it is required for execution of animal experiments.

Adenovirus purification by Vivapure AdenoPACK 20

According to the manufacturer's protocol two flasks of infected HEK 293 cells were detached from the flask's bottom and separated from the medium by centrifugation. Adenoviral particles were released from cells by freeze-thawing and centrifuged together with the retained supernatant (1,800 g, 5 min, 4°C). Free DNA was degraded by provided benzonase digestion (1h, 37°C) and major particles removed by filtration (0.45 µm). The resulting volume was mixed with 1/9 volume of 10x loading buffer while constantly shaking to minimize osmotic shock. The solution (~ 20 ml) was applied to an equilibrated AdenoPACK 20 Maxi Column and centrifuged (500 g, 5 min, 4°C) for membrane adsorption (Delmdahl, Nature Methods 3, (2006)). After washing twice with 1x washing buffer (18 ml) bound viral particles were eluted by 750 µl of the provided elution

buffer. The run-through solution was loaded again and incubated at RT for 10 min before centrifugation. Eluted particles were subsequently concentrated using the Vivaspin 20 centrifugal concentrator. Volume decreased from about 1,500 µl to 300 – 500 µl and was determined approximately by pipette. For long time storage at -20°C the virus solution was mixed with the same volume of 2x storage buffer and stored under S2 conditions. Viral titers were determined for total and infectious particles.

5.5.1.3 Determination of Adenovirus titer

The infectious titer of the produced Adenoviruses was determined using the antibody-based Adeno-X Rapid Titer Kit (Clontech). According to the manufacturer's protocol HEK 293 cells were used and infected with diluted virus solutions. After 48 h infected cells exposing Adenoviral hexon protein were immuno-stained by a specific antibody against hexon protein and a secondary antibody coupled to HRP. Stained cells were monitored in a bright field microscope, the number of primary infected cells per field of vision counted and the concentration of infectious viral particles (pfu/ml) calculated by the following formula:

$Titer_{Inf}$ = (stained cells x field factor) / (V in ml x dilution factor)

Field factor is provided by Clontech and depends on the object lens applied (79 for 10x, 331 for 20x).
In addition, the total number of viral particles (infectious and non-infectious) was determined by photometric measurement. 25 µl virus stock solution were solved in 475 µl 0.1 % SDS and incubated at RT for 15 min to free DNA from particles. Optical density (OD) was determined and the total amount of Adenovirus particles calculated:

$Titer_{particles} = OD \times dilution \times 10^{12}$

The ratio of total particles / infectious particles was used as a quality feature of the preparation. Usually at about 50 – 80 the ratio means only 1-2 % of purified viral particles form plaques in target cell layers.

5.5.1.4 Determination of oncolytic potency (oncolysis assay)

Target cells were seeded in 24 well plates ($8x\ 10^4$ cells or $1.6x\ 10^5$ when doxorubicin was added) and grown over night. Certain virus solutions (100 µl) were applied to the cells for infection (8 h after chemotherapeutic treatment) at multiplicity of infections ranging from 5 to 0.0005. Cells were incubated until cytopathic effect was clearly visible by microscopic analysis (depending on the cell line 7 – 11 days). Viable cells were stained by crystal violet staining.
Cells were washed with 1x PBS and fixed in 10 % formalin in 1x PBS for 10 min at RT. After washing with water cells were exposed to 0.1 % crystal violet (in 10 % ethanol) and incubated for 30 min on a shaker. After washing and drying wells could be analyzed for cell lysis. Stained wells represent viable cells (without oncolysis).

5.5.2 Vesicular Stomatitis Virus (VSV)

5.5.2.1 Preparation and storage

Infectious viral particles of VSV were applied to confluent layers of the BHK-21 derivative cell line BSR T7/5. Cells were incubated for 24 h, then, supernatant was collected and purified from cells and cell debris by centrifugation. Aliquots were prepared (~300 µl) and stored at -80°C.

5.5.2.2 Determination of VSV titer

The determination of VSV titers was usually conducted by $TCID_{50}$ assay. To verify the obtained data plaque assay was run a few times.

5.5.2.3 $TCID_{50}$

BSR-T7/5 cells were seeded in 96 well plates at a density of $1x\ 10^4$ cells per well (100 µl) and grown over night. Single dilutions (10^{-5} – 10^{-12}; 90 µl) of prepared infectious supernatants were applied to one row (12 wells) and incubated for 2 – 4 days. Wells displaying a cytopathic effect were counted and processed according to Kärber's statistical method. The results represent three independent measurements and are expressed as plaque forming untis per ml (pfu/ml).

5.5.2.4 Plaque assay

To determine VSV titer by plaque assay BSR T7 cells were seeded at $1.2x\ 10^6$ cells per 60 mm plate and incubated over night. After infection with dilutions of infectious supernatant cells were incubated 2 h at 37°C and then washed twice with 1x PBS to remove residual VSV particles. 3 ml of 1 % low melting agarose in growth medium were added to the plate and incubated at 4°C for 1 h to solidify the agarose layer. After returning to a 37°C incubator this layer maintains its gel-like constitution and prevents viral spreading over larger distances. Within 2-4 days visible plaques indicate cytopathic effect by VSV. These plaques were counted. The amount of plaques was multiplied with the dilution step and divided by the initial VSV containing volume of medium (in ml) to calculate the viral titer in pfu/ml.

5.5.3 Retrovirus

5.5.3.1 Cloning

In this work constructed retroviral vectors were exclusively based on Clontech's pQCXI plasmids with resistance genes for neomycin (pQCXIN) or puromycin (pQCXIP). Resulting viral particles are non-replicative and allow a rapid generation of stably expressing cell lines.

The mentioned plasmids control expression of a desired gene by the CMV immediate early promoter. Genes were inserted by *Eco*RI and *Bam*HI enzymes into the MCS succeeding the promoter. The expression of the selection marker was coupled by IRES to the expression of the genes of interest. Final DNA constructs were prepared as LPS free plasmids for transfection.

5.5.3.2 Production and application

For production of non-replicative retroviral particles a 293 based amphotropic packaging cell line (phoenix cells) was utilized that provides certain retroviral genes (gag-pol, env) in *trans*. Resulting particles are infectious and integrate in the host cell's genome but lack replicative capacity.

Phoenix cells were seeded in two 60 mm plates (9x 10^5) and grown over night. The retroviral plasmid containing the gene of interest together with a plasmid coding for the glycoprotein of VSV were co-transfected using PEI. VSV-G allows transduction of a broad range of target cells. 1 h before transfection chloroquine (final concentration: 25 µM) was applied to the cells to increase transfection efficiency. After transfection fresh medium was added that was supplemented with 2 % FCS only. 24 h later medium of the first plate was filtered (0.45 µm) and added to the target cells (about 25 % confluence) together with polybrene (final concentration: 8 µg/ml). After 4 h the process was repeated with the second plate of packaging cells. When viral transduction rate was insufficient larger volumes of virus solution were prepared and concentrated by ultracentrifugation. After the last transduction step medium was replaced by growth medium supplemented with the selecting chemical (neomycin or puroycin).
After 14 days of selection cells were used for experiments. Expression of the inserted gene was controlled by western blot analysis.

5.5.4 Lentivirus

In contrast to retroviral vectors lentiviruses are usually used to transduce non-replicating cells. In this work a lentiviral vector based on pLVTHM (Trono Didier, EPFL, Lausanne, Switzerland) – called NW4739 – was used to stably express shRNAs in human cells. NW4739 lacks H1 promoter and Tet operon between *Bam*H I and *Cla* I.

5.5.4.1 Cloning

The GFP gene of NW4739 was replaced by the neomycin resistance gene from pQCXIN (pS5757) to allow for permanent selection of transgenic cells. In this vector the U6-promoter-shRNA-cassettes were inserted by *Bam*HI and *Xba* I restriction enzymes. The resulting vectors were prepared as LPS free plasmids for transfection.

5.5.4.2 Production and application

To produce infectious lentiviral particles 293T cells were co-transfected with the lentiviral construct and three helper plasmids (pMDL g/p RRE, pMD-G, pRSV-Rev) in a 10 cm dish. 24 and 48 h after transfection supernatants were collected, combined, filtered (0.45 µm) and centrifuged (SW32, 15,000 rpm, 90 min, 4°C) to increase concentration of viral particles. Huh-7 cells (60 mm plate, ~20 % confluence) were infected with the remaining lentiviral supernatant over night. Then, fresh medium containing neomycin was added to the target cells.
Two weeks after the start of selection cells were used for experiments. The effect of the inserted shRNAs was checked by western blot analysis.

5.6 Animal experiments

5.6.1 Application number

Experiments regarding VSV were performed according to the German legal requirements (TierSchG). Number of application: 08/1569.

5.6.2 Tumor inoculation

6 – 8 weeks old nude mice from the Animal Research Institute of the MHH were anesthetized by diethyl ether and each mouse received $1x10^7$ A549 cells in a total volume of 150 µl (adjusted with growth medium) subcutaneously in the right flank applying a syringe. Tumors were grown to an approximate size of 250 mm^3 before experiment was started.

5.6.3 Application of virus and chemotherapy

Xeno-grafted mice were treated with 100 µl Doxorubicin (20 µg), $1x\ 10^7$ infectious particles wt-VSV (in 150 µl) or both treatments twice a week over an indicated period. Application of Doxorubicin occurred intravenously. 6 h later mice were infected intratumorally under diethyl ether anesthesia.

5.6.4 Determination of tumor size

Tumor size was measured every 5 days using a digital caliper and tumor volume was calculated by using the equation V (tumor) = (length x width2) / 2.

6. Results

6.1 Development of conditionally replicating Adenoviruses harboring a self-destruction switch

6.1.1 In vitro analysis of I-*Sce* I-cleavage capacity

The major aim of this project was to design and construct conditionally replicating Adenoviruses that are degraded in p53-positive cells by the yeast endonuclease I-*Sce* I. First, to basically understand I-*Sce* I-mediated cleavage and its properties to be applied in an adenoviral setting the enzyme and its activity were analyzed on plasmid basis under cell culture conditions. I-*Sce* I was derived from an expression vector containing a full length CMV promoter kindly provided by Toni Cathomen (Berlin). Since planned adenoviral target constructs for I-*Sce* I-mediated cleavage are DNA molecules situated in the cell nucleus, the enzyme should be efficiently translocated to this compartment since it is provide with an N-terminally nuclear localization sequences (NLS). Intracellular localization was determined by separation of nuclear and cytoplasmic compartments and subsequent western blot analysis. As shown in figure 5A, the majority of the endonuclease could be detected in nuclear extracts. Importantly, expression of I-*Sce* I did not impair cell morphology or growth indicating a non-genotoxic character of I-*Sce* I expression for human cells what is in concordance with literature data [82].

In preliminary experiments, reporter plasmids were designed encoding only a single I-*Sce* I recognition sequence. Unfortunately, co-transfection of reporter and I-*Sce* I-encoding plasmids failed to result in a detectable cleavage activity. I-*Sce* I-induced linearization was probably reversed by rapid and potent cellular DNA repair activities [152]. In order to unravel I-*Sce* I-cleavage activity, the experimental setup had to be changed to prevent the rapidly occurring repair of I-*Sce* I-mediated DNA cleavage. Thus, the I-*Sce* I-target plasmids were modified, then encoding two I-*Sce* I-recognition sequences.

Therefore, three constructs were designed to detect I-*Sce* I cleavage activity (figure 5B). In a control vector, a CMV promoter was separated from a luciferase gene by a single I-*Sce* I recognition sequence. In two other plasmids, the CMV promoter controlled the expression of an EGFP gene that was enframed by two I-*Sce* I-cleavage sites in directed (termed Sce_dir) or inverted (termed Sce_inv) direction.

Downstream of these sequences an intact luciferase gene was located. As cellular DNA repair mechanisms obviously efficiently reverse I-*Sce* I-mediated DNA-double strand breaks, it was hypothesized that cleavage and repair of a reporter vector in a single-molecule-reaction resulted in increased luciferase activity.

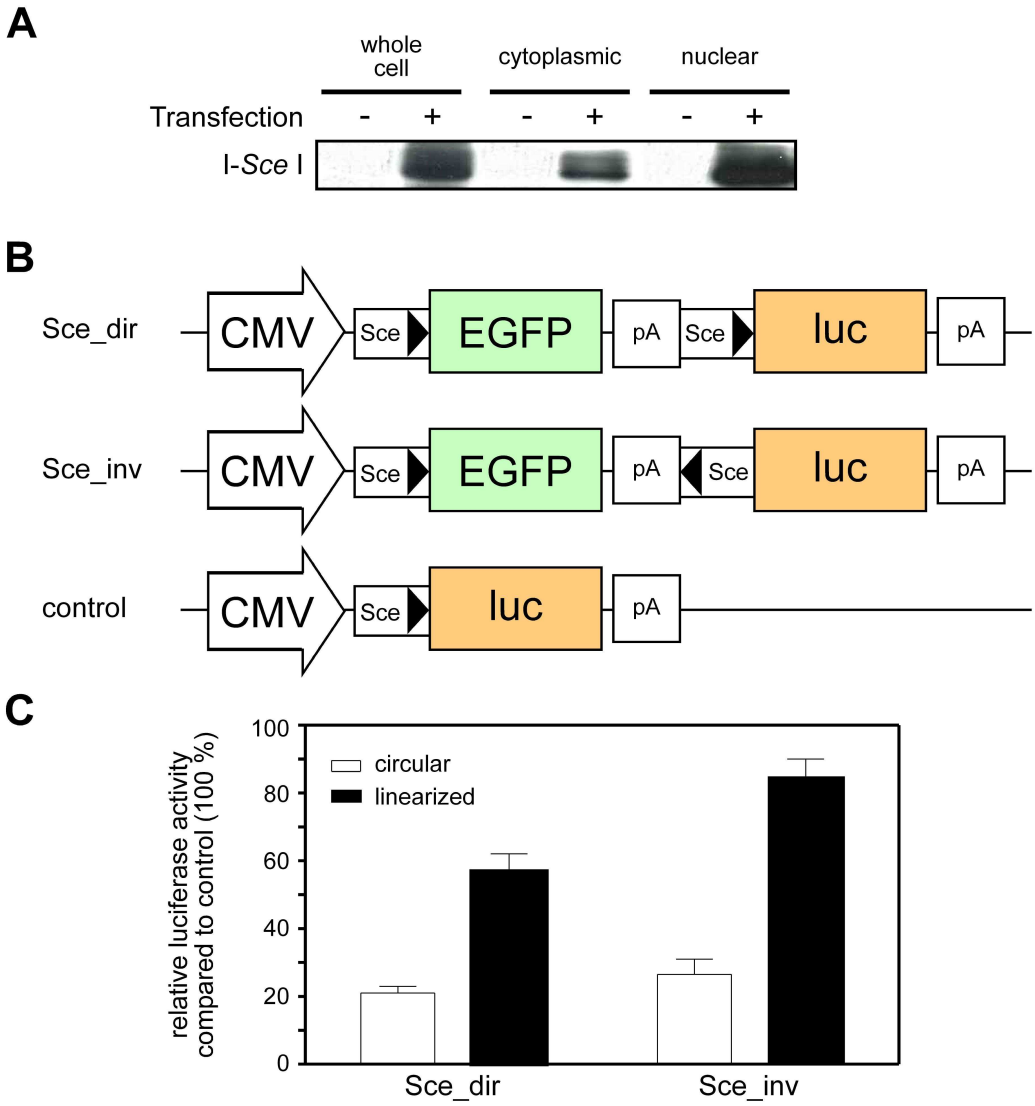

Figure 5. *In vitro*-analysis revealed nuclear localization and cleavage activity of I-*Sce* I. (A) Cells were transfected with an plasmid encoding I-*Sce* I (+) or mock transfected (-). Nuclear and cytoplasmic compartments were separated and analyzed for I-*Sce* I presence by western blot analysis. **(B)** Schemes of I-*Sce* I-sensitive reporter vectors that were generated to act as targets for I-*Sce* I-cleavage. **(C)** HepG2 cells were co-transfected with an I-*Sce* I expression vector and an indicated reporter vector in circular or linearied form. LacZ-normalized luciferase values were compared against the control (100 %) and are depicted in relative values (mock-transfected cells resulted in relative luciferase activity of 0.2%) (data presented are means ± standard errors of the means (SEM).

For this purpose, HepG2 cells were co-transfected with an I-*Sce* I-encoding plasmid, one of the three I-*Sce* I-sensitive reporter vectors and an LacZ-encoding plasmid for normalization. With regard to the planned application (the adenoviral genome is linear), all reporter plasmids were tested in both circular and linearized form. Cells were harvested after 48 hours and luciferase activity was determined subsequently. In figure 5C, I-*Sce* I-expression is supposed to lead to excision of the EGFP gene in the reporter vectors containing two I-*Sce* I-cleavage sites. Religation of the promoter to the luciferase gene would allow expression of functional luciferase protein. In contrast, cleavage of the control vector results in rejoining of the promoter to the luciferase gene. Thus, measured activity from the control vector was set 100 %. Luciferase activities from the EGFP-containing vectors Sce_inv and Sce_dir were compared to the control plasmid. Circular Sce_inv and Sce_dir plasmids generated a markedly increased luciferase activity of 20 – 30 % of the control. Importantly, cells transfected with pBluescript instead of I-*Sce* I-encoding plasmid (mock-transfection) did only exhibit relative activities of 0.2 %. Thus, ligation of the luficerase gene to the CMV promoter implies I-*Sce* I-mediated excision of the EGFP gene. Backbone-cleavage of plasmids resulted in linearization to resemble the conditions in the adenoviral genome. As compared to the control vector, up to 85 % luciferase activity were measured.

Results indicate that the expressed enzyme was functional since it specifically cleaved its recognition sequences. Interestingly, arrangement of the two I-*Sce* I-sites relative to each other seems to be important for this setting as Sce_inv yielded significantly higher luciferase activity values than the plasmid containing the directed setup.

6.1.2 Concept of I-*Sce* I-mediated destruction of the adenoviral vector in a p53-selective manner

From results shown in figure 5, a concept was derived that is presented in figure 6. In an E1/E3-deleted adenoviral backbone an I-*Sce* I-recognition sites-enframed E1 cassette was inserted. Downstream of the E1 region, a p53-dependent promoter (prMin-RGC) was inserted controlling the expression of the I-*Sce* I gene (figure 6A).

In this concept the adenoviral vector shall be inactivated by I-*Sce* I-mediated cleavage in the presence of transcriptionally active p53. As p53 allows expression

of I-*Sce* I, the enzyme recognizes its target sequences within the viral backbone. Ideally, complete cleavage should lead to separation of the E1A-controlling promoter from the E1A gene thus blocking E1A functions, and of the E1 cassette from the residual backbone (figure 6C). In contrast, in cancer cells with deleted or impaired p53 – resulting in loss of p53 transcriptional activity – I-*Sce* I is not expressed, the viral backbone remains intact and E1A is transcribed normally to allow for unaltered replication. Thus, due to the expression of E1A the infected cell initiates viral replication finally leading to release of adenoviral particles by cell lysis (figure 6C).

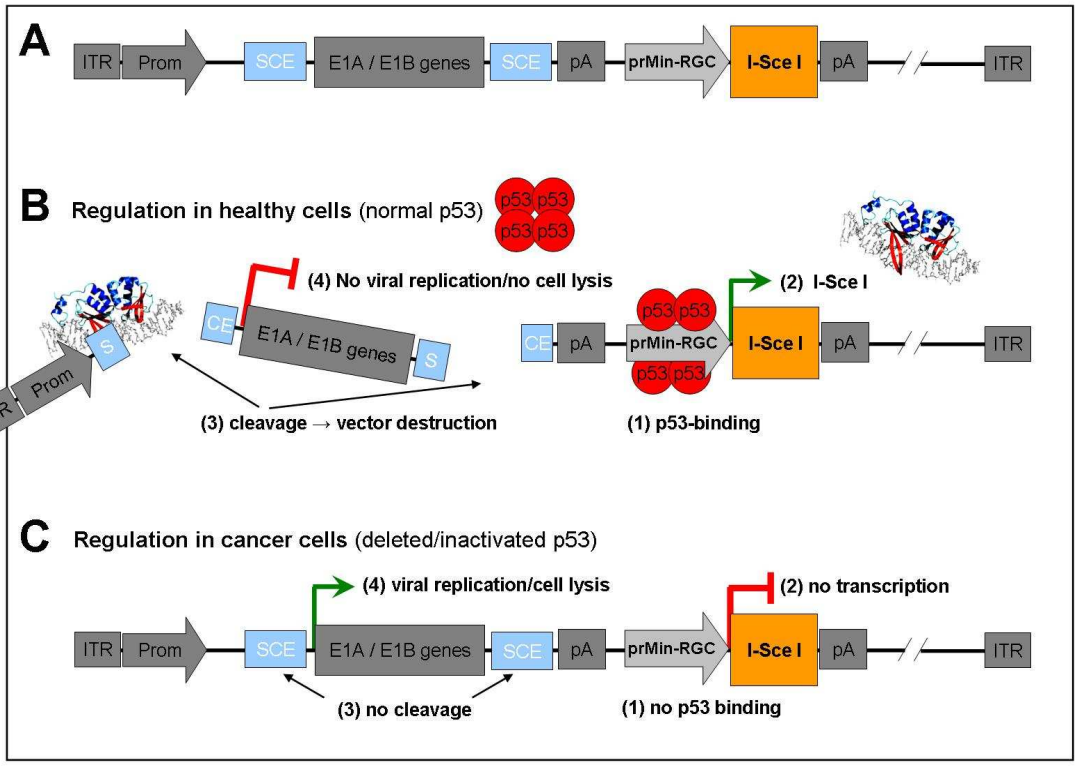

Figure 6. Concept of a conditionally replicating adenoviral vector that harbors a p53-dependent self-destruction switch. (A) Linear adenoviral DNA of an E1/E3-deleted adenoviral vector harboring a modified E1 region. **(B)** In normal cells, p53 is transcriptionally active, thus binding to the p53-dependent promoter prMin-RGC (1) and allowing transcription (2) of the adenovirally encoded DNA-cleaving enzyme I-*Sce*. I-*Sce* I cleaves the DNA binding sequence (SCE) framing the adenoviral E1 genes within the viral backbone (3). I-*Sce* I-mediated cleavage disintegrates the viral backbone and consequently averts viral replication and cell lysis (4). **(C)** In tumor cells, p53 is often functionally inactivated or deleted. The p53-dependent promoter is silent and I-*Sce* I will not be expressed (1, 2). The viral backbone cannot be cleaved and remains functional (3). E1A is expressed and viral replication proceeds to final cell lysis (4).

6.1.3 Generation of conditionally replicating adenoviral vectors

The concept described above was realized employing the ligation method introduced by Mizuguchi and Kay [153]. The backbone vector pAdHM4 contains

an E1/E3 deleted adenoviral genome flanked by *Pac* I recognition sites to produce a linear adenoviral DNA [153]. In this vector, recombinant E1 regions were inserted (figure 7A, B). In all viruses, the native E1A gene was replaced by an N-terminally truncated mutant (deletion of 66 bp) lacking amino acids 3 – 25 to inhibit binding to CBP/p300 and p53. E1A transcription was controlled either by a small CMV (230 bp) promoter or an hTERT promoter. Human cancer cells frequently activate the hTERT promoter. Since this is in stark contrast to normal, differentiated human cells it can be exploited to increase selectivity of oncolytic viruses (reviewed in [154]). Further, these promoter fragments were attached to binding sites of Gal4. Resulting promoters have been reported to be sensitive to the expression of the fusion protein Gal4-KRAB leading to inactivation of the expression unit. While the Gal4-domain mediates specific DNA-binding, Krüppel-associated box (KRAB) domains are potent transcriptional repression modules [74]. This construction allows for combination of the destruction switch concept with the p53-dependent transcriptional repressor termed Gal4-KRAB via an internal ribosomal entry sequence (figure 7B). The complete E1 region (E1A and E1B genes) was flanked by two inverted I-*Sce* I recognition sequences. Thus, complete I-*Sce* I-cleavage would excise the region of the adenoviral genome that is essential for initiating viral replication.

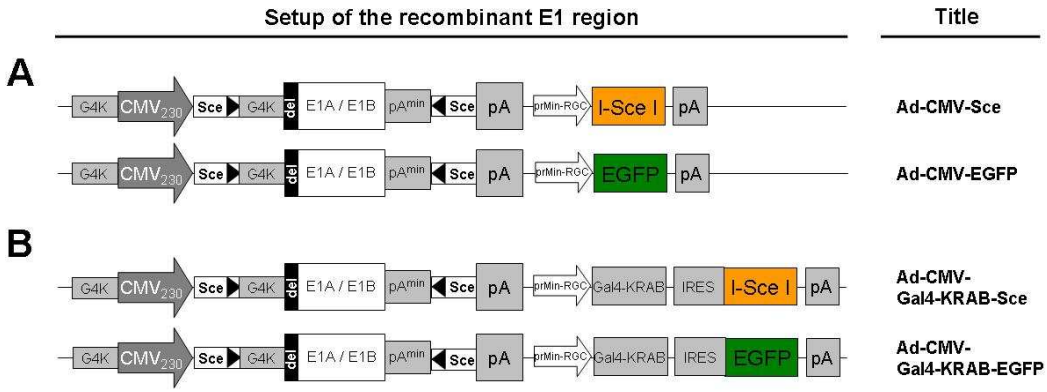

Figure 7. Basic setup of the E1 regions of conditionally replicating Adenoviruses harboring a self-destruction switch or EGFP. E1 genes are flanked by inverted I-*Sce* I-recognition sites (Sce) and are controlled by a CMV_{230}-promoter. Alternatively, instead of the CMV fragment a hTERT promoter was used (not shown; entitled analogously). Instead of the native E1A gene a N-terminally deleted mutant (del 66 bp) is used for all viruses. **(A)** The p53-dependent prMin-RGC promoter controls the expression of I-*Sce* I or EGFP. **(B)** In an additional setup, the I-*Sce* I-concept is combined with the application of the transcriptional repressor Gal4-KRAB that silences the promoter controlling the E1A gene. This artificial promoter contains 15 target sequences (G4K) for the Gal4-DNA binding domain of the fusion protein. Transcriptional repression is mediated by the KRAB domain.

Stepwise construction of the heterologous E1 region was examined by restriction enzyme cleavage and functional assays (data not shown) to ensure integrity of the final constructs. After production of infectious viral particles from the resulting plasmids the expression patterns of the resulting viruses were analyzed by western blotting (figure 8). Both I-*Sce* I and EGFP (in the control vectors) were expressed properly. Gal4-KRAB was correctly expressed by the viruses encoding the transcriptional repressor.

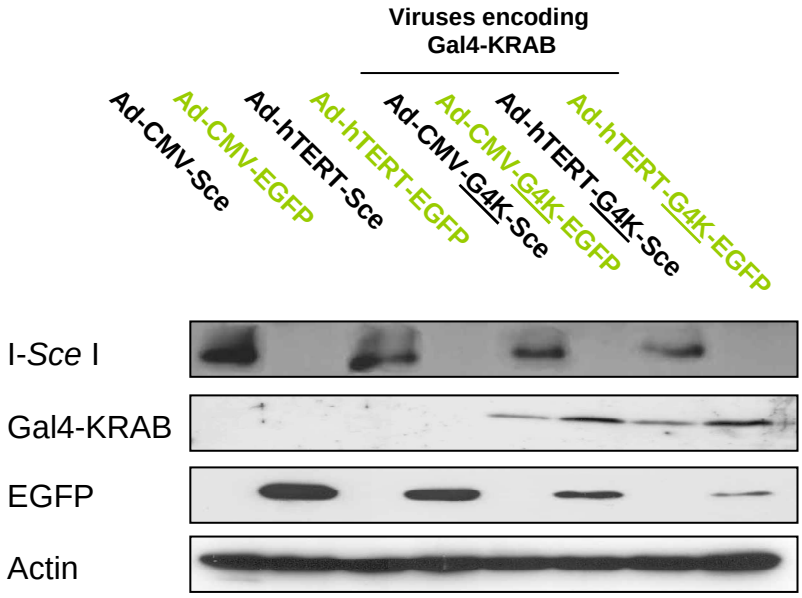

Figure 8. Expression patterns of the recombinant adenoviral vectors. p53-positive A549 cells were transduced with recombinant Adenoviruses as indicated at MOI = 5. Protein expression was controlled 40 hours post infection by western blot analysis. (In viruses, Gal4-KRAB is abbreviated by G4K.)

In summary, eight conditionally replicating Adenoviruses were generated that express certain regulatory proteins including the yeast meganuclease I-*Sce* I and the fusion protein Gal4-KRAB in a p53-selective manner.

6.1.4 Adenovirus-encoded I-*Sce* I recognizes and cleaves its target sequences within the viral backbone

As shown above in figure 5, the capacity of I-*Sce* I to recognize and cleave its 18 bp long recognition sequence was analyzed in cell culture on plasmid level. To determine the activity that was originated from virus-encoded I-*Sce* I, it was assumed again that mammalian cells rapidly react on generation of DNA double strand breaks in the above described manner. Figure 9A shows examples of

reorganized adenoviral DNA after I-*Sce* I-cleavage and repair. It was hypothesized that the E1 region following excision could preferably be circularized in an intra-molecular reaction by DNA repair mechanisms. This molecule is theoretically detectable by a specific PCR where primers will be elongated across the religation point (figure 9A) thus rendering the reaction unique for this circular DNA molecule.

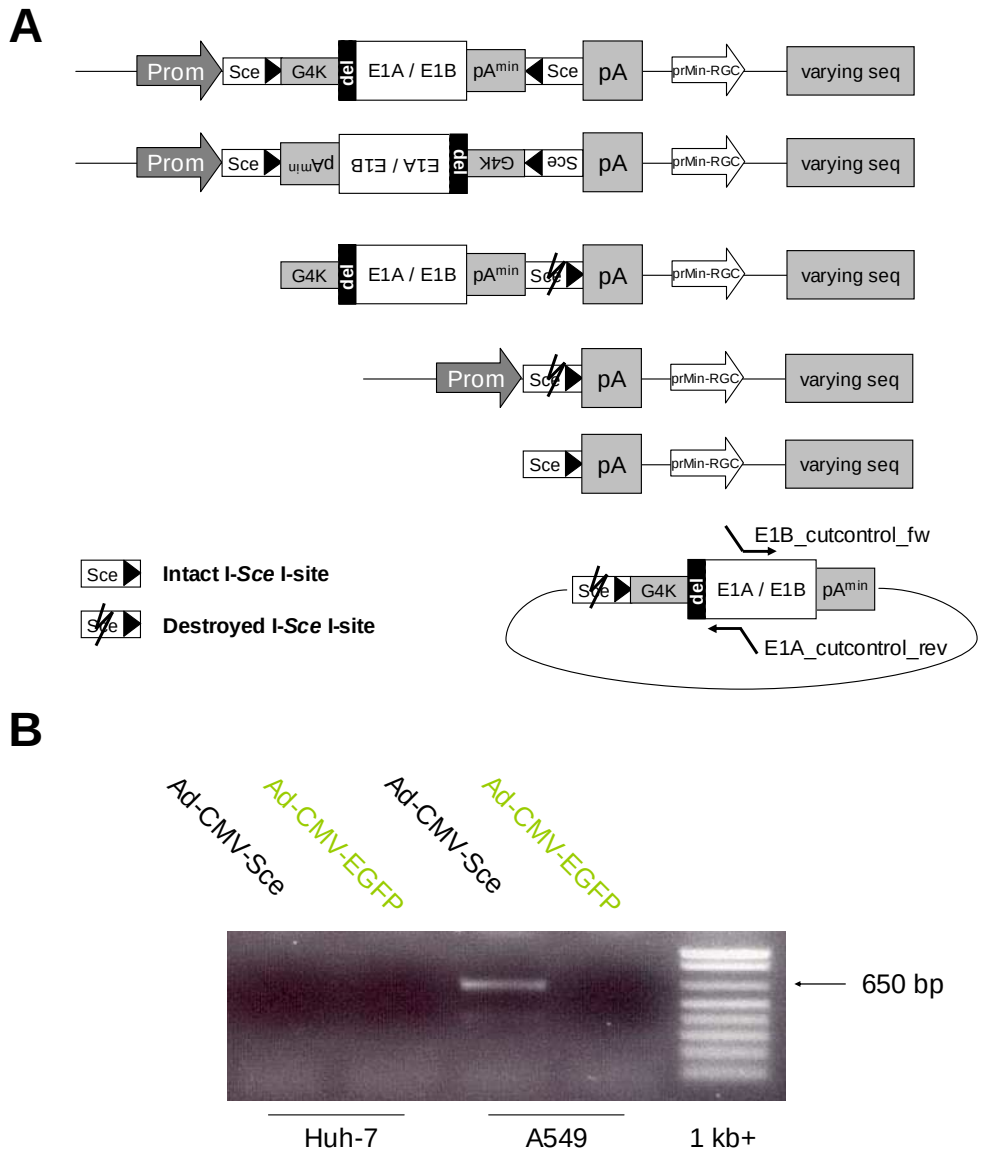

Figure 9. Adenovirus-encoded I-*Sce* I is expressed in p53-positive cells and cleaves its recognition sequence. (A) Cleavage of recombinant adenoviral DNA molecules activates cellular DNA repair mechanisms finally leading to a variety of possible cleavage-religation products. The majority of these products should remain linear except of excised E1 regions. These about 3,000 bp large fragments can be religated to circular DNA molecules in an intra-molecular-reaction (size not to scale). Design of a PCR that amplifies sequences across the religation point (about 650 bp product size) allows for detection of these molecules. **(B)** Agarose-gel analysis of a PCR that was performed on purified DNA from cells infected with an I-*Sce* I-encoding virus for 24 hours. Subsequent DNA sequencing confirmed the nature of the resulting DNA fragment.

Figure 9B represents the results of the experiment to determine I-*Sce* I-activity. p53-positive A549 cells and Huh-7 cells that harbor a transcriptionally inactive p53 mutant were infected with a low MOI of Ad-CMV-Sce or the control virus Ad-CMV-EGFP. DNA was extracted 24 hours after infection and subjected to PCR analysis. Whereas neither adenovirally infected Huh-7 cells nor Ad-CMV-EGFP infected A549 cells produced any detectable PCR product, a DNA fragment of about 650 bp could be amplified from A549 cells infected with the I-*Sce* I-encoding virus (figure 9B). Product size matched calculated length and thus, further was analyzed by DNA sequencing revealing the 650 bp fragment to contain E1A and E1B sequences fused to an I-*Sce* I-site.

Taken together, it was demonstrated that I-*Sce* I-harboring adenoviral vectors can be cleaved specifically by the encoded endonuclease. Thus, certain cleavage products have been detectable for Sce-viruses but not for EGFP-encoding control viruses. Furthermore, application of the prMin-RGC-promoters resulted in a limitation of endonucleolytic activity to p53-positive cells. These results provide evidence that I-*Sce* I can be successfully applied in the designed concept.

6.1.5 I-*Sce* I-encoding Adenoviruses are superior to their EGFP controls in terms of selectivity regarding p53-selectivity

In this work, recombinant adenoviral vectors were designed that replicate p53-selectively based on a novel regulation mechanism. Data obtained in preceding experiments (figure 8) revealed that constructed viruses did show the correct expression patterns of regulatory proteins. Furthermore, expression of the virus-encoded endonucleolytic enzyme I-*Sce* I was capable to cleave certain inserted target sequences within the adenoviral backbone in p53-positive cells (figure 9). In a subsequent experiment is was analyzed whether these data finally lead to a restriction of amplification of the recombinant vectors in p53-positive cell lines (A549, HepG2) compared to p53-dys-functional cells (Huh-7, H1299, Hep3B). Oncolytic activity was assessed 7 to 10 days after infection by crystal violet staining. In cells that possess only inactivated or even no p53 all viruses exhibited a lytic behavior (Figure 10, left panel). Importantly, corresponding I-*Sce* I and EGFP-control viruses did not show differences in their lytic capacities. Nonetheless, viruses using a CMV promoter to control the E1A gene exhibited a slightly higher lytic power than the hTERT-viruses. Since I-*Sce* I was expressed in

a p53-dependent manner, a regulation could only be expected in the p53-positive cell lines depicted in figure 10, right panel. In A549 cells, Ad-CMV-Sce lysed cells only at much higher employed MOIs than its control vector Ad-CMV-EGFP. The results correspond to hTERT-vectors where I-*Sce* I-expression led to reduced oncolysis. In HepG2 cells, the combination of I-*Sce* I with the transcriptional control of E1A by the hTERT promoter resulted in higher selectivity. Surprisingly, this was not observed in A549 cells.

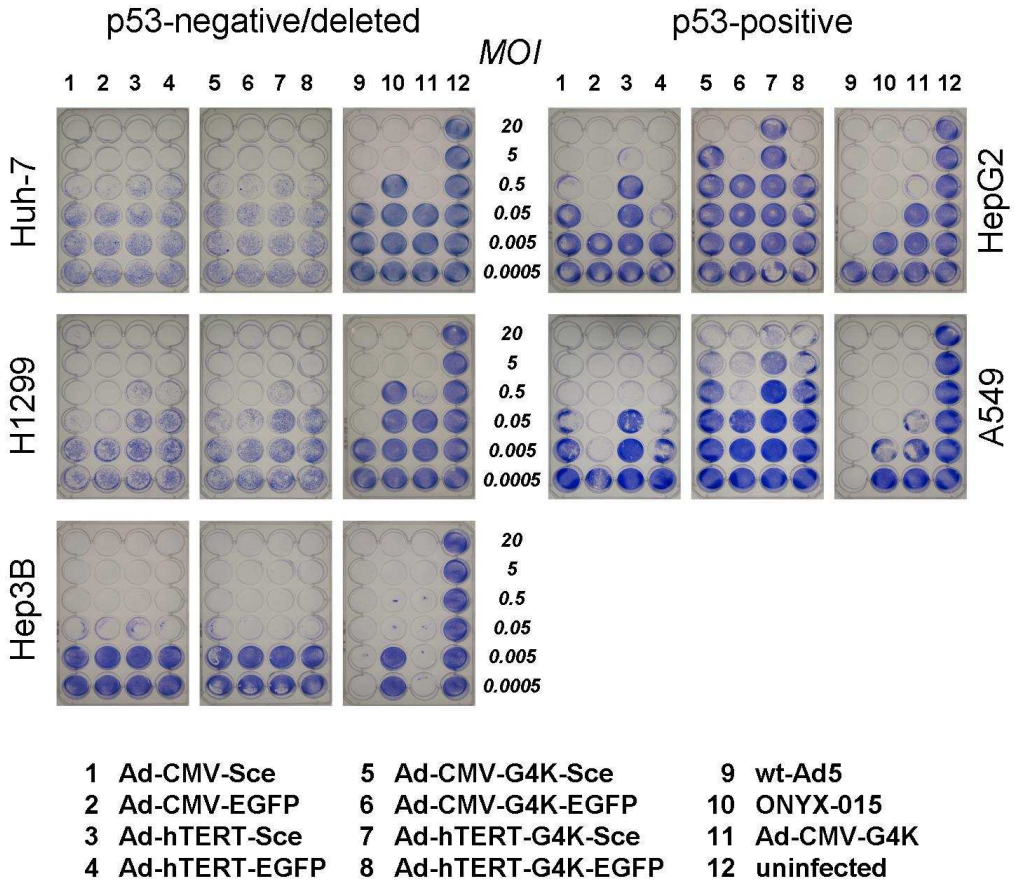

Figure 10. Oncolytic activity of recombinant adenoviral vectors in cells with transcriptionally active and impaired p53. Human cancer cell lines were seeded in 24 well plates and infected with decreasing amounts (resulting in MOIs 20 – 0.0005) of the indicated viruses. 8 hours prior to infection cells were supplemented with 50 ng/ml Doxorubicin to activate p53 protein. Repeated infection cycles were allowed for 7 – 11 days depending on the cell line. Cell staining was performed with crystal violet. Blue areas indicate non-lysed cells whereas unstained wells indicate extensive viral cytolysis. (Huh-7, mut-p53; H1299 and Hep3B, p53-deleted; A549 and HepG2, wt-p53)

Vectors applying the additional regulatory mechanism of Gal4-KRAB did exhibit an I-*Sce* I-regulation as well (in A549, HepG2). However, compared to I-*Sce* I-only viruses the extent was lower (half to one log step). Noteworthy, combining

the Gal4-KRAB and the I-*Sce* I regulation mechanisms resulted in a gain of selectivity.

Induction of a cytopathic effect in Huh-7, H1299 and Hep3B cells differs markedly. Beside replication, the infectivity of a particular cell type contributes to the oncolytic effect of a given vector [155]. Considering the results of the control viruses (wt-Ad5, ONYX-015, Ad-CMV-Gal4-KRAB) indicates that Huh-7 cells can not be infected as good as H1299 or Hep3B cells by adenoviral vectors. Importantly, there were no or only very limited (hTERT-viruses in H1299) differences between an I-*Sce* I-encoding virus and its corresponding EGFP-control virus.

In conclusion, recombinant adenoviral particles have been constructed that were demonstrated to be regulated by a p53-dependent endonucleolytic switch. Resulting, viral replication is highly restricted in wt-p53 cells, whereas oncolytic potency was not impaired in cell lines harboring dys-functional p53. This clearly indicates the functionality of the regulation concept employing I-*Sce* I.

6.2 Vesicular Stomatitis Virus

In a second project the virus-host interaction of a natural tumor virus – Vesicular Stomatitis Virus (VSV) – were analyzed in human cancer cell lines. Special interest was paid to the action of the Bcl-2-family members that are involved in regulating apoptotic cell death.

6.2.1 VSV-mediated decrease of Mcl-1 in human cancer cell lines

In order to analyze levels of proteins of the Bcl-2-family following VSV-infection, human cancer cell lines were infected with wild type VSV (Indiana) at MOI 0.1 and protein lysates were prepared (up to 48 hours post infection) with special interest on apoptosis-related proteins. Within the first 6 – 10 hours after infection a rapid decline of cellular levels of myeloid cell leukemia 1 (Mcl-1) protein was observed (figure 11A) in all tested cell lines. In contrast, levels of all other proteins including pro- and anti-apoptotic members of the Bcl-2 family as well as p53 remained unchanged. Elimination of Mcl-1 seems to be independent of the p53 status of an infected cell since p53 positive (HepG2, A549), mutant-p53 expressing (Huh-7) and p53-negative (H1299, data not shown) cell lines were affected similarly in a comparable time frame. Unfortunately, it was not possible to detect transcriptional activation of the pro-apoptotic genes Puma (p53 target gene) and Noxa (by a p53 independent mechanism) that has been described elsewhere [94;116;156].

Mechanisms that lead to the destruction of Mcl-1 could be the following: first, it has been reported that Mcl-1 is specifically cleaved by caspase-3 at Asp-127 and Asp-157 inhibiting its pro-survival function [157-161]; further, Mcl-1 protein levels are reported to be regulated by ubiquitin-mediated proteasomal degradation through the ubiquitin E3 ligase MULE/LASU1 [162;163]; additionally, an enzyme related to growth factor pathways, glycogen synthase kinase 3 beta (GSK-3β), targets Mcl-1 for destruction by specific phosphorylation at Ser159 located within the PEST domain of Mcl-1 that subsequently leads to proteasomal degradation via a different E3 ligase called β-TrCP [164]. Additionally, it can not be excluded that VSV proteins directly degrades Mcl-1 or targets Mcl-1 for degradation. To test caspase and proteasome dependence of the VSV-mediated Mcl-1 decrease, A549 cells were infected with VSV (MOI = 0.1) and concurrently treated with potent

pan-caspase (z-VAD-fmk) or proteasome inhibitors (MG132) for up to 12 hours (figure 11B). Z-VAD-fmk-treated cells showed kinetics of Mcl-1 decrease comparable to cells infected with VSV only. In contrast, proteasomal inhibition strongly stabilized the protein indicating that following VSV-infection Mcl-1 is not cleaved by activated caspases but is preferentially targeted for degradation via the ubiquitin-proteasome pathway by an unspecified E3 ubiquitin ligase.

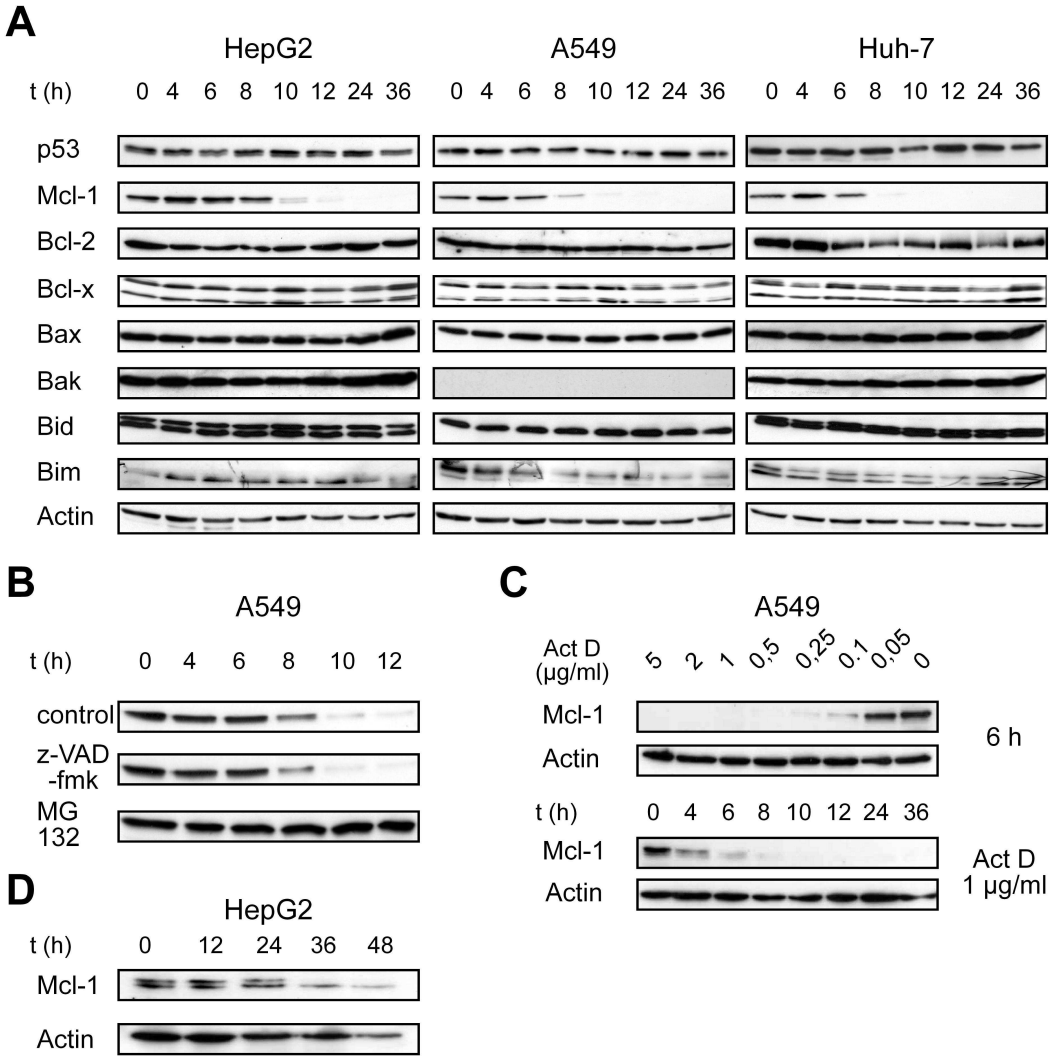

Figure 11. Decrease of Mcl-1 protein levels following VSV infection depends on VSV-mediated block of *de novo* proteins synthesis and proteasomal degradation of Mcl-1. (A) Human cancer cell lines were infected with wt-VSV (MOI = 0.1) and levels of apoptosis-related proteins were determined by western blotting. **(B)** A549 cells were infected with wt-VSV (MOI = 0.1) alone (control) or in combination with the pan-caspase-inhibitor z-VAD-fmk (20 µM; z-VAD) or the proteasome inhibitor MG132 (20 µM). Cell lysates were analyzed by western blotting. **(C)** A549 cells were treated with various concentrations of the transcription inhibitor Actinomycin D (Act D) and harvested after 6 h. Alternatively, A549 cells were treated with 1 µg/ml Actinomycin D, harvested after indicated times (h) and then subjected to western blot analysis. **(D)** HepG2 cells were infected with mutant VSV (attenuated virus 1, AV1) at MOI = 0.1 and harvested after the indicated times. Protein extracts were prepared and analyzed by western blotting for Mcl-1 and actin.

In general, Mcl-1 is rapidly turned over and, thus possesses a short half-life of smaller than 2 hours depending on the cell line [165-168]. VSV is known to block cellular protein biosynthesis globally by several mechanisms. This disrupted production of host cell proteins in conjunction with a constitutive protein degradation via the proteasome leads to rapidly declining levels of short-lived proteins – explaining Mcl-1's elimination following VSV infection. In an attempt to investigate inhibition of cellular protein biosynthesis separated from other viral effects, A549 cells were treated with different concentrations of the transcription inhibitor Actinomycin D (Act D) and harvested after 6 hours or after up to 36 h (figure 11C). Resulting data clearly showed that effects of Act D-induced block of transcription on cellular Mcl-1 protein levels resemble those elicited by VSV. Comparable data have been achieved for Huh-7 cells (data not shown).

The attenuated VSV mutant AV1 is unable to inhibit export of host mRNAs from the nucleus to the cytoplasm [94]. Following, the protein expression of AV1-infected cells was demonstrated to act normally [94]. In order to analyze the effect of AV1 on the Mcl-1 level of human cancer cells, HepG2 cells were infected with AV1 (MOI = 0.1) and protein lysates were prepared every 12 hours (0 – 48 h). Western blot analysis clearly showed that AV1 – in contrast to wt-VSV – does not induce a decline of cellular Mcl-1 protein levels (figure 11D). Thus, it can be assumed that VSV-mediated block of cellular protein synthesis in conjunction with normal proteasomal degradation leads to the observed Mcl-1 decrease.

Collectively, these data for the first time show that wt-VSV infection leads to a rapid decline of Mcl-1 protein level based on rapid proteasomal degradation. A similar decrease could not be observed for other pro- or anti-apoptotic Bcl-2-family members in these experiments.

6.2.2 VSV induces apoptosis via a strong activation of caspase-3

It has been demonstrated previously, that wt-VSV infection leads to induction of apoptosis via caspase-9 and caspase-3-like proteases [124;169]. This indicates the involvement of the intrinsic pathway in wt-VSV-mediated apoptosis induction.

To test whether VSV is capable to induce apoptosis in the used human cell lines caspase-3 activation was measured (figure 12). In HepG2 and Huh-7 cells caspase-3 activities reached comparable top levels at 24 to 36 hours while those of uninfected controls remain low. In contrast, infected A549 cells exhibited only

about 10 % of these activities culminating at 36 hours. A typical cytopathic effect of VSV is rounding of the infected cell after induction of apoptosis [170]. This effect was detectable by bright field microscopy for all three cell lines.

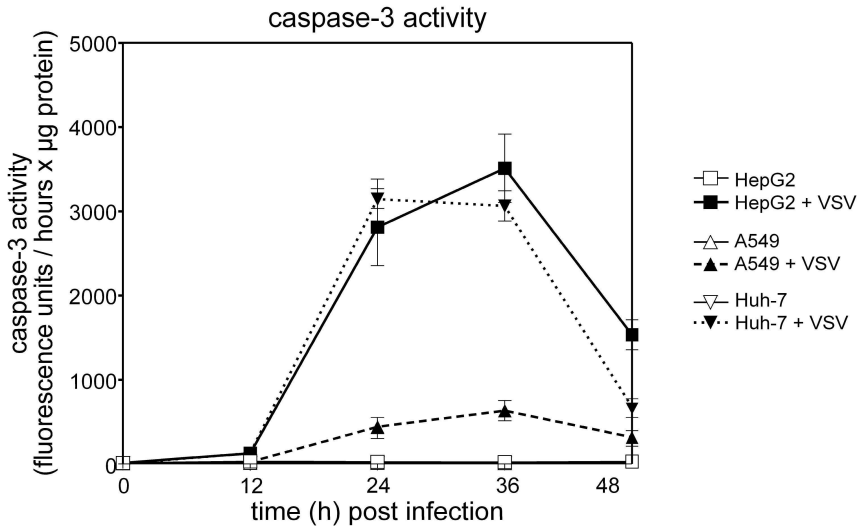

Figure 12. Human cancer cell lines induce apoptosis by activation of caspase-3 following infection with wt-VSV. A549, HepG2 and Huh-7 cells were infected at low MOIs (MOI = 0.1) of wt-VSV (black marks) or left uninfected (open marks) and harvested after indicated times. Caspase-3 activity was calculated in fluorescence units per µg protein and hour. Data represent results of three independent measurements and are presented as means ± standard errors of the means (SEM).

6.2.3 VSV mediates cleavage and subcellular relocalization of LC3 indicating induction of autophagy

Accompanied with apoptosis, is was reported that VSV induces autophagy in plasmacytoid cells and mouse embryo fibroblasts [171;172]. Autophagy is not only another programmed cell death mechanism but is additionally known to be part of the innate and adaptive immune response against viral infections [171]. To analyze induction of autophagy in human cancers the fusion protein of LC3 and YFP was applied. LC3B is a mammalian autophagy associated protein 8 (Atg8) homologue that is widely used as marker for autophagy. Induction of autophagy is known to coincide with cleavage of LC3 into LC3-I and -II and subsequent relocalization to nascent autophagolysosomal structures [151].

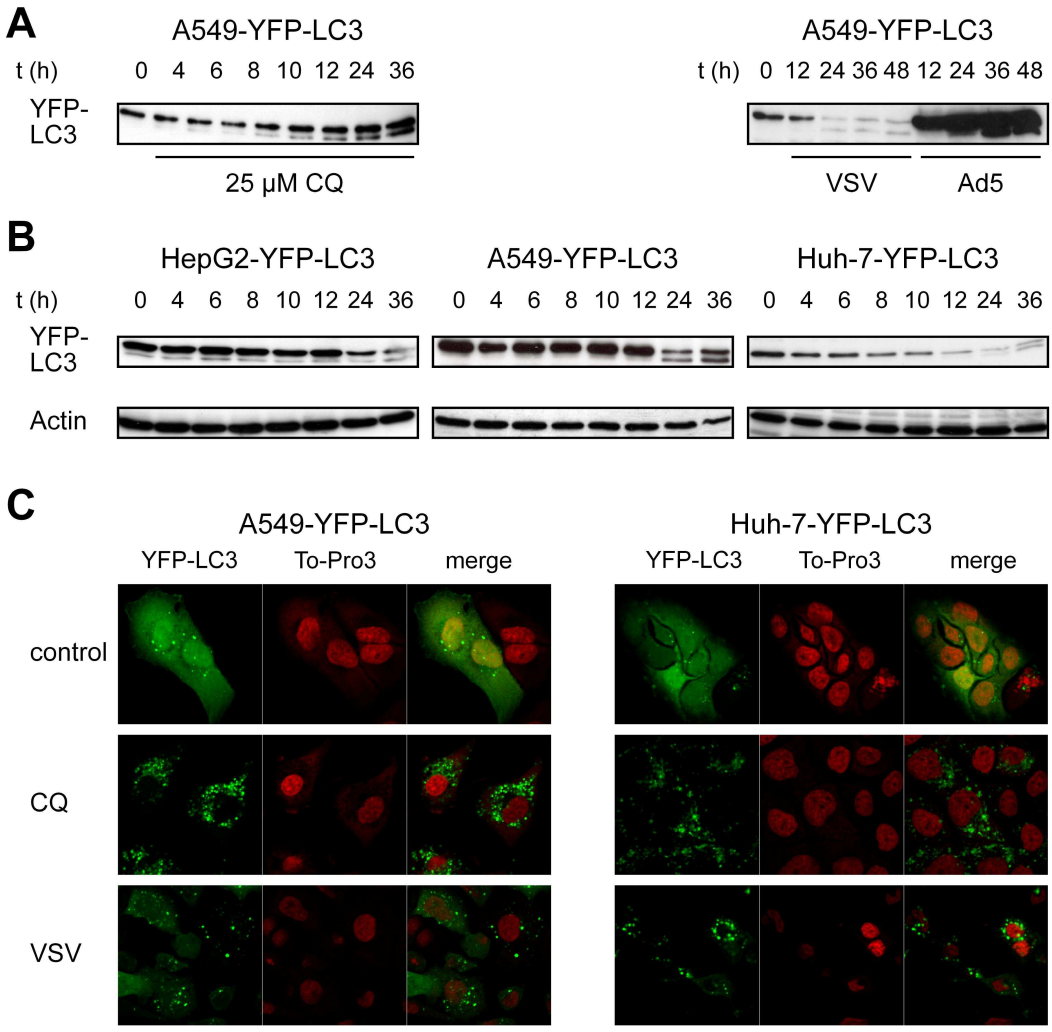

Figure 13. VSV infection induces autophagy in human cancer cell lines. Transgenic cells expressing the fusion protein YFP-LC3 were treated with wt-VSV (MOI = 10), wild type human Adenovirus type 5 (MOI = 10) or the autophagy-inducing agent chloroquine (25 µM). **(A)** Time courses of autophagy induction by chloroquine, VSV and wt-Ad5. After treatment cells were analyzed by western blotting. Generation of cleaved LC3-YFP (lower band) indicates induction of autophagy. **(B)** Human cancer cell lines stably expressing YFP-LC3 were infected with wt-VSV (MOI = 0.1) and protein extracts were analyzed for YFP-LC3 cleavage by western blotting. **(C)** Transgenic human cancer cell lines A549 and Huh-7 were treated like indicated (control = untreated, CQ = 25 µM, VSV-MOI = 0.1) and analyzed by confocal laser scanning microscopy after 12 h. YFP-signals (green), nuclear staining with To-Pro3 (red) and merged pictures are shown. Formation of autophagolysosomal structures can be observed by emergence of YFP-LC3-foci in contrast to diffuse distribution in untreated cells (control).

Following treatment with the potent autophagy inductor chloroquine (25 µM; CQ) LC3 was rapidly cleaved in stably transfected A549 cells (Figure 13A, left image). Infection with VSV or human wt-Adenovirus type 5 at MOIs = 10 revealed LC3-cleavage and, thus induction of autophagy (figure 13A, right image). Weaker protein levels in case of VSV (for 24 – 48 hours) are an effect of the virus-mediated block of protein production impairing the levels of the YFP-LC3.

Cleavage of LC3 could be detected in all three cell lines analyzed (figure 13B) even when low viral multiplicities (MOI = 0.1) were used. In addition to western blot analysis, induction of autophagy by VSV was confirmed by confocal laser scanning microscopy (CLSM)-aided monitoring of the relocalization of YFP-LC3 to focused dots representing autophagolysosomes (figure 13C). In summary, the data indicate that VSV-induced apoptosis in human cancer cell lines is accompanied by induction of autophagy.

6.2.4 Effects of Mcl-1 on activation of apoptosis and VSV amplification

Previous results indicated that proteasomal degradation is responsible for Mcl-1 elimination from VSV-infected cells (figure 11). To analyze more in detail the underlying mechanisms that target Mcl-1 for degradation an ubiquitination-resistant mutant of Mcl-1 (mut-Mcl-1) was used (kindly provided by Xiaodong Wang, Dallas, TX, USA) where crucial lysine residues (targeted by MULE/LASU1) were replaced by alanines [162]. Cells harboring a Mcl-1 protein that can not be ligated to ubiquitin by MULE/LASU1, will not be normally processed by proteolytic activity of the proteasomes [162]. Then, A549 and Huh-7 cells stably over-expressing wild type- or mutant-Mcl-1 were infected with wt-VSV and Mcl-1 protein levels up to 36 hours post infection were compared (figure 14A). While in parental cell lines Mcl-1 decreased rapidly, wt-Mcl-1-over-expressing cells showed a markedly prolonged presence of the protein. Thus, mut-Mcl-1 was detectable in VSV-infected cells up to 36 hours. The anti-apoptotic effect of Mcl-1 was analyzed by monitoring VSV-induced apoptosis in parental and transgenic (wt-Mcl-1, mut-Mcl-1, EGFP) A549 and Huh-7 cells. Results represented in figure 14B showed an impressive decrease of caspase-3 induction for both wt- and mut-Mcl-1-over-expressing cells in contrast to parental and EGFP-over-expressing controls. While A549 cells reached highest activation levels around 36 hours, Huh-7 values culminated earlier at about 24 hours post infection. To test whether increased Mcl-1 protein levels affect virus amplification, supernatants of infected cells were harvested every 12 hours to monitor produced viral progeny for a given period of time (figure 14C). Mcl-1 over-expression increased the produced VSV level at a statistically significant basis for all three time points. This might be directly attributed to lower levels of apoptosis induction for cells over-expressing mut-Mcl-1 (figure 14B).

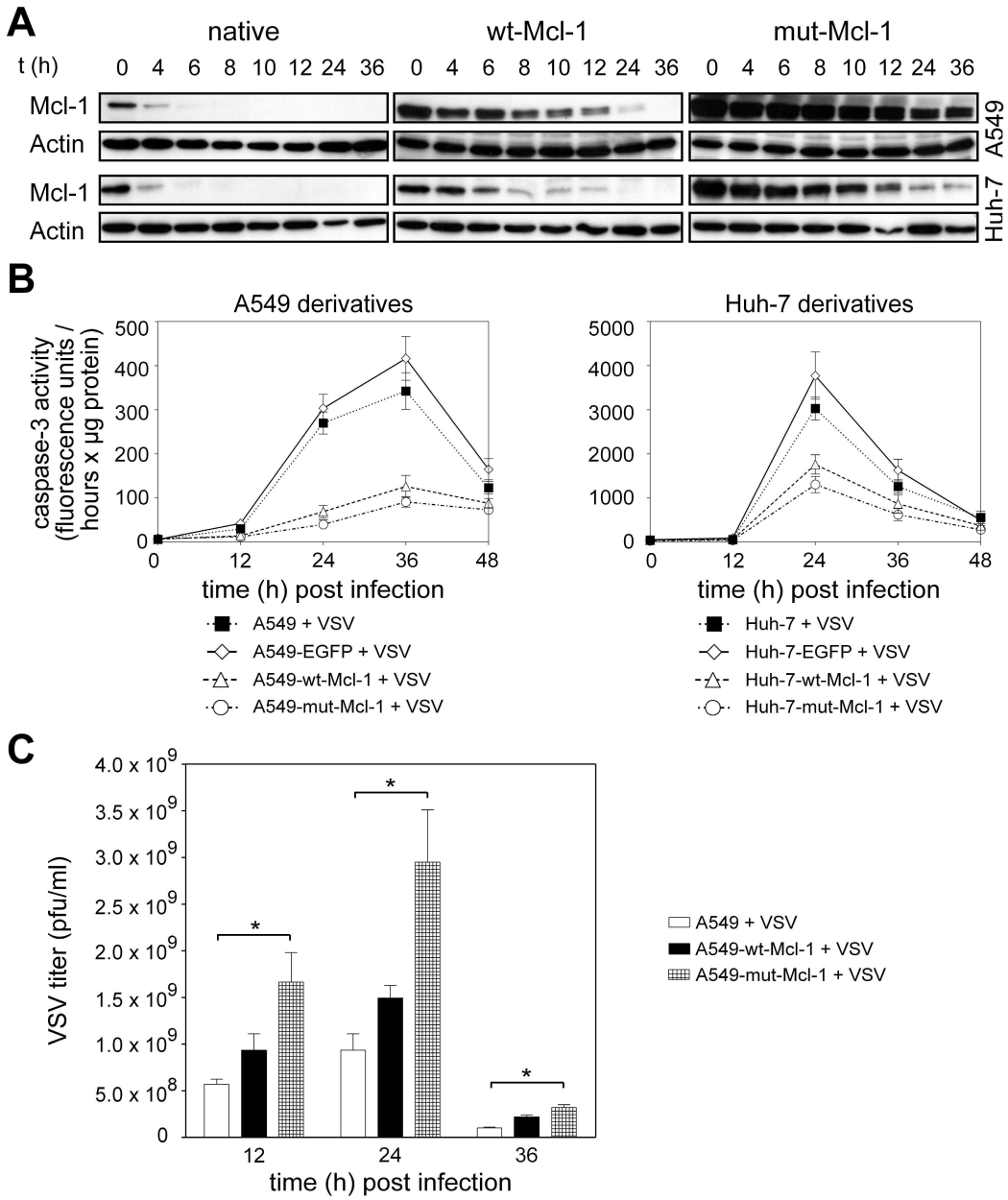

Figure 14. Over-expression of wt-Mcl-1 or mut-Mcl-1 impairs induction of apoptosis in wt-VSV-infected human cancer cells. (A) Native A549 and Huh-7 cell lines and wt- or mut-Mcl-1-over-expressing derivatives were infected with wt-VSV (MOI = 10). Protein levels of Mcl-1 and actin were analyzed by western blotting to determine effect of VSV-infection on cellular levels of the over-expressed Mcl-1 proteins depending on the time post infection. **(B)** Parental and transgenic A549 and Huh-7 cells expressing wt-Mcl-1, mut-Mcl-1 or EGFP were infected with wt-VSV (MOI = 0.1) and harvested after the indicated time points. Caspase-3 activity indicating induction of apoptosis was determined (data presented are means ± standard errors of the means (SEM)). **(C)** Parental and indicated transgenic A549 cells were infected with wt-VSV (MOI = 0.1). Supernatants were collected after the indicated times and viral titers were determined by $TCID_{50}$ analysis. Statistically significant differences were calculated from three experiments by Mann-Whitney-Test (*; $p < 0.05$).

Taken together, over-expression of wt-Mcl-1 and a stabilized ubiquitination-resistant mutant of Mcl-1 led to an increased protein level of Mcl-1 in VSV-infected human cancer cells. Increased protein levels subsequently impaired

induction of apoptosis measured by caspase-3 induction. Protein stabilization and less activation of the apoptotic cell death pathway coincided with higher viral titers.

6.2.5 Mcl-1 does not influence VSV-induced autophagy

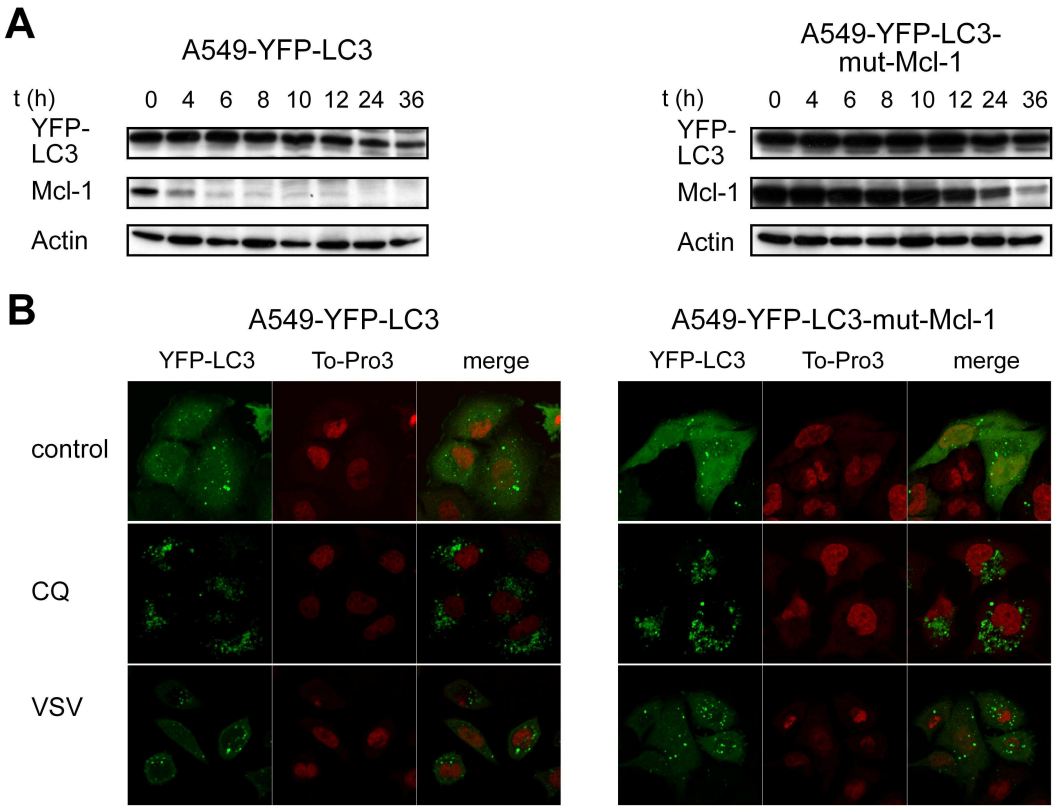

Figure 15. Induction of autophagy by wt-VSV is not influenced by over-expression of wt- or mut-Mcl-1 in human cancer cells. (A) Transgenic A549 cells expressing YFP-LC3 or YFP-LC3 and mut-Mcl-1 were infected with wt-VSV (MOI = 10). Western blot analysis determined LC3-cleavage and the Mcl-1 status. **(B)** The same transgenic cells were infected with VSV (MOI = 0.1), treated with chloroquine (CQ, 25 µM) or left untreated (control). Formation of autophagolysosomes indicating induction of autophagy was monitored by confocal laser scanning microscopy after 12 h (MOI = 0.1). Depicted are YFP-signals (green), nuclear staining with To-Pro3 (red) and merged pictures.

Beside apoptosis, VSV was observed to induce autophagy in human cancer cell lines (figure 13). As Mcl-1 significantly influences VSV-induced apoptosis (figure 14B), its actions on autophagic cell death were analyzed as well. For this purpose, A549 cells were stably transfected with YFP-LC3 alone or YFP-LC3 and the ubiquitination-resistant Mcl-1 mutant. Western blot analysis revealed no significant differences regarding the emergence of the LC3-cleavage product (figure 15A). To underpin this result, both cell lines were infected with low MOIs

of wt-VSV and 12 h post infection monitored for YFP-LC3-localization using CLSM. The typical formation of focused YFP-LC3 signals occurred in both cell lines independent of the Mcl-1 status (figure 15B). Whereas, untreated controls did not show any differences in both transgenic A549 cell lines, the number and intensity of the YFP-LC3-dots appeared to be minimally increased in CQ- and wt-VSV-treated A549-YFP-LC3-mut-Mcl-1 cells compared to A549-YPF-LC3 cells. Thus, microscopic data support the notion that Mcl-1 does not or not detectably influence induction of autophagy by wt-VSV.

6.2.6 Combination of VSV-virotherapy and Doxorubicin chemotherapy

Over-expression of Mcl-1 is known to confer resistance to chemotherapeutic treatment in different human cancers [173;174]. Hence, virotherapy-mediated reduction of Mcl-1 protein levels would greatly improve treatment opportunities. As VSV infection rapidly leads to decreased levels of Mcl-1 (figure 11) it should be tested if the potential of combined treatment with VSV and Doxorubicin to induce apoptosis exceeded that of single treatments.

A549 and Huh-7 cell lines over-expressing EGFP or mut-Mcl-1 were treated either with 100 ng/ml Doxorubicin or VSV (MOI = 0.1) or with a combination of Doxorubicin and VSV. Activation of caspase-3 was determined 24 h post infection (figure 16A). In general, expression of mut-Mcl-1 reduced apoptosis induction as already shown in figure 14B. A549 and Huh-7 cells expressing mut-Mcl-1, application of Doxorubicin did not or only slightly amplify the caspase-3-signal. In contrast, in control cells expressing EGFP instead of Mcl-1 caspase-3 values of combined treatment outstripped those of VSV single treatment of about 25 % in A549 and 50 % in Huh-7. This difference was statistically significant.

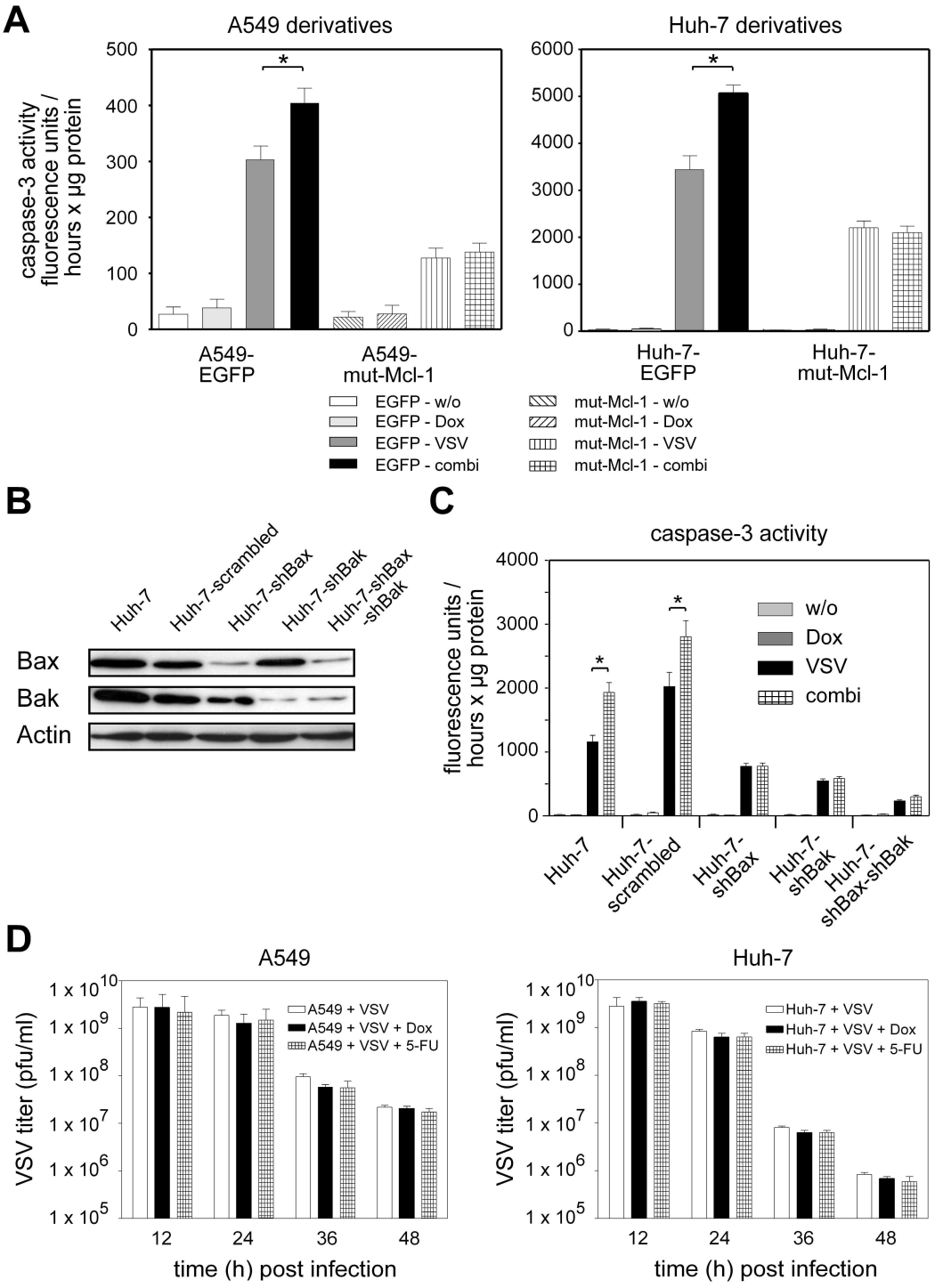

Figure 16. Combination of VSV-virotherapy and chemotherapy. (A) Transgenic A549 and Huh-7 cells expressing EGFP or mut-Mcl-1 were exposed to 100 ng/ml Doxorubicin (Dox), VSV (MOI = 0.1), a combination of both or left untreated and activation of caspase-3 was determined after 24 h. **(B)** Knock-down transgenic derivatives of Huh-7 cells expressing shRNAs against Bax, Bak or both were generated and effect on the indicated protein levels was determined by western blot analysis. **(C)** Knock-down transgenic derivatives of Huh-7 cells were subjected to caspase-3 activation analysis under indicated treatment strategies (100 ng/ml Doxorubicin, VSV-MOI = 0.1). Significance of differences was calculated using Mann-Whitney-Test (*; $p < 0.05$). **(D)** A549 and Huh-7 cells were treated with 100 ng/ml Doxorubicin (Dox) or 10 µg/ml 5-Fluorouracil (5-FU) in combination with VSV or exclusively with VSV (MOI = 0.1). Supernatants were collected every 12 hours and viral titer was determined by $TCID_{50}$ analysis (result of three measurements is shown). In general, data presented are means ± standard errors of the means (SEM).

Mcl-1, in concert with its pro-apoptotic binding partners Bak and Bax, regulates the permeability of the mitochondrial outer membrane. Therefore, the involvement of Bax and Bak in VSV-mediated apoptosis and chemotherapy-induced caspase-3 activation was analyzed in Huh-7 cells stably expressing shRNAs directed against Bax, Bak or both proteins (figure 16B). Silencing of Bax and Bak resulted in a strong reduction of caspase-3 activation in VSV or combination-treated cells (figure 16C). In contrast to control cells (parental, scrambled shRNA), knock-down of Bax and/or Bak led to an impaired sensitization of virally transduced tumor cells against chemotherapy.

An important prerequisite for the application of oncolytic viruses in combination with conventional chemotherapy is an unimpaired viral replication to maintain virotherapeutic efficacy. The influence of chemotherapy on viral amplification was assessed for Doxorubicin (100 ng/ml) and 5-Fluorouracil (10 µg/ml) by determining viral offspring in the supernatants of infected cells produced in 12 hours-periods in the course up to 48 hours post infection. Results of figure 16D demonstrated that VSV replication is not altered by therapeutically relevant concentrations of the applied chemotherapeutic agents.

In summary, VSV-mediated decrease of cellular Mcl-1 protein level could be rescued either by stabilization of Mcl-1 or by knock-down of the pro-apoptotic Mcl-1 binding partners Bax and Bak. It could be demonstrated that chemotherapeutic treatment does not interfere with viral replication.

6.2.7 Combination of VSV and chemotherapy enhances treatment efficacy of xeno-transplanted human tumors *in vivo*

Data obtained *in vitro* as described above suggest a relation between VSV and Mcl-1 that can be therapeutically exploited by additional chemotherapy to enhance apoptosis. Confirmation of this principle *in vivo* would greatly support the significance of *in vitro* results. For this purpose, A549 tumors were inoculated in nude mice and grown tumors were treated with a combination of 20 µg Doxorubicin intravenously and 1x 10^7 particles VSV intratumorally twice a week. Figure 17A illustrates the treatment success by development of the tumor size. Untreated or Doxorubicin-treated tumors constantly grew without a detectable difference. In contrast, tumors treated with VSV showed a marked delay of growth. Only application of the combined virochemotherapy was capable to

induce shrinkage of tumor size (of about 10 %) demonstrating the therapeutic succes. These results were supported by HE stained tumor sections (figure 17B) that were prepared from the treated tumors after day 40. VSV alone caused areas of cytopathic effects (no cell nuclei detectable, loss of normal cell morphology) that were significantly enlarged when tumors were treated with VSV and Doxorubicin in combination.

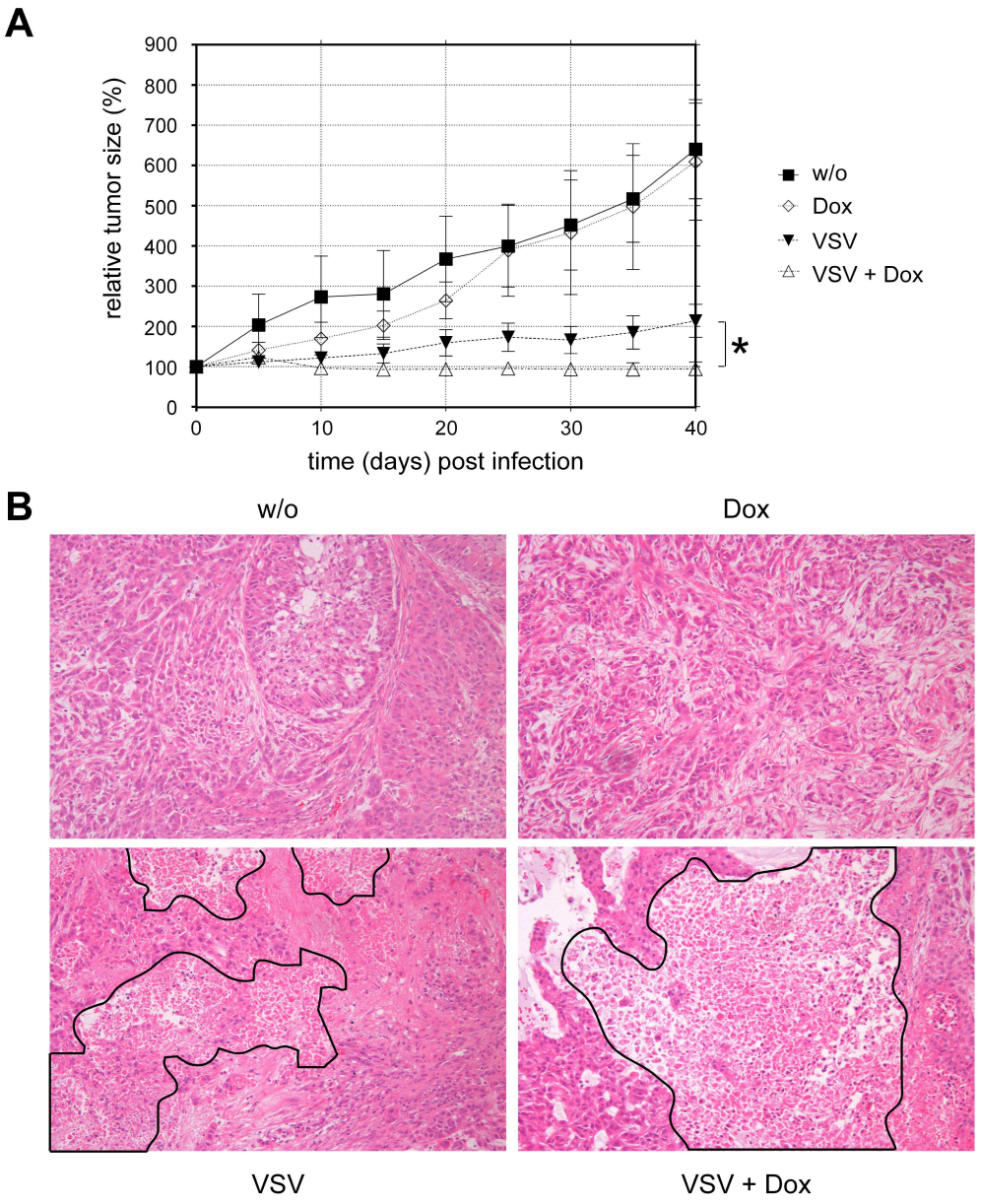

Figure 17. Destruction of xeno-transplanted A549-tumors on nude mice is superior for combination over single treatments. A549 tumors were implanted on nude mice and treated twice weekly with 20 µg Doxorubicin intravenously and/or 1x 10^7 infectious VSV particles intratumorally or left untreated (four groups each of 6 animals). Tumor growth was assessed every 5 days by a digital caliper. **(A)** Tumor volume was calculated and is depicted in relative representation (day 0 = 100 %), statistical significance was determinded by Mann-Whitney-Test (*; $p < 0.05$). Data presented are means ± standard errors of the means (SEM). **(B)** Mice were sacrificed at day 40 and tumors were embedded in paraffin and sections stained with hematoxylin/eosin. Areas displaying cytopathic effects are encircled.

Further experiments were performed to analyze this effect more in detail. Detectable effects of VSV treatment on the molecular level *in vivo* such as apoptosis induction or viral spreading are only hard to investigate when tumors were infected for several times over a long period of time like described for figure 17. Consequently, the experimental setting was altered. A549 tumors on nude mice were treated with Doxorubicin and VSV alone or in combination. In contrast to the formerly depicted experiment (figure 17), treatment was performed only once and tumors were harvested 1, 3 or 5 days later. Immuno-histochemical staining (anti-VSV-G-staining) of tumor sections was performed. In uninfected tumors, VSV-G was not detectable (figure 18A). In tumors that had been treated with VSV with or without additional Doxorubicin application the resulting staining reveals the presence of VSV-G protein. However, VSV-G appeared to be rather weak and can not be satisfyingly detected. HE staining from figure 17B demonstrated extensive cytopathic effects for VSV-infected tumors, so presence of VSV is necessary for this effect. Staining would be expected to be near to or at the rim of the destroyed tissue area. It might be speculated whether VSV in general spreads very slowly or cytopathic effect occurs only after repeated virus application. Other reports depicting vast intra-tumoral spreading of VSV were performed in rats and applied monoclonal mouse-anti-VSV-G antibodies [136;175-177]. This antibody would not be applicable in the used mouse model. Surprisingly, literature provides only very few references demonstrating histological staining of intra-tumoral VSV-spreading in mice by applying polyclonal antibodies against VSV-G. However, presented images have not achieved quality of results obtained within rats [149]. Thus, presumably intratumoral VSV-G detection is limited by technical reasons indicating the need for a potent, non-murine antibody for IHC applications. Consequently, *in vivo*-data could not prove differences between treatment strategies regarding viral spreading (figure 18A). On the other hand, this would be in concordance with results obtained from cell culture experiments where viral replication was not affected by chemotherapy (figure 16D).

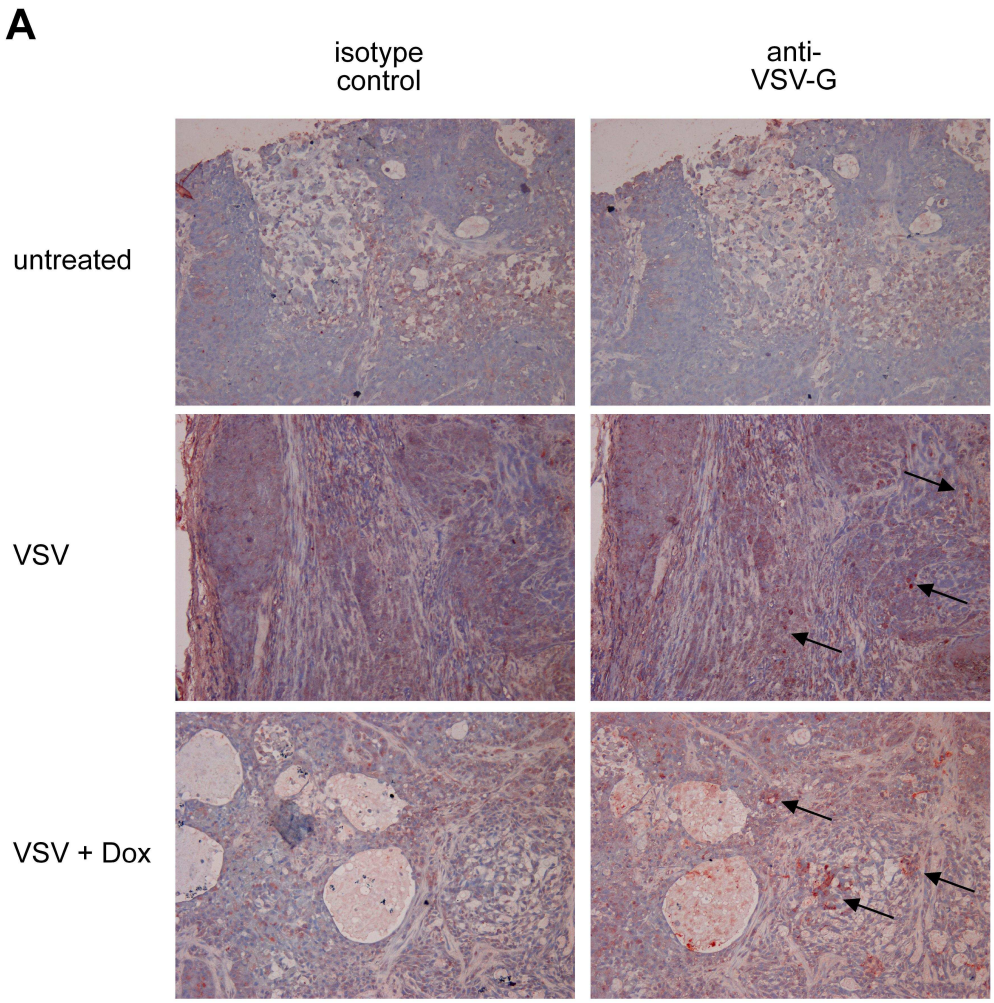

Figure 18. Biochemical analysis of tumor sections. A549 tumors were implanted on nude mice, treated once as indicated ($1x10^7$ VSV particles intratumorally, 20 µg Doxorubicin (Dox) intravenously), harvested after 1, 3 or 5 days and embedded in paraffin. **(A)** Paraffin sections (day 5) were stained for VSV surface glycoprotein compared to an isotype control. Brown dots (marked with arrows) indicate areas of VSV presence. (figure is continued on next page)

Induction of apoptosis *in vivo* was assessed by detecting apoptotic cells by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (figure 18B). Untreated tumors (control) did not show any TUNEL-positive signals. Doxorubicin-treated tumors exhibited only a small fraction of cells that underwent apoptosis (figure 18B, left column), however, TUNEL-signals increase up to day 5. VSV is a potent inducer of apoptosis in xeno-transplanted tumors since figure 18B demonstrated an impressive TUNEL-signal already 24 hours after VSV infection (middle column). Yet, in concordance with *in vitro* results, combination of chemotherapy and virotherapy was demonstrated to induce apoptosis at an even higher fraction of cells (figure 18, right column, days 3 and

5). As TUNEL signals multiply for days 3 and 5 compared to day 1 viral spreading must have occurred. Together, both results of apoptosis induction (TUNEL assay) and the observation of an amply cytopathic effect by HE staining contrast the results from immuno-histochemical analysis of infected tumors.

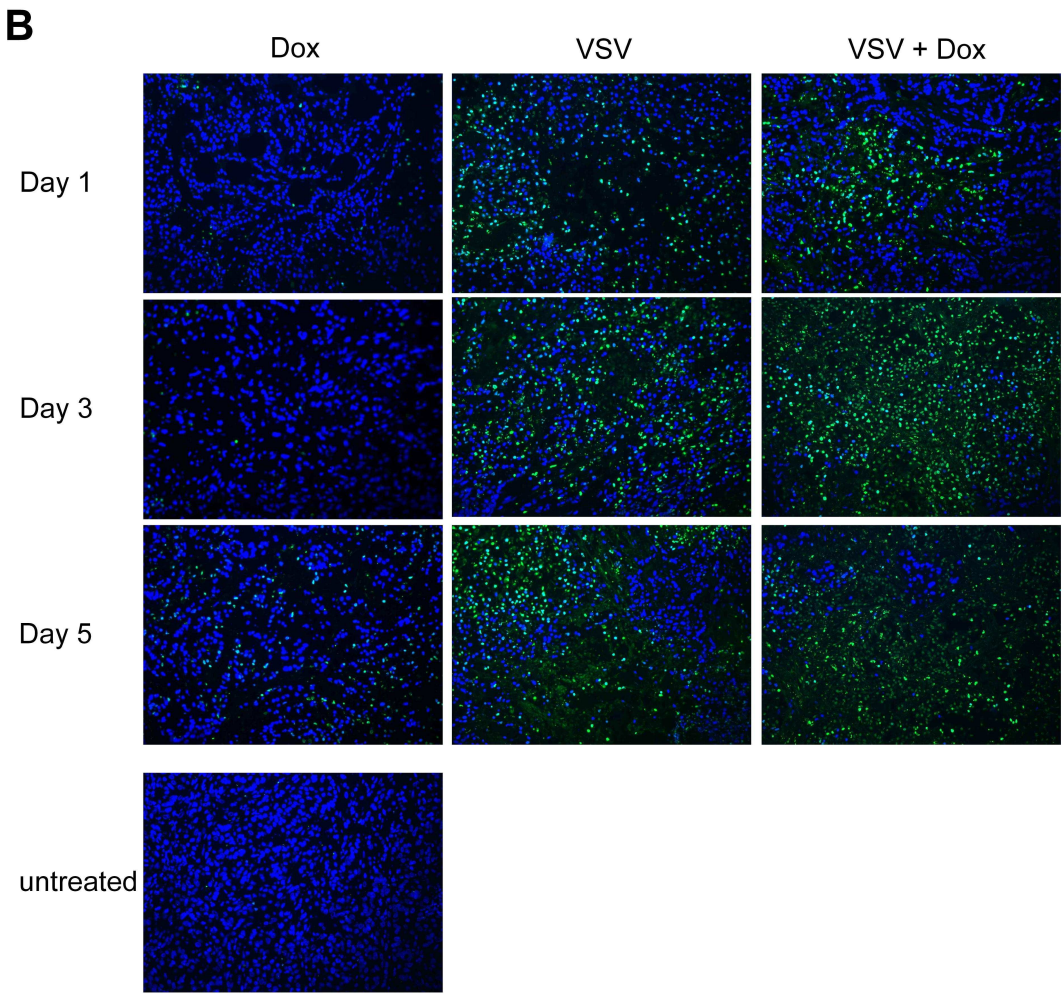

Figure 18. Biochemical analysis of tumor sections (continued). (B) Detection of apoptotic cells was performed by staining DNA nicks by TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling) (green signals), cell nuclei were colored by DAPI-staining (blue). Representative pictures were chosen for display.

Concluding, results from the here illustrated *in vivo* experiments demonstrate that VSV-mediated down-regulation *in vivo* can be exploited to sensitize human tumors to standard chemotherapy, since combination of VSV and Doxorubicin treatment led to higher extent of apoptotic areas and areas with cytopathic effects within tumor sections compared to VSV or Doxorubicin treatment alone. Thus, results obtained *in vivo* underpin *in vitro* data (figure 16). VSV spreading appeared not to be impaired *in vivo* (figure 18A) indicating that increased apoptosis

originates from the combined effect of VSV and Doxorubicin treatment and not from improved viral spreading.

7. Discussion

Oncolytic viruses represent a novel class of anti-cancer agents. Development of replication-competent viruses addresses the limited capacity of standard therapies to cure solid human cancers. Therapeutic success of oncolytic agents finally will depend on a thorough understanding of both the biology of the therapeutic virus and its interaction with cancerous and non-cancerous tissue. In the thesis presented here, virus-host-interactions between oncolytic viruses and human cancer cell lines were investigated to tightly restrict oncolytic virus infection to tumor cells. In a first project, a novel concept was established that for the first time refers to the destruction of oncolytic viruses in normal cells, thus aiming to prevent generation of viral break-through long time after treatment. This approach involved the application of a highly specific DNA endonuclease from yeast (I-*Sce* I) that was expressed by the oncolytic Adenovirus in a p53-selective manner. Secondly, the Vesicular Stomatitis Virus (VSV) is a long studied animal virus but only for about 35 years its meaning as anti-tumor agent is unraveled. Research is focused on wild type, mutant and engineered viruses. Nonetheless, the interaction of VSV and mammalian cells appears to be rather complex especially when considering that the virus only encodes five proteins and that a major part the matrix protein is responsible for manipulating the infected cell. Here, special emphasis was set on the meaning of proteins of the Bcl-2-family in VSV infected tumors.

7.1 Recombinant adenoviral vectors

The literature provides a wealth of concepts to restrict adenoviral replication and subsequent cell lyses to cancerous cells while normal tissue is spared (reviewed in [42]). In practice, infection of tumors with a self-replicating agent eventually will lead to infection of normal cells as well. Frequently, this issue has been addressed by development of mechanisms that prevent replication of viral therapeutics in non-target cells. According to the underlying strategies, four different regulation principles can be defined: transductional, transcriptional, translational and apoptosis targeting [26].

The modification of attachment proteins on the virus surface can be applied to target tumor cells exposing receptor molecules that are exclusively or at least preferentially expressed on tumor cells, e.g. the Her2/neu tyrosine kinase receptor

or the epidermal growth factor receptor (EGFR) [144;178]. This so called transductional targeting leads to preferential virus entry into cancer cells. This strategy includes the activation of viral surface proteins by proteases that are abundant in tumor-specific microenvironments [179] and expression of virus-encoded soluble fusion molecules [180].

Another concept (apoptosis targeting) involves the modification of viral genes whose products are essential for virus propagation in normal cells but are dispensable in tumor cells [37]. Deletion of adenoviral E1 genes that inhibit apoptosis and cell cycle arrest in infected cells renders Adenoviruses sensitive for p53 and RB induced apoptosis in normal cells. In contrast, in tumor cells with frequently occurring non-functional p53 and RB proteins E1-deleted Adenoviruses still are capable of generating infectious offspring without inducing cell death [181;182].

DNA viruses like Herpes Simplex Virus or Adenovirus can be transcriptionally restricted to tumor cells by inserting promoters that are primarily active in tumor tissue and regulate the transcription of genes that are essential for viral replication. In the case of Adenoviruses, promoters of the prostate specific antigen (PSA) [183] or the human telomerase reverse transcriptase (hTERT) [184] allowed a conditional replication in tumor cells. In recent years, development switched from rather simple promoter arrangements to more complex gene networks to limit virus replication to cancer cells. One example was introduced by our group applying a p53-sensor and a transcription repressor element [74].

It was suggested recently that genes responsible for cancer development are involved in anti-viral defense mechanisms as well [14;15]. A prominent example is the interferon system that represents a key module of innate anti-viral immunity and is often inactivated in tumor cells [36]. This difference between normal and cancerous cells for example can be exploited by the naturally tumor-selective virus Vesicular Stomatitis Virus (translational targeting). Normal VSV-infected cells initiate a strong IFN response leading to a global shut down of translation in neighboring cells. In contrast, cancer cells with impaired IFN signaling pathways remain susceptible to VSV infection since biosynthesis of VSV proteins is not inhibited. Based on this concept VSV vectors were created that show an increased specificity for cells with impaired IFN-pathways. In a first study, mutation of a

viral key protein (VSV-M) restored interferon release [94] while in another approach an IFN-β gene was inserted into the virus genome [147].

However, these regulatory circuits are observed to be overcome by physiological circumstances finally allowing the emergence of viral particles from normal tissue ("leakiness") [185;186]. This was attributed to several mechanisms such as "auto-activation" feedback loops of regulated viral genes and limited attenuation of the applied tissue-specific promoters that control viral genes essential for replication [186-189]. Additionally, infection of non-target cells with high amounts of infectious particles will allow for undesired viral replication. Thus, more sophisticated regulation concepts are required to increase safety profiles of existing oncolytic viruses.

The concept presented here (figure 6) provides a novel approach to render adenoviral vectors permanently non-functional by specific destruction of the viral genome. Central tool to achieve vector DNA destruction is the yeast endonuclease I-*Sce* I. In contrast to other endonucleases, the rare-cutting enzyme I-*Sce* I displays promising properties for the application in this concept. It has been reported that expression of the enzyme is well tolerated by mammalian cells suggesting that these complex genomes do not or only to a very limited extent provide I-*Sce* I-recognition sequences [82]. Importantly, the 18 bp long recognition sequence can not be found in the genomic information of human Adenovirus type 5.

To learn about functional properties of I-*Sce* I, first experiments were performed on basis of plasmids in cell culture. It could be observed that the enzyme is properly localized to the cell nucleus of human cancer cells and that it does possess a detectable capacity to specifically cleave circular or linear target reporter vectors (figure 5). However, data suggest a strong reaction of human cells on I-*Sce* I-mediated cleavage as they efficiently repair DNA double strand breaks ([152], personal communication with F. Pagues, Cellectis SA). To enable proper monitoring of I-*Sce* I-cleavage, it appeared to be reasonable to insert more than a unique I-*Sce* I-recognition site in the viral genome. Since I-*Sce* I is reported to be more efficient when concentration of target sequences was low [190] the number of recognition sequences in the adenoviral backbone was limited to two I-*Sce* I-sites. Furthermore, two I-*Sce* I-sites can be placed in inverted or directed orientation in an adenoviral genome because the 18 bp long recognition sequence is non-palindromic. In general, excision of a recognition site-framed adenoviral

DNA region was supposed to lead to a rapid circularization of the resulting fragment. This circularization would prevent a latter recruiting as a possibly "missing piece fragment" into other cleaved fragments to render an inactivated adenoviral backbone functional again. Noteworthy, fusion of DNA ends with no or only little sequence homology by the non-homologous endjoining (NHEJ) pathway leads to destruction of intact cleavage sites. Thus, adenoviral backbones encoding the inverted recognition site setup upon cleavage and religation would result in an excised, circular DNA fragment that could not be rescued by I-*Sce* I-cleavage since the recognition site is destroyed during the religation step [152]. In contrast, the directed orientation could result in re-cleavable fragments. Nonetheless, these mechanisms have to be further investigated in detail.

Since E1A has been demonstrated to be the major regulator of adenoviral replication it was chosen as target for transcriptional regulation in a variety of studies. Most prominently, heterologous promoters are utilized to restrict E1A expression to a desired cell type or tissue [58-62;184]. Therefore, one cleavage sites of I-*Sce* I was inserted 5' of the E1A gene to separate the gene from its driving promoter upon I-*Sce* I cleavage (figures 6, 7). The second recognition sequence was situated downstream of the E1B genes. This setup allows the excision of the whole E1 gene family that is essential for initiation of adenoviral replication – and for cellular transformation. Integration of an intact E1 DNA region into a mammalian genome has previously been reported to be sufficient for transforming human embryonic kidney cells [50].

Upon generation, recombinant adenoviral particles were analyzed for their protein expression patterns to check functional integrity of the constructed genomes. Correct expression of regulatory proteins was a prerequisite for the functioning of the concept. Subsequently, the activity of virus-encoded I-*Sce* I protein was analyzed. To this end, it was hypothesized that – within a pool of very different cleavage-religation products – circularized E1 fragments might have emerged that could be detected by PCR indicating I-*Sce* I cleavage. As a matter of fact, expected PCR products were exclusively detected in p53-positive cells. In contrast, for EGFP-encoding viruses or in p53-impaired cells, the cleavage activity could not be examined. This underlines the selectivity of the p53-dependent promoter prMin-RGC and its use in the provided concept.

After exploring certain characteristics of the regulation system, the test of the constructed viruses provided evidence for proof of the concept in oncolysis assays applying p53-positive and p53-deleted/impaired cell lines. Viruses being regulated by a prMin-RGC-I-*Sce* I-switch were more selective to p53 than their control vectors (factor 10 – 100) while exposing comparable lytic levels in p53-impaired cell lines. Surprisingly, the CMV-setup provided a higher selectivity than the hTERT-viruses in A549 cells. In contrast, in HepG2 cells the hTERT viruses were superior to CMV-viruses regarding restriction. Activation of hTERT expression is an essential element of cancer development in humans while this is not observed in normal differentiated cells [65]. Thus, integration of an hTERT promoter in an adenoviral setup to control E1A expression adds an additional level of selectivity to the replication regulation mechanism. According to this, it seems questionable that differences regarding hTERT status of HepG2 and A549 might explain the differing results. Rather, an experiment including non-transformed cells would yield reasonable results on this issue.

Similar results were observed as well when I-*Sce* I-switch and an additional transcriptional regulation by Gal4-KRAB were combined. For these viruses (Ad-CMV- or Ad-hTERT-Gal4-KRAB-I-*Sce* I) gain of selectivity showed comparable values (factor 10 – 100) when compared to the corresponding EGFP-controls. As viruses harboring both regulation principles showed an almost similar lytic pattern in cells with impaired p53 status, it can be concluded that combining these regulators renders the viruses more selective. This is in concordance with former reports about combining two separate regulatory elements [61;66].

The first and very prominent oncolytic adenoviral vector, Ad*dl*1520 (Onyx-015), was tested as control vector. Originally, replication of ONYX-015 was reported to be exclusively dependent on the p53 status of an infected cell. This assumption was based on deletion of the E1B-55k gene that is known to inactivate functional p53 protein. While ONYX-015 replicated normally in p53-dys-functional cells it did not propagate in p53-positive cell lines. However, following reports questioned the role of p53 on selectivity of ONYX-015. Rather, differential late viral mRNA export seems to be determining selectivity of Onyx-015 [72]. Independently of these discussions, ONYX-015 has maintained its meaning as important standard vector since it is currently subject of clinical trials. Compared to ONYX-015 all I-*Sce* I-encoding viruses exhibited a higher restriction in p53-positive cells, thus

demonstrating functionality of the I-*Sce* I-concept. Importantly, I-*Sce* I-encoding viruses performed equally or even better in cells with deleted or mutated p53 genes (figure 10).

Taken together, the application of a genome-destruction switch that acts p53-selectively and specifically on adenoviral DNA provides a new tool for restricting viral replication to cancer cells. Nonetheless, based on the provided data questions arise that will have to be answered to demonstrate an advantage over previously described regulative mechanisms. First, results for selectivity have to be verified in other additional lines to provide evidence for a generalized application. Performing experiments in isogenic cell lines only differing in the transcriptional status of p53 [191] would greatly enhance validity of the so far obtained data. Next, it can be speculated whether excision of a genomic region that is essential for viral replication results in decreased levels of mRNA and protein of E1A since cleavage separates the driving promoter from the E1A gene. It can be assumed that there is a competition between prMin-RGC-promoter and the promoter controlling E1A. If transcription of the E1A gene is initiated rapidly and leads to high amounts of functional protein, I-*Sce* I-cleavage will not result in block of adenoviral replication. This can be observed for high applied MOIs in oncolysis assays (figure 10, MOIs = 5 – 20, A549, HepG2). Importantly, these examinations should be performed within the early phase after infection.

As alternative to this “transcriptional explanation”, in a replication-based theory it could be argued that decreased numbers of adenoviral genome templates (lacking the essential left ITR) are responsible for the observed effects. Analysis of the number of genomic DNA templates in p53-positive and –negative cell lines would address this question.

Thirdly, the concept claims to inactivate viruses in normal cells. Generation of free DNA-ends by destruction of adenoviral DNA activates DNA repair pathways that could activate cell death programs [152]. Thus, survival of infected normal cells has to be tested to exclude apoptosis-targeting as underlying regulation mechanism.

If all these questions are answered, a fourth point seems worth to be explored: cleavage products of adenoviral DNA might be subjected to error-prone repair and can be incorporated into the cells genome. Integration of the E1 region should be prevented as the combination of these genes possesses transformative potential

[50]. It has been previously shown that I-*Sce* I-mediated excision of an EGFP gene from a plasmid vector leads to integration into *Xenopus* genomes [192]. This indicates that integration of DNA fragments is not only a theoretical challenge. Additionally, this observation supports the strategy to limit the number of I-*Sce* I-recognition site to minimize the amount of cleavage products. In order to prevent insertion of functional adenoviral genes into the host genome, an alternative positioning of the I-*Sce* I-cleavage sites – such as within the E1A gene – might pose a solution.

In addition to the potential transformative potential of the generated cleavage products, there occurs another problem based on the action of DNA repair processes. Cleavage and subsequent religation of adenoviral cleavage products might result in functional adenoviral backbones that were rendered I-*Sce* I-insensitive by destruction of one or both inserted cleavage sites. To limit the emergence of these I-*Sce* I-insensitive break-through mutants, it might be promising to insert additional I-*Sce* I-sites in the adenoviral backbone. Alternatively, and in conjunction with the aim to reduce the transformative potential of this concept, altered positioning of cleavage sites within the E1A gene would be a smart solution. Since DNA repair processes were demonstrated to be highly error-prone (deletions and insertions of nucleotides were reported) [152], this would inactivate the E1A gene e. g. by frame-shift-mutations.

Noteworthy, this concept requires novel virus production processes. Usually, adenoviral vectors are propagated in 293 cells. These cells are stably transfected with an intact adenoviral E1 region that leads to degradation of p53. However, monitoring virus infection clearly demonstrates residual p53 function since prMin-RGC promoter allowed expression of EGFP in control viruses. Thus, it has to be assumed that I-*Sce* I is expressed as well. This certainly increases the risk of amplification of I-*Sce* I-insensitive mutant particles that impair safety of the vector. However, preliminary experiments with p53-deficient H1299 and Hep3B cells resulted in unsatisfying viral titers thus requiring different cell lines.

In summary, it can be concluded that the application of the yeast meganuclease I-*Sce* I represents a promising strategy to degrade conditionally replicating Adenoviruses in p53-selective manner. The provided results demonstrated a high selectivity and efficacy profile of the constructed vectors that is superior to other

established regulation mechanisms. Future efforts will be made to promote the established concept by providing valid data on the mechanism of action.

7.2 VSV-mediated Mcl-1 destruction

The natural tumor virus Vesicular Stomatitis Virus (VSV) is known to selectively infect and destroy human cancer cells while normal cells are spared from cell lysis. Great effort has been made to investigate underlying mechanisms for tumor selectivity. Several studies have demonstrated a strong dependence of VSV on an impaired interferon system [34;93]. Other cellular components like the tumor suppressor protein p53 have also been reported to play a role in handling VSV infection in mammalian cells [14;116]. Importantly, oncolytic viruses destroy infected cells subsequent to infection. In this process induced apoptosis seems to play a more or less important role for the replication cycle of different therapeutically exploited viruses. Usually, induction of apoptosis limits viral amplification, and therefore viruses have developed different strategies to prevent premature apoptotic cell death prior to the completion of the viral life cycle [193;194]. On the other hand, virus-induced apoptosis can not simply be interpreted as anti-viral defense mechanism. Rather, it was described that apoptosis might facilitate release of viral offspring from infected cells to enhance viral spreading [195;196]. For VSV the process and meaning of induced apoptosis have been investigated in several studies. VSV was reported to trigger apoptosis at early stages in the viral life cycle [125]. Shortly after infection and uncoating of virions but prior to synthesis of viral proteins and start of replication, apoptosis is induced. Surprisingly, even UV-inactivated viruses were capable to induce apoptotic cell death. This observation is not unique for VSV but is in concordance with results obtained for other viruses like Sinbis virus [197], reovirus [198] and vaccinia virus [199]. For VSV, a crucial role for the viral matrix protein was suggested in inducing apoptosis since transfection of mRNA of the viral M-protein is sufficient to elicit apoptosis in human neural cells in the absence of other viral components [126;127]. In the same study, for BHK cells the contribution of another unknown viral component that contributes to induction of apoptosis has been proposed [127]. On the molecular level it has been described that VSV

infection leads to activation of caspase-3 and caspase-3-like proteases [124;126;127]. For murine fibroblasts an Apaf1-mediated activation of caspase-9 was observed [200]. Following VSV infection, caspase-9 is activated in simian kidney fibroblast Vero-76 cells [169], HeLa and BHK cells [201]. While for wild type VSV caspase-8 activation has been challenged [169], certain mutant VSV strains exclusively induce apoptosis via the extrinsic pathway employing caspase-8 [139]. In the here presented study, caspase-3 activation was measured to evaluate induction of apoptosis. For the analyzed human cancer cell lines HepG2, A549 and Huh-7 caspase-3 activity markedly increased 24 hours post infection at MOI = 0.1. In concordance, for Vero-76 cells an increase of caspase-3 activity has been reported 2 – 3 hours post infection, though cells were infected with much higher amounts of virus (MOI = 50) [169].

In HeLa cells caspase-8 and -9 activities were reported to be reduced by over-expression of the anti-apoptotic Bcl-2 protein [202]. Furthermore, over-expression of Bcl-2 was shown to inhibit downstream effects of VSV-mediated activation of caspase-3 such as PARP-cleavage and DNA fragmentation [124]. Additionally, the cellular level of Bcl-xL declines in VSV infected Vero-76 cells [169]. These results demonstrate an important role for members of the Bcl-2 family of proteins in virus-host-interaction. In order to investigate the impact of VSV on this protein family, human cancer lines were infected with low MOIs of wild type VSV and subsequently analyzed for levels of proteins associated to apoptotic pathways. In line with earlier results Bcl-2 and Bax protein levels were not altered following VSV infection, whereas the decline for Bcl-xL was not reproducible in these experiments [124;169]. Surprisingly, one member of the Bcl-2 family of proteins, myeloid leukemia cell 1 (Mcl-1) protein, showed a marked decrease of protein levels when cells are infected with wild type VSV (figure 11A). This decrease was not demonstrated to be dependent of the p53 status of the infected cells. Additionally, this decrease is independent of caspase cleavage since general inhibition of caspase activity by the pan-caspase inhibitor z-VAD-fmk was unable to stabilize Mcl-1 protein levels. In contrast, application of MG132 resulted in significant recovery of Mcl-1 protein levels suggesting a role of the proteasome system in Mcl-1 degradation. This is consistent with former results where E1A- or DNA-damage-mediated Mcl-1 destruction could be blocked by application of MG132 or the structurally different proteasome inhibitor epoxomicin [167;203].

Block of general protein biosynthesis by the transcription inhibitor Actinomycin D led to a drop of cellular Mcl-1 levels that displayed a noticeable similarity to VSV-mediated Mcl-1 elimination (figure 11C). Furthermore, in a very recent report it was demonstrated, that the VSV mutant AV1 – that lacks the capacity to efficiently block host cell protein biosynthesis due to a mutation in the viral M-protein – did not influence Mcl-1 levels what is in striking contrast to the wild type virus [129]. Preliminary experiments with HepG2 cells (figure 11D) confirm that AV1 does not influence Mcl-1 protein levels like wt-VSV.

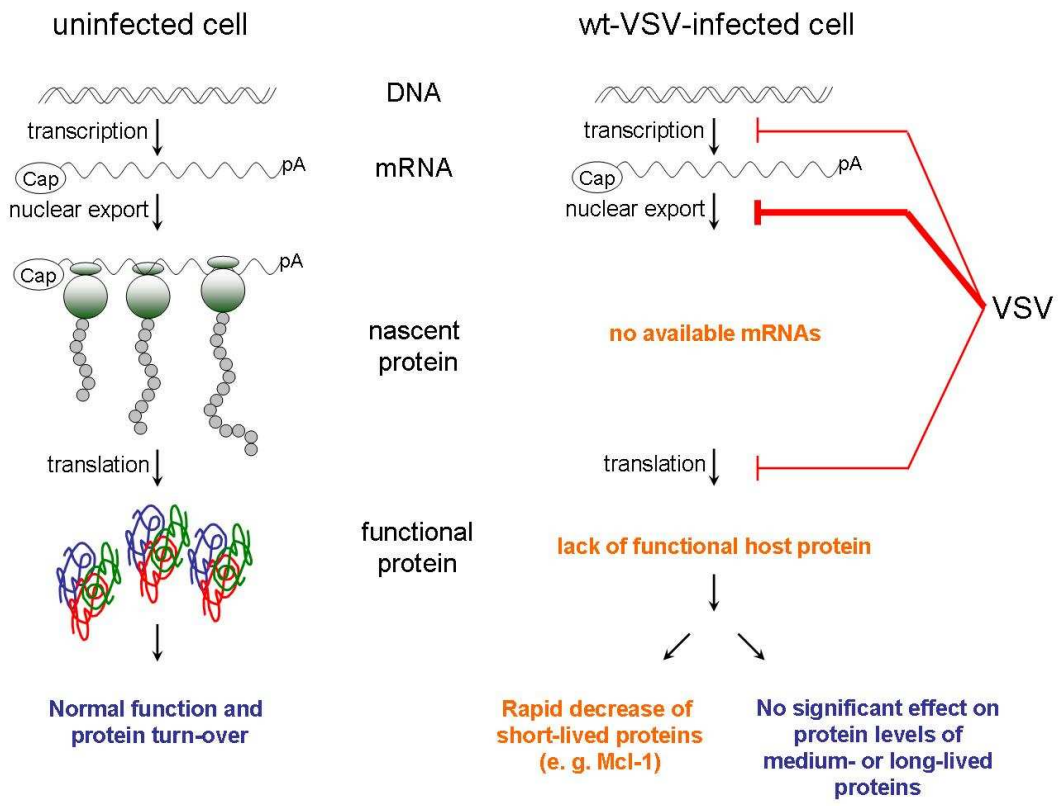

Figure 19. Influence of wt-VSV on host cellular protein biosynthesis. Uninfected cells (left side) perform protein biosynthesis by transcribing genes into mRNA, subsequent ripening, export and translation of these mRNAs in the cytoplasm of the cell. After protein folding and post-translational modifications proteins are turned over in a specific manner. Infection of cells with wt-VSV (right side) is followed by a rapid block of certain steps (so far reported for transcription, translation, and especially mRNA-export) of the host cellular protein production machinery to inhibit actions of anti-viral pathways. This results in lack of new functional host (but not viral) proteins and leads to a detectable decline of short-lived host cell proteins.

In summary, these data provide evidence that VSV infection leads to a rapid decline of Mcl-1 protein based on the combination of blocked *de novo* protein synthesis by VSV and continuous proteasomal degradation of Mcl-1 (figure 19). It has been demonstrated that a Mcl-1 mutant that can not be ubiquitinated is

significantly more stable following VSV infection (figure 14A). This indicates that proteasomal degradation is mediated by the Mcl-1 ubiquitin-E3-ligase MULE/LASU1 [162]. However, the results described here do not exclude the possibility that VSV directly targets Mcl-1 for proteasomal destruction. Furthermore, it could not be evaluated whether GSK-3β is involved in targeting Mcl-1 for ubiquitination. Although it can not be ruled out, a contribution of GSK-3β appears rather unlikely as this enzyme today is primarily known to play important roles in energy metabolism, cell development and body pattern formation [204]. The recently demonstrated link between GSK-3β and the intrinsic apoptotic pathway [164] might hold promise for further investigations including a possible role of GSK-3β in fighting viral infections via Mcl-1 phosphorylation.

The Mcl-1 protein, among Bcl-2, Bcl-xL, Bcl-w and A1 is a member of the pro-survival subgroup of the Bcl-2 family of proteins, although differing in certain structural and functional characteristics from the other proteins. Mcl-1 is situated in the outer mitochondrial membrane and inhibits release of cytochrome c [158;166]. Pro-apoptotic Bim, Bid, Puma and Noxa proteins are known binding partners of Mcl-1 [203;205-209]. The interaction of pro-apoptotic Bak protein and Mcl-1 suggests a scenario where sequestration of the Bak protein by Mcl-1 prevents oligomerization of the pore-forming Bak molecules. However, mechanisms by which Mcl-1 blocks the onset of apoptosis are not yet fully understood and are still under constant debate.

In contrast to other Bcl-2-family members, Mcl-1 is an early-response gene that can be rapidly induced and turned over [166;210;211]. This property makes Mcl-1 an ideal early-response element in the apoptotic signaling cascades, thus playing a protective role against DNA damage, adenoviral infection, growth factor withdrawal, and treatment of cytotoxic agents [167;203;212-217]. Disappearance of Mcl-1 has been reported to contribute to the onset of apoptosis and is provoked by combining blockade of protein synthesis and continued protein degradation [167;203]. Consistently, when cells over-expressing wild type or a stabilized mutant of Mcl-1 were infected with VSV levels of apoptosis induction dropped markedly (figure 14B). This inhibition of apoptosis coincides with elevated levels of viral progeny that is produced within the first 36 hours after infection (figure 14C). Comparable results for virus titers were reported for Jurkat cells over-expressing Bcl-2 [124]. According to the data mentioned, it might be speculated

whether Mcl-1 is a general anti-viral switch that facilitates induction of apoptosis in RNA and DNA virus-infected cells. Based on its short half-life, Mcl-1 is ideal for sensing viral hijacking of the translation machinery. Involvement in anti-viral defense is further supported since Mcl-1 has been reported to be down-regulated by other viruses (Adenoviruses, infectious pancreatic necrosis virus) as well [203;218].

While the role of Mcl-1 in the apoptotic pathways is getting clearer much less is known about its involvement in autophagy. Autophagy is an ancient cellular degradation process for proteins, organelles and cytoplasm, that promotes both cell death and survival and participates in innate and adaptive anti-viral immune responses [219]. Recently, a weak interaction between Mcl-1 and Beclin-1, a Bcl-2 interacting protein that promotes autophagy has been reported [220]. Since VSV was observed (figure 13) and reported to induce autophagy [171;172] it was interesting to investigate a possible contribution of Mcl-1 to autophagy. The over-expression of a stabilized Mcl-1 mutant in VSV-infected cells did not significantly alter the observed state of autophagy compared to infected parental cells indicating that Mcl-1 neither directly nor indirectly is involved in autophagy in VSV-infected cells. The contribution of autophagy to cancer and virus infection is ambivalent. Autophagy was reported to promote or to restrict cancerous development [221;222]. In virally infected cells, autophagy might provide a defense strategy of the infected cell or, in contrast, a viral mechanism to gain access to cellular resources [223;224]. Experiments carried out here can more or less suggest an exclusion of a decisive role for Mcl-1 in VSV-induced autophagy.

In a variety of human hematopoietic and lymphoid cancers, including B-cell lymphoma, chronic lymphocytic leukemia, chronic myeloid leukemia, mantle cell lymphoma and multiple myeloma, Mcl-1 has been shown to be over-expressed [225-230] suggesting a role in cancer development for Mcl-1. Additionally, cells with elevated Mcl-1 levels exhibit a more resistant phenotype to conventional chemotherapeutic treatment than cells with normal Mcl-1 protein levels [173;174]. Thus, several strategies to target Mcl-1 have been described including small molecule inhibitors [209;231-234], anti-sense molecules and RNA interference [235;236]. In this work it was shown that VSV leads to Mcl-1 clearance from infected cells. Therefore, the possibility was investigated that these infected cells are rendered more susceptible to apoptotic stimuli in particular conventional

chemotherapy (Doxorubicin). As a result, combined VSV-virochemotherapy induced apoptosis to a higher extent than VSV or Doxorubicin treatment alone. This result could be observed *in vitro* and *in vivo* (xeno-transplanted nude mice). Based on the results of different apoptosis assays (caspase-3 activation, TUNEL), it can be concluded that the enhanced anti-tumor effect of combined virochemotherapy depends on higher levels of apoptotic cell death. In concordance with former reports applying different Mcl-1 inhibition strategies [209;230-236], this is caused by VSV-mediated sensitization of cancer cells to Doxorubicin treatment.

Several viruses such as alpha and gamma herpesviruses or adenoviruses are known to express homologues of Bcl-2 family members to prevent premature cell death by inhibiting release of cytochrome c from mitochondria [237;238]. A well illustrating example appears to be Adenoviruses that harbors E1B-19k [239]. E1B-19k takes over anti-apoptotic function from Mcl-1 (binding Bax and Bak) that is degraded in consequence of adenoviral E1A expression [203]. It has been reported that VSV produces higher levels of progeny when Bcl-2 protein is stabilized [124]. Additionally, in the here presented work Mcl-1 was shown to be a cellular factor that is degraded following VSV infection. Stabilization of Mcl-1 led to increased VSV titers like demonstrated for Bcl-2 stabilization. Based on the mentioned data, the question might be raised why VSV did not acquire the ability to express a Bcl-2 homologue during evolution as observed in other viruses? VSV infects a wide range of organisms thus lacking a clear host specificity. Expressing an additional gene product like a Bcl-2-homologue on one hand might improve virus amplification to a limited extent. Concomitantly, this might be accompanied by loosing fitness as a longer genome probably will demand more resources (time, energy) to replicate thus reverting the before mentioned positive effect. Importantly, in contrast to Adenoviruses, expression of Bcl-2 or Mcl-1 did not completely abrogate apoptosis while viral replication proceeded. While the adenoviral replication cycle takes about 48 hours, VSV amplifies in less than 24 hours. Therefore, inhibition of apoptosis might be irrelevant for VSV. Taken together with the fact that induction of apoptotic cell death is not required for VSV replication [124], additional suppression of apoptosis by Bcl-2 homologues simply might not tremendously improve viral amplification rates.

This study is the first to describe a physiologically important relationship between VSV and Mcl-1 in apoptosis with implications for cancer therapy demonstrating that Mcl-1 can be down-regulated by VSV infection in a proteasome dependent manner. Furthermore, as the VSV-mediated Mcl-1 decrease can be exploited therapeutically, this study represents a potential advancement in the understanding of oncolytic VSV therapy of human cancers. Though several findings point to protein biosynthesis shut-down to be responsible, further direct signaling to Mcl-1 can not be excluded, and could represent an interesting issue for further studies.

7.3 Outlook

The present work focused on investigations on the interactions of oncolytic viruses with human cancer cell lines. The data provided here demonstrate that application of highly specific DNA endonucleases represents a powerful tool to control replication and perhaps maintenance of oncolytic DNA viruses. Furthermore, cleavage of viral backbones not only allows for selective destruction of oncolytic viruses in normal cells but prevents a later break-through of underlying replication control mechanisms. To test this hypothesis further research on stress response pathways and an involvement of apoptosis in the established concept is necessary.

The natural tumor virus VSV initiates the destruction of the anti-apoptotic Bcl-2 family member Mcl-1 in human cancer cell lines rapidly after infection. Data indicate that the Mcl-1 protein is a key component of the cell's defence against VSV infection. Additionally, the obtained data provide strong evidence that this anti-viral mechanism can be successfully exploited to enhance anti-tumor therapy in combination with conventional chemotherapy *in vitro* and *in vivo*.

Independently of the here presented work, it has to be noted that oncolytic virotherapy – the underlying concept of this thesis – can only be one component of a mosaic of amultimodal therapeutic strategies that might finally achieve to cure cancer. In recent years, simple reliance on oncolytic vectors has been replaced by concepts activating the patient's innate and adaptive immunity against neoplastic lesions by the help of viruses. Thus, pure virotherapeutic approaches might end up as part of a bigger picture, though, having paved the road for novel therapeutics to treat solid human cancers.

8. Literature

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9. Appendix

9.1 List of figures

9.2 Abbreviations

Ad	Adenovirus
APS	ammonium persulfate
ARF	another reading frame
ATP	adenosine triphosphate
ATCC	Amercian Tissue Culture Company
ATP	adenosine triphosphate
AV1	attenuated virus 1 (mutant VSV)
β-gal	β-galactosidase
Bcl-2	B-cell lymphoma 2
bp	base pairs
BSA	Bovine serum albumin
CMV	Cytomegalovirus
CO_2	carbon dioxide
CPE	cytopathic effect
CQ	chloroquine
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides (dATP, dGTP, dCTP, dTTP)
DTT	dithiothreitol
E.*coli*	Escherichia coli
E1A	Adenovirus early region 1A
E1B	Adenovirus early region 1B
EDTA	ethylenediaminetetraacetic acid
eIF2α	eukaryotic initiation factor 2 alpha
EGFP	enhanced green fluorescent protein
EtOH	ethanol
FCS	fetal calf serum
fw	forward
g	acceleration of gravity
h	hour
H2O	water
HCC	hepatocellular carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
hTERT	human telomerase reverse transcriptase
i.t.	intratumoral
i.v.	intravenous
I-*Sce* I	intron-encoded meganuclease from the *Saccharomyces cerevisiae*
IL	interleukin
IRES	internal ribosomal entry sequence
kDa	kilodalton
KRAB	Krüppel associated box
lacZ	β-galactosidase
LB	Luria Broth
LC3	microtubule-associated protein 1 light chain 3
luc	luciferase
M	molar

Mcl-1	myeloid cell leukemia 1
MCS	multiple cloning site
mg	milligram
MHH	Hannover Medical School
min	minute
ml	millilitre
mM	millimolar
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
MOI	multiplicity of infection (= viral particles per cell)
NSCLC	non-small cell lung cancer
OD	optical density
ONPG	ortho-nitrophenyl-β-galactoside
ONYX-015	conditionally replicating Adenovirus of ONYX Pharmaceuticals, Inc.
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
prMin-RGC	p53-dependent promoter
rev	reverse
RNA	ribonucleic acid
RNase	ribonuclease A
RNAi	RNA interference
rpm	rounds per minute
RT	room temperature
S.D.	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamid gel electrophoresis
shRNA	short-hairpin RNA
TAE	Tris/acetate/EDTA buffer
TBS	tris-buffered saline
TBST	tris-buffered saline plus Tween20
TE	Tris/EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	2-Amino-2,2-hydroxymethylpropan-1,3-diol
TUNEL	deoxynucleotidyl transferase (TdT)- mediated dUTP nick end labeling
UTR	untranslated region
VSV	wild type Vesicular Stomatitis Virus
VSV-G	glycoprotein of the Vesicular Stomatitis Virus
VSV-L	Vesicular Stomatitis Virus polymerase
VSV-M	matrix protein of the Vesicular Stomatitis Virus
VSV-N	nucleocapsid protein of the Vesicular Stomatitis Virus
VSV-P	phosphoprotein of the Vesicular Stomatitis Virus
v/v	volume per volume
w/v	weight per volume
w/o	without
wt	wild type
YFP	yellow fluorescent protein

9.3 Acknowledgements

Without the help and support of others this work would not have been possible in the here presented form. A multitude of people provided support, ideas, research and their invaluable time! Therefore, I feel indebted to everyone who helped me to bring my thesis alive without getting lost along this long way.

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Pursuing a PhD thesis sometimes can make one feel being exhausted and panic-stricken, or like being lost in a forlorn mission. On the other hand, a simple band in a western blot or agarose gel can provide energy for weeks. Now that I write these words, I passed this emotional roller coaster. So, I truly want to thank the ones who encouraged me to stick to my projects and go ahead. Verena and Silke, you've really enriched my time at the MHH. Thanks for providing your professional help, for rescuing me from lack of material, for the coffee session, for playing badminton, for setting up the student's kolloq, and for reading my work!

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9.4 Curriculum Vitae

Personal data

Name	Peter Schache
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Nationality	German

Education

2005 – 2008	**PhD thesis** as scholarship holder of the "Stiftung der Deutschen Wirtschaft" (sdw; Foundation of the German Economy) at the Department of Gastroenterology, Hepatology and Endocrinology at Hannover Medical School with Prof. Dr. Stefan Kubicka and Dr. Florian Kühnel
2006 – 2007	**Additional business studies for natural scientists**, Distance University Hagen, First Diploma
1997 – 2004	**University studies in biochemistry/molecular biology** at FSU Jena and Universidad de Granada, Spain Diploma thesis „Analysis of the Mechanism of the Humane Topoisomerase I-DNA-Complex-Mediated Apoptosis"
1996	**University entrance exam** at the special school for natural sciences Carl-Zeiss-Gymnasium (Abitur)

Community Service German Red Cross, Camburg (10/1996 – 11/1997)

Research working experience

04/2002 – 10/2002	Institute for Molecular Biotechnology, IMB-Jena, Dept. „Molecular Biology", Jena
05/2001 – 10/2001	Bayer-Yakuhin, Ltd., Kyoto, Japan
06/1999 – 09/2000	Quantifoil Micro Tools GmbH, Jena
05/1998 – 07/1998	Hans-Knöll-Institute for Research on Natural Substances, Jena
06/1997 – 12/1997	Medical Center of the Friedrich-Schiller-University Jena, Dept. „Molecular Cell Biology", Jena

9.5 List of publications

9.5.1 Scientific journal articles

Schache,P., Gürlevik,E., Strüver,N., Woller,N., Malek,N., Zender,L., Wirth,T., Kühnel,F., Kubicka,S., (**2009**). VSV VIROTHERAPY IMPROVES CHEMOTHERAPY BY TRIGGERING OF APOPTOSIS DUE TO PROTEASOMAL DEGRADATION OF MCL-1 (in press).

Gürlevik,E., Woller,N., Schache,P., Malek,N., Wirth,T., Zender,L., Manns,M., Kubicka,S., Kühnel,F. (**2008**). P53-DEPENDENT ANTIVIRAL RNA-INTERFERENCE FACILITATES TUMOR-SELECTIVE REPLICATION OF ONCOLYTIC VIRUSES (submitted).

Gürlevik,E., Schache,P., Strüver,N., Wirth,T., Zender,L., Manns,M.P., Kubicka,S., Kühnel,F. (**2008**). THE ADENOVIRAL VECTOR ADSENSOR-P53T PROVIDES A HIGHLY SELECTIVE TOOL FOR ONCOLYTIC VIROTHERAPY OF TUMORS WITH ACTIVATED TELOMERASE AND ABERRATIONS IN P53-TRANSCRIPTIONAL ACTIVITY (manuscript).

Søe K., Rockstroh A., Schache P., Grosse F. (2004). THE HUMAN TOPOISOMERASE I DAMAGE RESPONSE PLAYS A ROLE IN APOPTOSIS. DNA Repair (Amst). **2004** Apr 1;3(4):387-93.

9.5.1 Poster presentations

Schache,P., Gürlevik,E., Strüver,N., Manns,MP., Kubicka,S., Kühnel,F.: GENERATION OF A CONDITIONALLY REPLICATING ADENOVIRUS HARBORING A P53-DEPENDENT SELF-DESTRUCTION SWITCH FOR TREATMENT OF P53-ALTERED TUMORS. EASL, Milan, **2008**.

Schache,P., Goez,A., Gürlevik,E., Strüver,N., Manns,MP., Kubicka,S., Kühnel,F.: GENERATION OF A CONDITIONALLY REPLICATING ADENOVIRUS HARBORING A P53-DEPENDENT SELF-DESTRUCTION SWITCH FOR TREATMENT OF P53-ALTERED TUMORS. DGVS, Berlin, **2008.**

Gürlevik,E., Schache,P., Strüver,N., Manns,M., Kubicka,S., Kühnel,F.,: THE ONCOLYTIC ADENOVIRUS AD-I-REP2 UTILIZES P53-DEPENDENT RNA INTERFERENCE FOR TUMOR-SELECTIVE REPLICATION. DGVS, Berlin, **2008**.

Gürlevik,E., Schache,P., Strüver,N., Manns,M., Kubicka,S., Kühnel,F.,: THE ADENOVIRAL VECTOR ADSENSOR-P53T PROVIDES A HIGHLY SELECTIVE TOOL FOR ONCOLYTIC VIROTHERAPY OF TUMORS WITH ACTIVATED TELOMERASE AND ABERRATIONS IN P53-TRANSCRIPTIONAL ACTIVITY. DGVS, Berlin, **2008**.

Gürlevik,E., Schache,P., Strüver,N., Manns,M., Kubicka,S., Kühnel,F.,: THE ADENOVIRAL VECTOR ADSENSOR-P53T PROVIDES A HIGHLY SELECTIVE TOOL FOR ONCOLYTIC VIROTHERAPY OF TUMORS WITH

ACTIVATED TELOMERASE AND ABERRATIONS IN P53-TRANSCRIPTIONAL ACTIVITY. EASL, Mailand, **2008**.

Woller,N., Schulte,B., Milewska,D., Schache,P., Gürlevik,E., Manns,MP., Kubicka,S., and Kuhnel,F.: TELOMERASE DEPENDENT CONDITIONALLY REPLICATING ADENOVIRUS (hTERT-AD) EXPRESSING CARexPTDS DISPLAYS ENHANCED ONCOLYTIC PROPERTIES IN NONPERMISSIVE TUMOR CELLS. EASL, Wien, **2006**.

9.6 Declaration

Herewith, I do confirm that I have written the present PhD thesis myself and independently, applying only the indicated materials and sources, and that I have not submitted it at any other university worldwide.

Hannover, December 18th 2008

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