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MultiHit Mice as a Tool to Identify Cooperating Ras Effector Pathways in Tumorigenesis

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1. Abstract

Cancer is the leading cause of death in the world. Over the last years, a lot of effort has been made to investigate human cancers and to design therapeutic drugs. Mouse models are an important asset in identifying gene mutations related to human diseases.

Cancer formation is a multi step process. Cells that have acquired several genetic and epigenetic changes are positively selected and ultimately induce tumor formation. However, mouse models are usually restricted to only one genetic change that can be investigated at a time. The examination of two or more hits requires complex breeding schemes. Even if the resulting mice are viable, this situation does not represent the selection process in cancer formation.

Being aware of these difficulties the MultiHit mouse model was designed. It contains three potential oncogenes or gene mutations previously related to cancer. These three genes are cloned in off orientation. Upon Cre-activation the modules start to flip between on and off until the Cre-activity ceases. The flipping leads to one of eight possible combinations of activated hits differing from cell to cell. Cells with cooperating hits will be selected and eventually be transformed into a tumor cell.

In this thesis the Ras effector (RasE) MultiHit mouse is characterized. Three mutations of the Ras protein are known to activate exclusively the MAPK, Ral or PI3K pathway, respectively. These mutations were used to identify cooperation between these three pathways.

Skin appendage tumors, lung adenocarcinomas and pancreatic ductal adenocarcinomas were observed after Cre-activation. The activation events in skin tumors and lung tumors were analyzed by in situ hybridization. More than 98% of all skin tumors had all three modules switched on. Only one tumor showed single activation of the PI3K pathway. In contrast, several different combinations were found in lung tumors. Again, tumors with all three modules activated were most frequent but tumors with PI3K only and combinations of MAPK/Ral and MAPK/PI3K were found as well. Interestingly the combination Ral/PI3K was never found which suggest that these two pathways together inhibit tumor formation.

The MultiHit model can be used answer different questions in cancer research and might represent a test system to evaluate newly identified mutations in human tumors.

2. Zusammenfassung

Krebserkrankungen sind mittlerweile die häufigste Todesursache weltweit. In den letzten Jahren wurde an verschiedendsten Krebsarten geforscht und eine Vielzahl von neuen Medikamenten entwickelt und getestet. Genetische Mausmodelle sind eines der wichtigsten Werkzeuge geworden, um Gene und Mutationen, die in Zusammenhang mit der Entstehung von Krebs stehen, zu identifizieren.

Die Entstehung von Krebs ist ein mehrstufiger Prozess. Zellen die mehrere genetische und epigenetische Veränderungen erfahren haben und dadurch einen Wachstumsvorteil bekommen, werden selektioniert und können einen Tumor bilden. Bis jetzt waren Mausmodelle auf die Untersuchung einer einzelnen, genetischen Veränderung beschränkt. Um mehrere Veränderungen zu erforschen, müssen diese Mäuse aufwendig gekreuzt werden. Auch wenn diese Mäuse überlebensfähig sind, spiegelt dieser Ansatz nicht den natürlichen Selektionsprozess bei der Krebsentstehung wider.

Um diese Schwierigkeiten zu umgehen wurde das MultiHit Mausmodell entwickelt. Es enthält drei potentielle Onkogene oder Genmutationen, die im Zusammenhang mit Krebs gefunden wurden. Diese drei Gene werden so kloniert, dass anfangs kein Genprodukt davon gemacht werden kann. Bei Aktivierung der Cre-Rekombinase springen die drei Gene zwischen dem inaktiven („off“) und dem aktiven Zustand („on“) hin und her. Lässt die Cre-Aktivität nach, stellt sich in jeder Zelle eine der acht möglichen Kombinationen ein. Zellen mit kooperiernden Veränderungen werden selektioniert und können letztendlich einen Tumor bilden.

Im Folgenden wurde die Ras Effector (RasE) MultiHit Maus charakterisiert. Es gibt drei Ras Mutationen, die jeweils spezifisch nur einen Signaltransduktionsweg anschalten. Diese drei Mutanten wurden verwendet um Kooperationen zwischen dem MAPK, dem Ral und dem PI3K Signalweg bei der Tumorentstehung zu identifizieren.

Tumore der Hautanhangsdrüsen, Adenokarzinome in der Lunge und ductuläre Adenokarzinome im Pankreas (Bauchspeicheldrüse) wurden nach Cre-Aktivierung beobachtet. Die Aktivierung der drei Expressionsmodule wurde mittels in situ Hybridisierung untersucht. Mehr als 98% aller Tumore der Hautanhangdrüsen hatten alle 3 Module gleichzeitig aktiviert. Nur ein einziger Tumor hatte den PI3K Signalweg alleine eingeschaltet. Bei den Lungentumoren wurden verschiedene Kombinationen gefunden. Tumore mit Aktivie-

rung von allen Modulen waren auch hier am häufigsten, aber auch PI3K alleine und Kombinationen von MAPK/Ral und MAPK/PI3K führen zur Tumorbildung. Interessanterweise wurde die Kombination von Ral/PI3K kein einziges Mal gefunden, was drauf hinweist, dass diese Kombination die Entstehung von Tumoren sogar inhibiert.

Mit dem MultiHit Model können verschiedenste Fragestellungen der Krebsforschung beantwortet werden und es kann dazu verwendet werden um neu identifizierte Mutationen zu untersuchen.

3. Introduction

3.1. Cancer: The most common and dreadful disease

Cancer is the most common cause of deaths world wide. In 2004 it has claimed 7.4 million deaths all around the world. This is nearly 13% of all deaths worldwide. In the United States cancer is the second most frequent cause of death. Only cardio vascular diseases draw more victims every year. It is estimated that in the US there will be 1.5 million new cancer cases and more than 500.000 cancer related deaths in 2010 alone[1].

Cancer is defined as a class of diseases where a group of cells breaks out of the normal growing habit and starts to grow in an uncontrolled manner. Tumors that invade adjacent tissue or spread through blood and lymph vessels are called malignant tumors. If tumor cells detach from the primary tumor and float through the blood system to another site in the body it is called metastasis. Usually lymph nodes next to the primary tumor are affected but also lung, bone, liver and brain are common metastatic locations for solid tumors[2]. In contrast benign tumors do not have these abilities and therefore they are much less dangerous to the body.

Generally cell growth and division are tightly regulated to avoid formation of abnormal tissue. Over the years two main groups of genes regulating proliferation and cell death were identified. Tumor suppressor genes protect the cell from uncontrolled growth and can promote apoptosis in the event of aberrant DNA replication. The tumor suppressor p53 and the retinoblastoma protein are the best known examples in this group. The loss of a tumor suppressor impedes important control mechanisms in the cell and can represent the first step in transforming a cell to a cancer cell[3]. In contrast, oncogenes are dangerous for the cell when they are mutated or expressed at high levels. Oncogenes derive from normal genes called proto oncogenes. They protect the cell from premature apoptosis and promote cell growth and differentiation in a tightly controlled manner. However, aberrant activation can set the foundation for transforming a cell[4].

Experiments in the early nineties have shown that tumor formation requires several genetic and epigenetic changes (hits) that occur stochastically in a given tissue[5,6]. Initial hits might lead only to increased cell division and hyperplasia. A higher division rate in a given

tissue increases the probability of new mutations to occur. Similar to the Darwinian process of evolution hits that cooperate with the initial change are positively selected and can eventually lead to tumor formation. The mutation rate of human genes is approximately 2.0 x10^{-7}/gene/division[7]. It is believed that 5 to 7 genetic changes are needed to transform a human cell into a tumor cell. The given mutation rate makes it very unlikely for a cell to accumulate several hits in cancer relevant genes. It may also explain the correlation between the incidence of cancer and age. However, if one of the first hits influences DNA damage control or related mechanisms, the probability of developing cancer rises dramatically. This effect is also known as mutator phenotype in early tumor cells[8].

The loss of a tumor suppressor or the activation of an oncogene opens the door for more genetic and epigenetic changes. Cancer formation in humans usually takes decades which gives a hint to the complexity of the processes required. This multi step process can be view as a modified Darwinian selection. Cells with higher proliferation or with less DNA damage control are more prone to produce offspring with another genetic change[6].

The analysis of a plethora of different human tumors has shown that a cell has to achieve several new capabilities in order to be fully transformed. Six capabilities are proposed to be shared by almost all tumor types[6].

1.) Self-Sufficiency in growth signals

2.) Insensitivity to antigrowth signals

3.) Evading apoptosis

4.) Limitless replicative potential

5.) Sustained angiogenesis

6.) Tissue invasion and metastasis

3.2. The role of Ras GTPases in Cancer

GTPases are enzymes with hydrolytic activity to bind and hydrolyze guanosine triphosphate (GTP). These proteins can be divided into to monomeric and hetero trimeric GTPases[9] . They have functions in signal transduction, control of cell growth and proliferation and act usually as molecular switches.

Monomeric GTPases are also called small GTPases because of their molecular weight of about 21kD. The Ras superfamiliy makes up for the biggest group of small

GTPases and is divided in at least 8 subfamilies called Ras, Rho, Rab, Rap, Arf, Rheb, Rad and Rib[10]. This list is not completed as there are still small GTPases found that do not fit into one of these subfamilies[11].

These proteins can act as molecular, binary switches for all kinds of cellular signaling. Ras proteins can be an inactive and in an active conformation. In the inactive state, Ras is bound to GDP, while in the active state it is bound to GTP. Guanine nucleotide exchange factors (GEFs) are able to load monomeric GTPases with GTP to activate them. Cellular events like cell division or proliferation have to be tightly regulated. The intrinsic GTPase activity is very low and the GTPase would be active for an undefined amount of time. Therefore GTPase activating proteins (GAPs) can stimulate the intrinsic GTPase activity to end the signal transduction. However, it was found that in more than 20% of all human tumors Ras is point mutated at the amino acid residue 12 or 61 and less frequently at residue 13. Structural analysis of the Ras protein showed that an amino acid exchange in these sites impedes the GTPase activity completely. GAPs are not able to inactivate the Ras signaling any more. Uncontrolled Ras signaling can lead to the transformation of the affected cell (Figure 1).

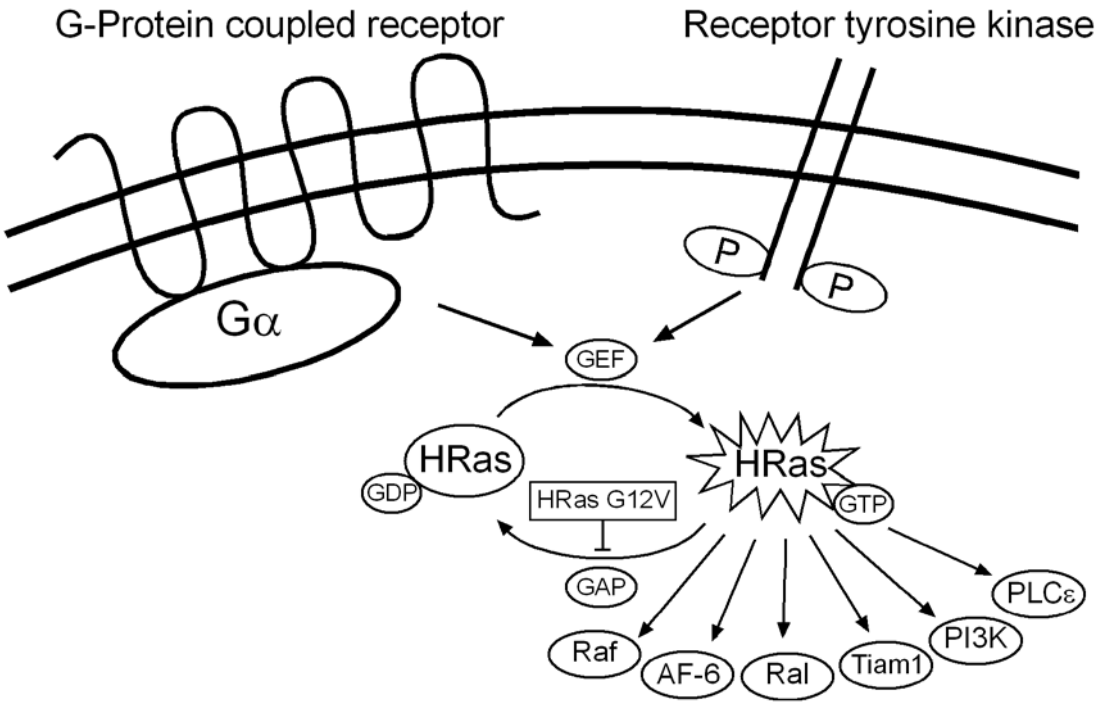


Figure 1: Ras effector pathways. Extracellular signal received and transmitted by G-Protein coupled receptors or receptor tyrosine kinases activate and recruit guanosine exchange factors (GEFs). Upon Ras activation it can activate several effector pathways. The G12V mutation impedes the deactivation of Ras by GTPase activation proteins (GAPs).

In the human genome there are three *Ras* genes encoding four different proteins. The proteins are HRas (Harvey rat sarcoma viral oncogene homolog), NRas (neuroblastoma rat sarcoma viral oncogene homolog) and KRas 1 and 2 (Kirsten rat sarcoma) which are two splice variants of the *KRas* gene. These four proteins are similar in structure and function[12]. Hydrophobic residues like farnesyl or palmitoyl anchor the Ras GTPases to the cytoplasmic face of the cell membrane. Ras proteins can be activated by extracellular signals received by receptor tyrosine kinases (RTK) or G-Protein coupled receptors. When activated, these receptors recruit GEFs to the membrane to activate Ras. Ras proteins have an effector loop for binding and activating downstream targets. Three pathways have turned out to be most important[13].

1.) The MAPK/Raf pathway

Figure 2: The MAPK/Raf pathway. Activated Ras can activate the Raf/MEK/Erk signaling cascade.

Upon activation of Ras the conformation of the effector loop changes and recruits the GTPase Raf (MAPKKK) to the membrane. Raf becomes active and phosphorylates a second kinase, called MEK (MAPKK). MEK is known to be a dual specificity kinase. It phosphorylates serin/threonine and tyrosine residues. MEK phosphorylates the extracellular signal-regulated kinases 1 and 2 (Erk1/2). The two activated Erks, in turn, phosphorylate a plethora of molecules that regulate cellular processes like cell growth and differentiation[14].

2.) The Ral pathway

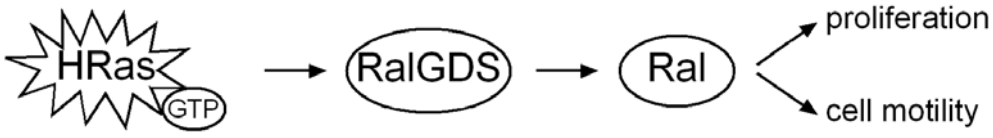


Figure 3: The Ral pathway. Activated Ras can activate the RalGDS/Ral signaling cascade.

Ral-A and Ral-B are Ras related GTPases with 58% sequence identity[15]. Upon Ras activation a Ral specific GEF is recruited to the membrane. Upon binding to Ras its conformation changes and enables RalGDS to activate the two Ral proteins. Ral-A and Ral-B have influence on the activity of Cdc42, Rac and other proteins important for cell motility and cytoskeleton organization[16].

3.) The PI3K pathway

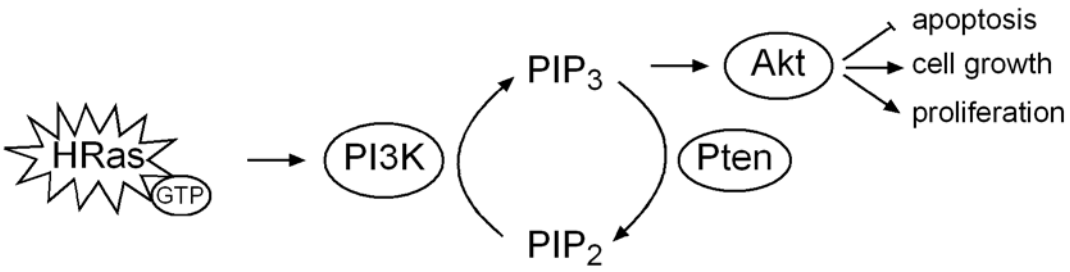


Figure 4: The PI3K pathway. Activated Ras can activate the PI3K/Akt signaling cascade..

The third main effector pathway activated by Ras is the phosphatidylinositol 3-kinase (PI3K) pathway. PI3K adds a third phosphate group to phosphadityl inositol-(4,5) diphosphate (PIP_2). The phosphatase Pten is the antagonist of PI3K because is cleaves the 3´-phosphate of PIP_3[17].

Similar to the mechanisms described before, activated Ras recruits PI3K to the membrane where it comes in near vicinity to its substrate PIP_2. Many proteins, like Akt (also called protein kinase B), can bind PIP_3 with a pleckstrin homology domain. Once phosphorrylated and tethered to the membrane Akt activates several proteins that have effects on cell survival and cell proliferation. Hence, it can stimulate the increase of the cell volume[18].

A lot of effort has been made to dissect the role of different Ras effector pathways. Mutation experiments of the Ras effector loop revealed several amino acid residues crucial for the interaction with certain effector proteins. The mutation of S35 in the effector loop exclusively activates the MAPK pathway[19]. Activation of other effector pathways is not possible anymore. The G37 mutation exclusively activates the Ral pathway[20]. The PI3K pathway is activated by the C40 mutation[21]. These three mutations display a valuable tool for the investigation of the numerous Ras effector pathways.

3.3. Lung cancer

Lung cancer is the deadliest form of all cancer related diseases. About 29% of male and 26% of female cancer death are related to lung cancer. In the year 2010 222.000 new lung cancer cases and 157.000 lung cancer related death are estimated in the US alone[1].

Two forms of lung cancer make up for more than 97% of human cases[22]. The non-small cell carcinomas (NSCLC) can be divided in three subgroups: the squamous cell lung carcinoma, adenocarcinoma and large cell lung carcinoma[23]. These three subtypes account for 80% of all lung cancer cases. Small cell carcinomas (SCSL) are responsible for approximately 17% of all lung cancers[24]. Carcinoid tumors[25], sarcomas[26] and other unspecified lung tumors are only a minority of new cases each year.

Smoking is by far the biggest contributor to lung cancer. About 87% of all lung cancer cases in the US are caused by the inhalation of carcinogenic substances in cigarette smoke[27]. 60 different substances that contribute to the carcinogenic potential of cigarette smoke have been identified by now[28]. The lifetime risk among smokers to develop lung cancer is about 17.2% for male and 11.2% for female smokers. That is dramatically higher compared to the nonsmoker risks of 1.4% and 1.3%, respectively[29].

Other risk factors for lung cancer are the inhalation of radon gas[30], contact with asbestos[31] and some viral infections[32].

3.4. Mouse models for lung cancer

Intensive genetic studies on thousands of lung cancer patients have revealed several novel mutations related to lung cancer. At the same time new ways were discovered to manipulate the mouse genome and use transgenic mice as models for human diseases.

In 1975 Shimkin and Stoner discovered that several inbred mouse strains differ in their susceptibility to chemically induced lung cancer[33]. A polymorphism in the *K-Ras* gene, found only in the most susceptible mice, is believed to influence the sensitivity to lung cancer[34]. Mice carrying the *K-Ras* G12D mutation were later shown to develop early onset lung cancer[35].

Besides chemical models there are different genetic approaches to investigate lung cancer in mice. The use of classical knock out models is limited as some tumor suppressor

knock outs are embryonic lethal. Other mouse strains with not lethal knock outs of tumor suppressors like p53, p16 and p19 usually succumb to tumors other than lung tumors[36].

Conditional or inducible expression of mutated alleles in specific tissues circumvents the formation of other tumors. One example is a mouse that has a Lox-Stop-Lox cassette in front of the *K-Ras* G12D allele. This mouse develops normally because the mutated allele is not active and one functional *KRas* copy is sufficient for embryonic development[37]. After inhalation of adenovirus particles expressing Cre-recombinase the Lox-Stop-Lox cassette is excised and the mutant allele becomes active[38]. This approach enables the investigation of lung tumor initiation and progression without the influence of other tumors.

3.5. The MultiHit concept

Common cancer mouse models work with knock-out or knock-in vectors where a single gene is mutated or turned off. However, only one genetic change can be investigated in these models. To analyze cooperation between two different hits these mice have to be crossed according to complex schemes. This is laborious and time consuming. It is hardly possible to investigate the cooperation between three hits because several breeding generations would be needed and resulting mice are possibly not even viable. Most importantly, the forced introduction of mutations does not mimic the evolution process of cancer that selects the most potent cooperating hits.

Being aware of these difficulties, the MultiHit mouse model was established. This model harbors a MultiHit BAC transgene that allows the randomized introduction of several hits in a given tissue. Cells with cooperating hits should be positively selected and give rise to tumors (Figure 5).

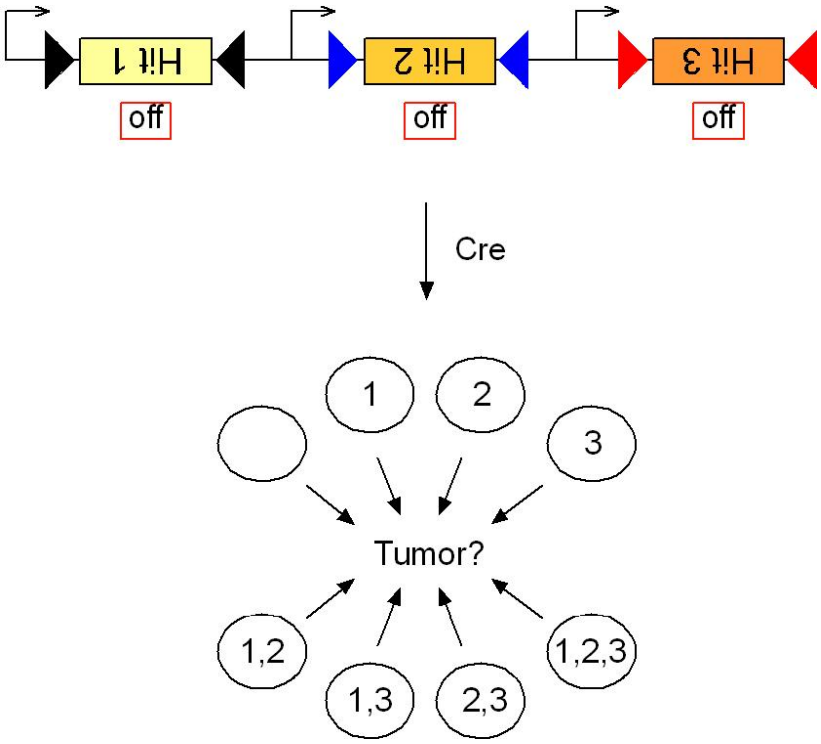


Figure 5: Principle of the MultiHit tumor mouse model. The scheme shows a MultiHit construct for three hits (upper drawing). The hits can represent an expression unit for an oncogene or an expression unit for the knock-down of a tumor suppressor gene. The expression units are not active since they are cloned in an inverted (off) orientation behind a CAGGS promoter (arrow). Each expression unit is flanked by loxP sites (triangles) that allow Cre-mediated activation (flipping) of the expression units. Upon transient induction of Cre-activity, the three expression modules flip forth and back until Cre-activity ceases. Then, they remain either in on- or in off-orientation. Cre-induction in a mouse harboring the MultiHit construct results in randomized activation of expression units creating eight genotypically different cell types (lower drawing). Combinations of hits that have a synergistic effect in tumorigenesis will be positively selected and can lead to tumor formation.

The MultiHit strategy can be employed in different ways to answer biological questions. The "downstream effector selection" approach has been used to identify requirement and cooperations between the MAPK, Ral and PI3K pathways activated by oncogenic Ras in different cancers. Oncogenic mutations that constitutively activate Ras proteins are commonly found in human tumors and have been used for the generation of cancer mouse models. Oncogenic Ras activates several downstream effector pathways. Among them, the MAPK, PI3K and Ral pathways are considered to be most important for Ras-induced tumor formation. However, recent data have suggested that the requirement for Ras effector pathways in oncogenic transformation is cell type specific[39].

We have established RasE (Ras effector) MultiHit mice to identify cooperating Ras effector pathways in cancer formation. The MultiHit BAC for RasE mice contains gene modules for three different H-RasV12 alleles (S35, G37, C40). These mutants carry not only the

oncogenic V12 mutation but also individual mutations in the HRas effector loop that allow selective activation of the MAPK (S35), Ral (G37) and PI3K (C40) pathways. Therefore, Cre induction gives rise to eight cell types with differential activation of Ras effector pathways. The three expression modules of the MultiHit construct will be called **M** (MAPK), **R** (Ral) and **P** (PI3K) to simplify the following explanations.

3.6. BAC technology for the assembly of the MultiHit construct

Bacterial plasmids can be used to clone and amplify heterologous pieces of DNA. The size of inserts is limited to several kilo bases. Larger DNA fragments are instable or impossible to ligate into the plasmid. Since the genetic mapping of whole genomes requires the analysis of thousands of DNA fragments, methods to handle bigger pieces of DNA were needed. Murray and Szostak described in 1983 the first yeast artificial chromosome (YAC)[40]. It is a linear, 55kb long plasmid containing all necessary sequences for replication and preservation in yeast. The three key features of a YAC are the centromeres, telomeres and replication origins. Together this ensures stable duplication and segregation. YACs can be used to clone fragments from 100 to 3000kb. However it is difficult to purify their DNA because the shape is very similar to normal yeast chromosomes and they have been found to be not to stable over many generations.

These problems led to a further search for alternatives to clone large DNA fragments. Bacterial artificial chromosomes (BACs) were the next logical step. The identification of several genes in the low copy F-plasmid led to the production of the first BAC[41]. It consisted of the regulatory genes *oriS*, *repE*, *parA*, and *parB*. The *oriS* and *repE* genes mediate the unidirectional replication of the F-plasmid while *parA* and *parB* maintain copy number at low level (one or two per E. coli genome). A selection marker, like an antibiotic resistance, enables the selection of cells that have taken up a plasmid after transformation. The usual insert size for a BAC ranges from 150 to 300kb, but can reach up to 700kb. BACs are stable for hundreds of generations and easy to purify with standard kits.

Recombineering stands for recombination mediated genetic engineering and is a method of manipulating BAC DNA[42]. ET recombination is homologous recombination in E. coli mediated by the phage protein pair *RecE/RecT*[43]. Inserts have to be flanked by homology arms with a minimum length of only 9bp[44].

The MultiHit construct was cloned in a two step process. It could not be assembled by standard cloning techniques because it consists of several repetitive elements. A two step cloning protocol was developed that allowed rapid assembly of BAC constructs for three hits (Figure 6).

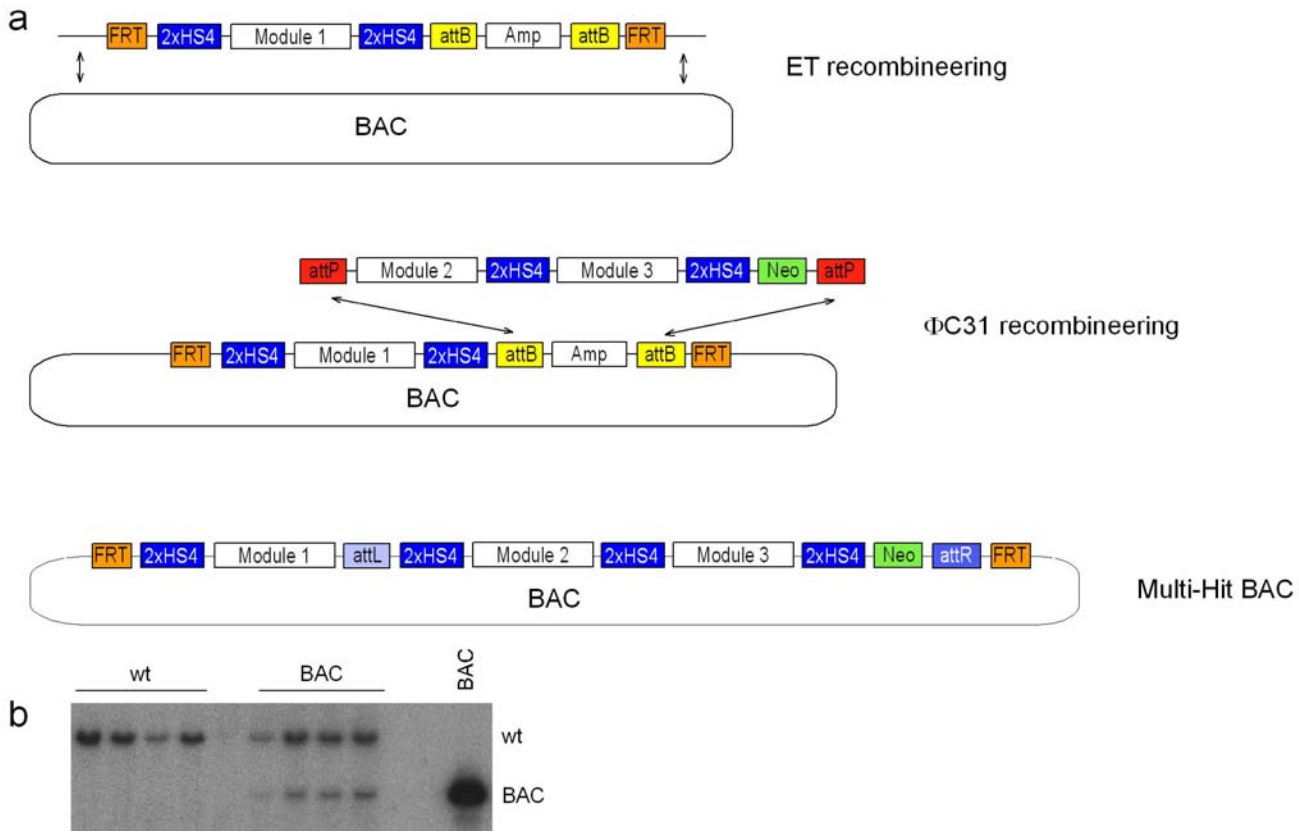


Figure 6: Scheme of the recombineering strategy to generate large MultiHit BAC constructs. (a) The first expression cassette, containing only module 1, was integrated in the Collagen 1a1 BAC via ET recombination. In a second, ΦC31 recombinase mediated, step the expression cassette containing module 2 and 3 was integrated. (b) Southern blot analysis using F1 offsprings from the RasE transgenic founder mouse Tg(RasE)290Biat and a probe matching the Collagen 1a1 locus. Tail DNA from four wild-type and four BAC-transgenic mice was used after PCR pre-screening. The BAC used for oocyte injection was loaded as control. This analysis demonstrates single copy integration.

In a first ET recombineering step, a vector containing expression module 1 is integrated via homologous recombination into a BAC backbone. A standard integration site in an open chromatin region of the Collagen 1a1 BAC was used. Module 1 is flanked by FRT recombination sites and chicken 2xHS4 insulator sequences[45,46] that should avoid promoter interference and promoter suppression effects from neighboring expression units. Together with the insulated module 1, an ampicillin resistance gene (Amp) flanked by ΦC31

attB recombination sites, is integrated. Therefore, recombinants can be selected for ampicillin resistance. In a second step, a vector containing expression modules 2 and 3 is integrated into the BAC via ΦC31 attP recombination[47]. This recombination event replaces the ampicillin resistance gene for the incoming sequence that contains a neomycin resistance gene (Neo). Positive recombinants can be screened for loss of ampicillin resistance and gain of kanamycin resistance. The resulting construct contains all three expression modules flanked by chicken 2xHS4 insulators. The whole locus is flanked by FRT recombination sites that allow FLP-mediated deletion of the whole construct which might be used to address the question of oncogene addiction[48]. The attL and attR sequences are generated by ΦC31 recombination between attP and attB sites. The generation of these two sites makes the recombination irreversible and therefore a valuable tool for the manipulation of large DNA fragments.

The transgenic MultiHit mouse was made via pronuclear injection. The completely assembled and Not1 linearized BAC was injected into the maternal pronuclei of fertilized oocytes. These oocytes were implanted into the uterus of a pseudo pregnant foster mother. The resulting founder mice were back crossed to Bl6 background to check for germline integration. The F1 offspring was analyzed with PCR. DNA from four wild type and four transgenic mice was isolated and used for Southern blot. A Collagen-A probe was used to determine if the mice have more than one BAC copy in their genome. Single copy integrants show a wild type band that is twice as strong as the BAC band because of the two collagen alleles. All four analyzed mice are single copy integrants. Single copy integration is essential since the presence of multiple copies would increase the background activation of Ras effector pathways in tumors.

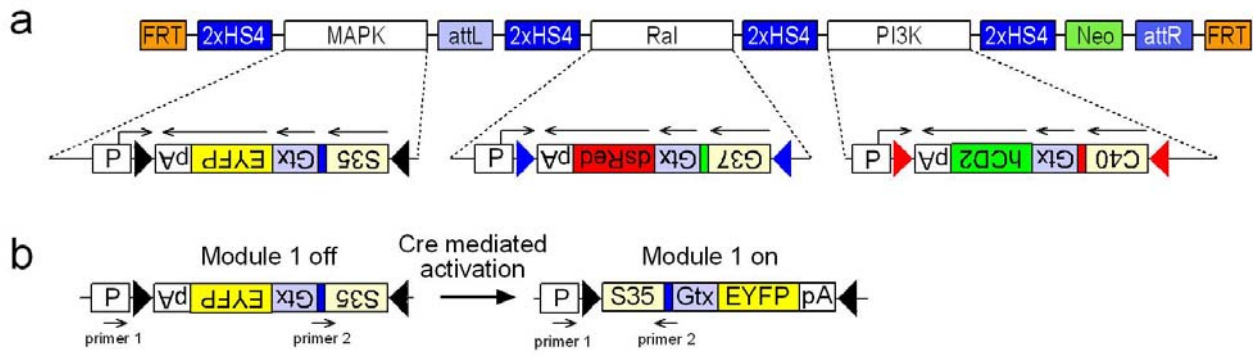


Figure 7: The RasE (Ras effector) MultiHit mouse model. (a) Scheme of the RasE MultiHit BAC construct (upper drawing) and detailed schemes of the individual expression modules (lower drawings). (b) Primer orientation in module 1 on and off. In the off orientation Primers 1 and 2 cannot form a PCR product because they are facing in the same direction. After Cre-mediated activation of the module a PCR product can be amplified.

Figure 7 shows the RasE MultiHit construct in detail. The expression modules consist of a CAGGS promoter (P), the HRasV12 effector mutants S35, G37 and C40 for activation of the MAPK, Ral and PI3K pathways respectively, an IRES (Gtx), a reporter gene (EYFP, dsRed, hCD2t) and a SV40 polyadenylation signal (pA). The Ras effector-IRES-reporter-pA sequences are flanked by heterotypic loxP sites (black triangles = canonical loxP, blue triangles = lox5171, red triangles = lox2272)[49,50]. Heterotypic loxP sites were used to avoid Cre-mediated deletions because they are unable to recombine with loxP sites of another type. Additionally the three modules are cloned in an inverse (off) orientation behind the promoter. Therefore, the Ras effector genes are not expressed in the RasE mouse model but can be activated by Cre-mediated inversion.

Activation of the three modules can be followed by PCR using unique signature sequences downstream of the Ras effector mutants (blue, green and red bars in Figure 3b). When the module is in off orientation primer 1 and 2 are facing in the same direction. Therefore no PCR product can be made. However, after Cre-mediated inversion of the module the primers can amplify a PCR product.

4. Aim of the thesis

RasE RosaCreER^{T2} mice develop several different tumors between 9 and 12 weeks after Tamoxifen injection. The most consistent tumors types were skin appendage tumors, lung tumors and pancreatic ductal adenocarcinomas. However, it was not know which combinations of the three expression modules contribute to these tumors.

The aim of the thesis is the determination of activation events needed to induce tumorigenesis in different organs. The activation of the three modules should be investigated on DNA, RNA and protein level. Southern blot and certain PCR strategies can be used to determine the state of the three modules on DNA level. To check if mRNA is produced from activated modules in situ hybridization and realtime PCR can be used. Activation of the three modules should lead to elevated Ras protein levels and higher expression of genes regulated by Ras. This can be analysis can be done by immunohistochemistry and Western blot.

To be sure that the activation of the three modules is a stochastic event that does not prefer some combinations it is necessary to test the activation in vitro, too. Therefore, mouse embryonic stem cells are transfected with the RasE MultiHi BAC. After cotransfection with a Cre-expressing plasmid single cell clones have to be tested for module activation. If the system is unbiased all possible combinations should arise with approximately the same frequency. Hence, if some combinations are selected in lung tissue they should not be over represented in the in vitro experiment.

5. Materials and Methods

5.1. Mice

All animal experiments were performed in accordance with Austrian and European laws and with the general regulations specified by the Good Scientific Practices guidelines of the University of Vienna. Following mouse strains were used:

- RasE MultiHit mice
- RosaCreER^{T2} mice[51]
- MxCre mice[52]
- p53 knock out mice[53]
- Pten flox/flox mice[54]

5.2. Mouse experiments

5.2.1. Cre-induction upon Tamoxifen injection

100mg Tamoxifen (Sigma T5648) are dissolved in 10mL sunflower seed oil (Fluka 88921). RasE RosaCreER^{T2} mice were injected intraperitoneally with 100µL Tamoxifen (= 100µg Tamoxifen total) at four to six weeks of age. If needed, mice were repeatedly injected on consecutive days three or five times.

5.2.2. Cre-induction upon topical Hydroxy-Tamoxifen application

100mg Hydroxy-Tamoxifen (OH-Tamoxifen, Sigma H7904) are dissolved in 10mL absolute ethanol (Sigma 16368). An area of about 1.5cm x 2.5cm is shaved on the back of a RasE RosaCreER^{T2} mouse. Using a Gilson pipette 100µL of the OH-Tamoxifen (100µg Tamoxifen total) solution is slowly dropped onto the shaved skin. The procedure has to be repeated on four consecutive days[55].

5.2.3. Cre-induction upon poly I:C injection

10mg poly I:C (Sigma P9582) are dissolved in 5mL sterile 0,9% NaCl solution. Before use the poly I:C should be heated to 37°C. RasE MxCre mice were injected intraperitoneally with 50µL poly I:C (= 100µg poly I:C total) at four to six weeks of age.

5.2.4. Lung specific Cre-induction upon AdenoCre inhalation

Lung application of AdenoCre was performed through intranasal instillation using an AdenoCre-$CaCl_2$ precipitate. For AdenoCre-$CaCl_2$ precipitation, 60µL MEM, 2.5µL AdenoCre solution (1010pfu/mL; University of Iowa, gene Transfer Vector Core) and 0.6µL $CaCl_2$ were mixed for each mouse and incubated 20min at room temperature[38]. AdenoCre inhalation was done by Daniel Schramek in the IMP/IMBA mouse house.

5.3. Tail DNA isolation

Tail digestion buffer:

50m M	Tris-HCl, pH.8
100mM	EDTA
100mM	NaCl
1%	SDS

0.5 mg/mL Proteinase K (Sigma P8044) (add to the buffer before use)

- Cut 2cm tail and add 500µl tail digestion buffer
- Incubate o/n at 56°C
- Mix 5min in an Eppendorf mixer
- Add 167µL 6M NaCl
- Mix 5min in an Eppendorf mixer
- Spin at 14000rpm for 10min
- Transfer 500µL supernatant to a new tube
- Add 334µL isopropanol, mix and spin at 14000rpm for 10min
- Discard supernatant properly
- Wash pellet with 1mL 70%EtOH, centrifuge (14000rpm, 10min) and discard supernatant the carefully
- Air dry pellet (5-10min)
- Dissolve DNA in 100µL distilled H_2O, for 1 hour at 37°C

5.4. Genotyping

Reagents used for PCR: Taq DNA polymerase (Roche 11 146 165 001), dNTPs (Fermentas), primers (MWG Biotech), DMSO (Sigma D4540).

Standard PCR mastermix for 1 reaction:

Template	1µL
Puffer (+$MgCl_2$)	2.5µL
DMSO	2.5µL
dNTPs (10mM each)	0.5µL
Primer1 (50ng/1µl)	1µL
Primer2 (50ng/1µl)	1µL
TaqPolymerase	0.2µL
H_2O	17.3µL (18.3µL if a primer mix is used)
	Σ 25.0µL

PCR Program:

94°C	2min	1 cycle
94°C	30sec	40 cycles
55°C	30sec	
72°C	1min	
72°C	10min	1 cycle

Routinely, DNA fragments were analyzed on agarose (Sigma, A9539) gels by electrophoresis. 2% gels were used for fragments between 300bp and 1kb. Bigger fragments can be separated in a 1% agarose gel. 1x TAE was used to dissolve the agarose and as running buffer as well.

50x TAE (Tris acetate EDTA) buffer:

242g Tris (Roth, 5429.2)

57.1mL Acetic acid

100mL 0.5M EDTA (AppliChem, A1103), pH 8.0

Fill up with distilled H_2O to 1L and autoclave.

Below, DNA sequences of primers used for gene-specific genotyping PCRs are listed. All oligonucleotides were obtained from MWG Biotech.

Primer Name	**Primer sequence**
Cre1	5′-CGGTCGATGCAACGAGTGATGAGG-3′
Cre2	5′-CCAGAGACGGAAATCCATCGCTCG-3′
Amplicon: 700pb	
Jun1	5′-CTCATACCAGTTCGCACAGGCGGC-3′
Jun2	5′-CCGCTAGCACTCACGTTGGTAGGC-3′
Amplicon: 450pb	
p53: Neo19 mut. rev.	5′-CATTCAGGACATAGCGTTGG-3′
p53: x7 common fwd.	5′-TATACTCAGAGCCCG-3′
p53: X6.5 wt rev.	5′-ACAGCGTGGTGGTACCTTAT-3′
Amplicon: wt: 450bp, knock out allele: 700bp	
Pten 1	5′-CTCCTCCTACTCCATTCTTCCC-3′
Pten 2	5′-ACTCCCACCAATGAACAAAC-3′
Amplicon: wt: 200bp, floxed allele: 300bp	
MultiHit1	5′-GCCATCACGAGATTTCGATT-3′
MultiHit2	5′-TGTGACACCCCAGCATTAGA-3′
Amplicon: 580bp	
MultiHit ON fwd.	5′-GCTAACCATGTTCATGCCTTC-3′
Ras S35 rev.	5′-CAGAAACACACCAAGTCTTCCA-3′
Ras G37 rev.	5′-ACAACGAGATGAGGACCAGAAT-3′
Ras C40 rev.	5′-TGTACTCAAAGCTGGAAGGTGA-3′
Amplicon: 750 bp	

Table 1: PCR primer sequences

5.5. Southern blot

Genomic DNA was digested with different restriction enzymes. To detect the activation of the three modules by Southern blot, labeled RNA probes against reporter genes were used. To check for single copy integrants after embryonic stem cell transfection a collagen probe was used.

Restr. enzyme	RNA Probe	Fragment length
XcmI	YFP-Probe	M1 off: 3536bp M1 on: 1774bp
XcmI	dsRed-Probe	M2 off: 3769bp M2 on: 1744bp
HindIII	hCD2t	M3 off: 3846bp M3 on: 2836bp

The probes were labeled with the Prime it II Random Primer Labeling Kit (Stratagene 300385), according to the manufacturer's protocol.

- Digest approximately 10µg of genomic DNA, load on a 0.8% agarose gel containing EtBr and run it in TAE at 90V
- Depurinate the DNA in the gel by rocking it in 0.25M HCl for 20 min.
- Denature the DNA in the gel by rocking it in 0.5M NaOH/1.5MNaCl for 30 min.
- Blot over night as follows:
- Set up the blot from bottom to top:
    - Plastic bag
    - 6 sheets Whatman paper soaked in 10xSSC
    - Gel
    - Parablot nylon membrane (GeneXpress) pre-soaked in 10xSSC
    - 4 sheets Whatman paper soaked in 10xSSC
    - 4 sheets dry Whatman
    - Paper towels, approximately 1kg weight

- Next day, soak membrane in 50mM NaPi for 20 min rocking
- Bake for 1 hour at 80°C
- Crosslink membrane with Stratalinker (Stratagene)

Membrane hybridization:

- Pre-hybridize membrane in hybridization buffer for 1h at 65 °C.
- Add probe and hybridize at 65°C overnight
- Wash membrane with Washing buffer 3 times 20min each, at 65°C
- Wrap membrane in plastic foil and expose at -80°C, 4-7days

<u>20xSSC:</u>

175,3g NaCl

88,2g Sodium citrate

Fill up with distilled H_2O to 1L and adjust to pH 7.0.

<u>0.1M NaPi (natrium phosphate buffer) pH 7.2:</u>

68.4mL 1M Na_2HPO_4

31.6mL 1M NaH_2PO_4

Fill up 1L with distilled H_2O.

<u>Hybridisation buffer:</u>

0.5M NaPi pH 7.2

7% SDS

1mM EDTA

<u>Washing buffer:</u>

40mM NaPi pH 7.2

1% SDS

5.6. RNA isolation and cDNA synthesis

RNA was isolated from organs and tumors with Trizol (Invitrogen) according to the manufacturer's protocol. Genomic DNA contamination in total RNA extracts was digested using 10U of DNAse I (Roche 04716728001), for 30 min at 37°C. After DNAse I inactivation at 70°C for 10min 1µg total RNA was transcribed into c-DNA using the "RevertAid H Minus First Strand cDNA synthesis kit" (Fermentas) following the manufacturer's instructions.

5.7. Real time PCR

An Eppendorf Realplex Mastercycler was used for real time PCR. In contrast to normal PCR SYBR Green (Roche Diagnostics) is added to the PCR reaction. It enables quantitative detection of the amplicons. RNA was analyzed in triplicates and the expression levels of transcripts were calculated with the comparative CT (threshold concentration) method. The individual RNA levels were normalized for GAPDH and are depicted as relative expression values.

Real time PCR mastermix for 1 reaction:

Template (cDNA 1:5 diluted in H_2O)	5µL
Puffer (+$MgCl_2$)	2.5µL
DMSO	2.25µL
dNTPs (10mM each)	0.5µL
Primer1 (50ng/1µL)	1µL
Primer2 (50ng/1µL)	1µL
CybrGreen (Roche), (diluted 1:100 in DMSO)	0.25µL
TaqPolymerase (5 Prime, 2200020)	0.1µL
H_2O	15.4µL
	Σ 25.0µL

Real time PCR program:

94°C	2min	1 cycle
94°C	30sec	40 cycles
55°C	30sec	
72°C	1min	
72°C	10min	1 cycle

Followed by melting curve (standard Eppendorf program)

Real time PCR primers

Primer Name	**Primer sequence**
Gapdh for.	5′-TGTTTGTGATGGGTGTG-3′
Gapdh rev.	5′-TACTTGGCAGGTTTCTC-3′
Amplicon: 251 bp	
Ras real fwd.	5′-AGCTGATCCAGAACCATTTTGT-3′
Ras S35 real rev.	5′-CAGAAACACACCAAGTCTTCCA-3′
Ras G37 real rev.	5′-ACAACGAGATGAGGACCAGAAT-3′
Ras C40 real rev.	5′-TGTACTCAAAGCTGGAAGGTGA-3′
Amplicon: 528 bp	

Table 2: Real time PCR primer sequences

The primer sequences were designed using an on-line available oligo design program (Invitrogen) and DNA oligonucleotides were obtained from a commercial supplier (MWG Biotech).

5.8. Western blot

Protein extraction (the whole procedure has to be carried out on ice):

- Resuspend homogenized tissue in an appropriate volume of Protein isolation buffer containing protease inhibitor cocktail Complete (Roche) and mix well by pipetting
- Shake vigorously at 4°C for one hour
- Spin extracts at 13000rpm for 15min
- Transfer protein lysates carefully into a new Eppendorf tube
- Measure protein concentration with the Bradford method using the Bio Rad Protein assay solution (BioRad, 500-0006) according to the manufacturer's protocol
- Before loading, add an apropriate volume of 6x sample loading buffer to the desired quantity of protein
- Immediately incubate at 95°C for 5min
- Store protein lysates at -80°C.

The proteins (10µg per gel lane) were separated by SDS-polyacrylamide gel electrophoresis (SDS-Page) on 10% or 12% gels, using a BioRad apparature. Proteins were transferred by semi dry technology (BioRad blotting chamber) onto Hybond C-Extra nitrocellulose membrane (Amersham) for big size proteins or Immobilon PVDF membrane (Millipore) for small size proteins, for one hour at 60mA per gel.

- After the transfer, incubate membrane in Ponceau S (Fluka, 78376) stain for 5 min at room temperature (to check transfer efficiency, equal loading and integrity of proteins)
- Rinse membrane several times with distilled water
- Incubate membrane with 5% skim milk/1xPBS/0,1%Tween20 or 5%BSA/1xPBS/0,1%Tween20 blocking solution, for one hour at room temperature

- Dilute the primary antibody in Blocking Solution and incubate o/n at 4°C, while rocking
- Wash membrane with 1xPBS/0,1%Tween20, 3 times, 10min each
- Dilute secondary antibody in blocking solution without NaN_3 (NaN_3 inhibits the activity of the horse radish peroxidase enzyme) and incubate membrane for one hour at room temperature
- Wash membrane with 1xPBS/0,1%Tween20, 3 times, 10min each
- Add ECL solution (ECL-Plus Western Blotting detection system, GE Healthcare), according to the manufacturers protocol
- Cover membrane with plastic wrap and expose to CL-xPosure Film (Thermo Scientific, 34090).

Western blot stripping:

- Add 700µL ß-mercapto-ethanol (Sigma, M7522) to 9,3mL Stripping Buffer
- Incubate membrane with this solution at 42°C for 30min
- Wash 3 times with 1xPBS/ 0,1%Tween20 to completely remove ß-mercapto-ethanol
- Block membrane in Blocking solution at room temperature for 30min
- Membrane can be re-probed with a different antibody.

Antibodies used for Western blotting:

Antibody name	Dilution	Source
PanRas	1:200	Santa Cruz Biotechnology, sc-6246
HSC 70	1:200	Santa Cruz Biotechnology, sc-7298

Protein isolation buffer:

25mM	HEPES pH 7.5
150mM	NaCl
10mM	EDTA
0.1%	Tween-20
0.5%	NP-40
10 mM	beta-Glycerolphosphate

Lower gel (for 2 small gels)	12%	10%
1.5M Tris pH 8.9	5mL	5mL
30% Bis-Acrylamid (Sigma)	8mL	6.66mL
10% SDS	200µL	200µL
10% APS	100µL	100µL
Temed	10µL	10µL
H_2O	6.5mL	7.84mL

Upper gel (for 2 small gels):	
1M Tris pH6.7	0.8mL
30% Bis-Acrylamid	0.8mL
10% SDS	60µL
10% APS	60µL
Temed	4µL
H_2O	4.3mL

6x sample loading buffer:

Add 6.05g Tris-base to 40 mL H_2O. Adjust to pH 6.8. Fill up up to 100mL with H_2O. Filtrate through a 0.45µm filter. Add 0.4g SDS and store at 4°C up to one month.

7mL	of the upper solution
3mL	glycerol
1g	SDS
0.93g	DTT
1.2mg	bromophenol-blue

Add H_2O up to 10mL if needed. Store at -20°C

5x SDS Electrophoresis buffer:

15.1g Tris-base

72g Glycin

5g SDS

Fill up to 1L with distilled water. Do not adjust the pH of the stock solution as the solution is pH 8.3 when diluted. Store at 4°C.

5x Transfer buffer:

15.15g Tris

72 g Glycin

Fill up to 1L with distilled water.

Stripping buffer:

62,5mM Tris HCl, pH 6.7

2% SDS

Ponceau S solution:

0.1% Ponceau S

5 % Acetic Acid

5.9. Histology

Fresh organs were washed with PBS and fixed in 4% buffered formaldehyde (Roti Histofix 4%, Promega P087.3) at 4°C o/n. After fixation tissues were dehydrated using graded ethanol. Dehydrated organs were embedded in paraffin. Solid paraffin blocks were stored at RT. 2µm thick sections of paraffin blocks were generated using a microtome (Shandon) and put on glass carrier slides (Superfrost Plus, Menzel-Gläser).

5.9.1. Haematoxylin and Eosin staining

- Bake paraffin slides for 1h at 60°C
- Remove paraffin 3 times, 10min in Xylene substitute (Shandon, 9990505)
- Rehydrate tissue in descending ethanol solutions (100%, 96%, 80%, 70%, 50%) for 30s each

- dH_2O for 5min
- Haematoxylin (Merck, 1:1 dilution in dH_2O) for 10min
- Wash with tap water
- Rinse with tap water for 7min
- Dehydrate tissue in ascending ethanol solutions (50%, 70% for 30s each and 80% for 20s)
- Eosin (Roth, 7089.2) 0.1% eosin in 80% ethanol and 0.25% glacial acetic acid) for 2.5min
- 96% ethanol for 30s
- 100% ethanol for 2min
- Xylene for 2min
- Mount with Eukitt (Fluka; 03989)

<u>Eosin staining solution:</u>

- 2mL 10% eosin
- 198mL 80% ethanol
- 500µL acetic acid

5.9.2. Immunohistochemistry (IHC)

Standard IHC Protocol:

- Bake slides at 60°C for 1h
- Remove paraffin for 3 times for 10min in Xylene substitute (Thermo Scientific)
- Rehydrate tissue in descending ethanol solutions (100%, 96%, 80%, 70%, 50%) for 30s each
- dH_2O for 5min
- For antigen retrieval, boil the sections for 10min in Citrate Buffer (pH 6.0) (DAKO S2369), antigen retrieval pH 9.0 (DAKO S2367) or do Proteinase K treatment according to the antibody requirements
- Wash 3x with PBS
- Peroxidase block with 3% H_2O_2 in PBS for 15min at room temperature (RT)
- Wash 3x with PBS

- Incubate with Avidin Block (Vector Labs) for 10min at RT
- Wash 3x with PBS
- Incubate with Biotin block for 10min at RT (Vector Labs)
- Wash 3x with PBS
- Incubate with Superblock (ID labs) for 7min
- Wash 3x with PBS
- Incubate with Mouseblock (Eubio MKB-2213) (2 drops in 2.5mL PBS)
- Wash 3x with PBS
- Incubate with the 1st antibody diluted in PBS/1% BSA overnight at 4°C in humid chamber.
- Wash 3x with PBS
- Incubate with secondary anti-polyvalent biotinylated antibody (ID labs) for 10min at RT
- Wash 3x with PBS
- Incubate with streptavidin-HRP (ID labs) for 10min at RT
- Wash 3x with PBS
- Develop with AEC Chromogen (ID labs, BP1108) according to manufacturer's protocol
- Counter stain with 1:5 diluted haematoxylin solution for 15s
- Mount with Aquatex (Merck)

Superblock, secondary anti-polyvalent biotinylated antibody and streptavidin HRP solutions are contained in the IDetect Superstain System kit (ID labs, IDST1007).

Proteinase K antigen retrieval protocol:

Proteinase K (Sigma, P2308) stock solution (20x, 400µg/mL):

Proteinase K	4mg
TE Buffer, pH 8.0	10mL

Mix well, aliquot and store at –20 °C.

Proteinase K working solution (1x, 20µg/mL):

Proteinase K Stock Solution (20x)	1mL
TE Buffer, pH 8.0	19mL

Mix well. This solution is stable for 1 month at 4 °C. For longer storage, aliquot and store at –20 °C.

Incubate tissue slides with the Proteinase K working solution for 10 min at 37°C and 10 min at RT. Wash 3x with PBS and continue with the Standard IHC protocol.

TE buffer:

10mM	Tris/HCl pH 8.0
1mM	EDTA pH 8.0

Antibodies used for IHC with the standard protocol:

Antibody name	Dilution	Source
Synaptophysin	1:50	Dako Cytomation, A0010
KI67	1:1000	Novocastra NCL-KI67-P
CD3	1:300	Neomarkers, RM9107
F4/80	1:100	AbD Serotec, MCA497G
Insulin	1:200	Santa Cruz Biotechnology, sc-9186
PanKeratin	1:100	ThermoScientific, MS-343-R7
CK7	1:500	Abcam, ab9021
CK19	1:100	Abcam, ab15463
CC-10	1:100	Santa Cruz Biotechnology, sc-25555
SPC	1:100	Santa Cruz Biotechnology, sc-13979

5.10. In situ hybridization

In order to obtain RNA probes for in situ hybridization the gene of interest has to be cloned into a vector between two RNA polymerase transcription sites (Figure 8). The plasmid was linearized with XbaI and HindIII respectively.

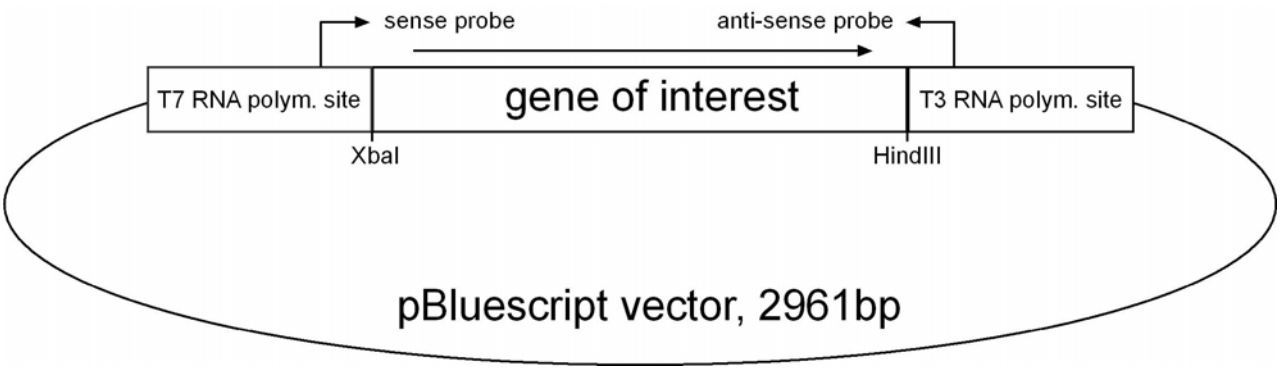


Figure 8: Scheme of a vector used for in vitro transcription of RNA probes for in situ hybridization. The gene of interest is inserted between a T3 and a T7 RNA polymerase site. For transcription of the sense probe the vector is linearized with HindIII and transcribed with the T7 RNA polymerase. For transcription of the antisense probe the vector is linearized with XbaI and transcribed with the T3 RNA polymerase.

DNA	20µg (in a max. volume of 16µL)
Enzyme	2µL
10x Restriction Buffer	2µL
Pure H_2O up to	20µL

Digest for 4h or o/n at 37°C. The completeness of the digest was checked on a 1% agarose gel containing EtBr.

RNA probe synthesis

linearized DNA (1µg/1µL)	1µL
5x Transcription buffer (Fermentas)	4µL
10x DIG RNA Labeling Mix (Fermentas)	2µL
RNase Inhibitor (Fermentas)	1µL
RNA polymerase (T3 or T7; Fermentas)	1µL
DEPC – H_2O	9µL (up to 20µL)

- Incubate at 37°C , for 2h
- Increase volume to 50µL with DEPC water and spun trough Pharmacia Sephadex g-50 pre-made columns.
- Check 2µL on a 1% agarose TAE gel
- Ratio should be 10:1 RNA to DNA template
- Aliquot and store the probes at -80°C

Preparation of slides for Hybridization

- Bake slides on a hot plate (60°C) for 30min
- Allow to cool just until the wax looks solidified

Use DEPC treated solutions for all steps prior to hybridization

- Dewax in xylene 2x 5min (under the hood)

Rehydrate to PBS:

- 100% EtOH 5min (hood)
- 100% EtOH 5min
- 75% EtOH (DEPC-H_2O) 5min
- 50% EtOH (DEPC-H_2O) 5min
- 25% EtOH (DEPC-H_2O) 5min
- PBS – DEPC 5min
- PBS – DEPC 5min

Hybridization:

- 4% PFA/PBS 10min
- PBT – DEPC (“1”) Rinse
- PBT – DEPC (“2”) Rinse
- PBT – DEPC (“3”) Rinse

Antigen Retrieval:

- Proteinase K (Sigma) in PBS 10min
  (20µL PK (10mg/mL) in 200mL PBS – DEPC)
- PBT – DEPC (“2”) 5min
- PBT – DEPC (“3”) 5min

Fixation:

- 4% PFA/PBS 5min
- PBT – DEPC (“1”) Rinse
- PBT – DEPC (“2”) 5min
- PBT – DEPC (“3”) 5min

Acetylation:

- Add 625µL acetic anhydride to 250mL 0.1M TEA (Sigma T1377) shake well and use immediately. 15min
- PBT – DEPC (fresh) Rinse
- PBT – DEPC (“2”) 5min
- PBT – DEPC (“3”) 5min
- Air dry slides 30min or use the oven (50°C)
- Prepare cassettes and hybridize solutions

For most RNA-probes prehybridization is not necessary, but in case of high background the procedure is as following:

- Put 100µL prewarmed (65°C) of hybridization solution per slide and coverslip
- Place slides into a humid chamber incubate at 65°C for at least 1h

Preparing of the probes:

- Mix 1µL probe – DIG with 100µL hybridization solution per slide and incubate at 85°C for 3min (not longer or the RNA might get degraded)
- Add probe and coverslip slides avoiding air bubbles
- Incubate at 65°C overnight (at least 16 hours) in the water bath

Post Hybridization Washes:

All washing steps are carried out in the water bath and solutions should be pre-warmed.

- Remove coverslips by rinsing in 5x SSC
- 1x SSC/50% Formamide 30min at 65°C
- TNE 10min at 37°C
- TNE + RNase A (20µg/mL) 30min at 37°C
  (400µL RNaseA 10mg/mL in 200mL TNE)
- TNE 10min at 37°C
- 2x SSC 20min at 65°C
- 0.2x SSC 20min at 65°C
- 0.2x SSC 20min at 65°C

Antibody incubation and detection

- MABT 5min at RT
- MABT 5min at RT

- Block in 20% HISS/MABT (400µL/slide) and incubate 1 hour at RT
- α–DIG–AP (1:2000, Roche) in 2% HISS/MABT (400µL/slide) incubate o/n at 4°C in a humidified box (aluminum foil wrapped box with soaked towels)

- MABT Rinse at RT
- MABT 5min at RT
- MABT 5min at RT
- MABT 5min at RT
- NTMT pH 9.5 10min at RT

- Add BM purple AP substrate (Roche)
  [as little as possible – just enough to cover the tissue]
- Incubate at RT for 1 hour – 3 days [at 4°C if the reaction is slower]

To stop the reactions:

- NTMT pH 9.5 Rinse at RT
- PBS 5min at RT
- PBS 5min at RT

- Postfix in 4% PFA/PBS for 10 – 20min
- Rinse in PBS 2 times and then in water
- Counterstain with nuclear fast red (Roth; N0069.1) for 1min, dehydrate in ascending EtOH solutions and embed with Eukitt (Fluka; 03989).

Solutions for in situ hybridization:

MPW-DEPC:

Add 1 mL DEPC/L distilled water, stir until clear and autoclave.

PBS-DEPC:

100mL 10xPBS + 900mL MPW-DEPC

Hybridization Solution:

10mM	Tris pH 7.5
600mM	NaCl
1mM	EDTA
0.25%	SDS
10%	Dextrane sulfate (Amersham; 17-0340-01)
1x	Denhadt´s
200µg/mL	yeast tRNA (Gibco)
50%	Formamide

Store at -20°C

50x Denhardt´s:

1% (w/v) Ficoll 400 (Sigma; F2637)

1% (w/v) Polyvinylpyrrolidone (Sigma, P5288)

1% (w/v) BAS

In MPW

10x TEA [1M]:

Add 149.19g TEA in a bottle and fill up to 1L with distilled water.

20xSSC:

175.3g NaCl

88.2g Sodium citrate

Fill up with distilled H_2O to 1L and adjust to pH 7.0.

5x TNE:

50mM Tris pH 7.5

2.5M NaCl

5mM EDTA

5x MAB

500mM Maleic acid

750mM NaCl

Adjust pH to 7.5 with 10M NaOH

1x MABT

200mL 5x MAB + 800mL distilled water, add 1mL Tween-20 (Sigma; P9416)

NTMT, pH 9.5

100mM NaCl

100mM Tris pH 9.5

50mM $MgCl_2$

0.1% Tween-20 (Sigma; P9416)

5.11. Transfection of embryonic stem cells with the MultiHit BAC

5.11.1. BAC purification

2mL of LB medium with Neomycin [20µg/mL] (LB-neo medium) were inoculated from an E. coli glycerol stock (at -80°C) containing the RasE MultiHit BAC. After over night incubation at 37°C 250mL LB-neo medium was inoculated and left shaking for 5-6h. Bacteria were harvested from the culture by centrifugation at 5000rpm for 15min at 4°C. The BAC construct was purified with the BAC 100 (Nucleobond, 740 579) kit according to the manufacturers protocol. The DNA concentration of the purified DNA was measured and test digests with 6 different restriction enzymes were made.

10µL BAC [c=0.4µg/µL]
3µL enzyme
3µL 10x buffer
14µL H_2O
Σ 30µL, digest o/n at 37°C

Enzymes used: EcoRI, SfiI, SrfI, SdaI, MluI, AscI (Fermentas).

The 6 digests were separated on a 1% agarose gel and the DNA fragments compared to a previous BAC preparation. All fragments showed the anticipated size.

Before the BAC can be used for electroporation it has to be linearized with the restriction enzyme NotI.

30µL BAC
6µL Buffer "O" (Fermentas)
3µL NotI (Fermentas)
21µL H_2O
Σ 50µL

5.11.2. Electroporation

One frozen aliquot of mouse embryonic fibroblasts was thawed. The cells were propagated in gelatin coated flasks in embryonic stem cell medium (ESC-medium). ECS-medium is DMEM (PAA, E15-101) supplemented with:

- 15% FCS (Sigma, F7524)
- 1x Penicillin/Streptomycine
- 1x nonessential amino acids (PAA, M11-300)
- 1x L-Glutamine
- 1x L-Pyruvat (Sigma, S8636)
- 1x β-Mercaptoethanol (1:000 stock in H_2O, dilution 1:138)
- LIF, 1.5mL of LIF containing supernatant

0.1% Gelatin:

Add 200mg gelatine from porcine skin [Sigma, G1890] to 200mL 1x PBS and autoclave.

The cells of a ~100% confluent 75cm^2 flask were trypsinized with 1x Trypsin (PAA, L11-004) and one half of the cells was centrifuged (10min at 1.000rpm). The pellet was taken up in 800µL sterile PBS and transferred to a 4mm electroporation cuvette (Biozym, 748040). 10µg linearized BAC is added and electroporation was carried out with the GenePulsor Xcell (Biorad) using the following program:

settings	**value**
Voltage [V]	230
Capacitance [µF]	500
Resistance [Ω]	∞
Cuvette	4mm

After the electroporation the cells were diluted 1:10000 with ESC-medium and plated on gelatin coated culture dishes. After 5-7 days single cell clones became visible. 24 different clones were picked and propagated in a gelatin coated 24well plate. When the cells reached ~ 100% confluences the cells were trypsinized and 50% was used for DNA isolation and 50% was propagated further. The DNA was used for Southern blot analysis to check for single copy integrants. Additionally the integrity of the three expression modules was checked by PCR.

One single integrant clone, that showed three intact expression modules, was grown to maximum density in a 75cm^2 flask. The cells were trypsinized, centrifuged (10min at 1.000rpm) and the pellet was taken um in 800µL sterile PBS. 10µg of plasmid of transient Cre-expression was added and the cells were electroporated with same program as before.

After the electroporation the cells were plated in low density (dilution between 1:1000 and 1:10000). The rest of the cells was propagated in a 25cm^2 flask and spilt 1:10 every other day. After 5 passages the cells were frozen in FCS containing 10% DMSO.

5.11.3. Genotyping of single cell clones

After 5-7 days single cell clones were visible. About 300 clones were picked and transferred to gelatin coated 96 well plates. When the medium turned yellow the cells were trypsinizied with 30µL 2x Trypsin (PAA, L11-400). After adding 180µL ESC-Medium to the wells, 70µL of the cell suspension was transferred to a new gelatin coated plate. The residual 140µL were transferred to a 96 well PCR plate. The plate was centrifuged for 10min at 5000rpm and the medium was removed carefully. 100µL NID buffer and 2µL Proteinase-K [10mg/mL] was added to each well.

<u>NID Buffer:</u>

50mM	KCl
10mM	Tris pH 8.3
2mM	$MgCl_2$
0.1mg/mL	gelatin
0.45%	Nonident P40 (Fluka, 74385)
0.45%	Tween 20 (Sigma, P9416)

The PCR plate was sealed and incubated in a PCR cycler with the following program

- 56°C for 3h
- 95°C for 10min
- Storage at 4°C

After centrifugation (10min at 5000rpm) to pellet the cell debris, 5µL of the obtained solution was used for PCR to check for the activation of the three modules.

5.12. Statistics

All values are represented as means ± s.e.m.. Statistical analysis was performed using the student's t test and a P value below 0.05 was considered significant. Calculation was performed using the GraphPad Prism 5 software.

6. Results and Discussion

6.1. Dosage dependent module activation in mice

To test the module activation in vivo, RasE Rosa26CreER^{T2} double transgenic mice were injected 1x, 3x and 5x with Tamoxifen. As a control one RasE MultiHit mouse without Cre was injected 5x with Tamoxifen and one double transgenic mouse was left untreated. Three days after the last injection, mice were killed and DNA was isolated from lung (lu), liver (li), spleen (s) and gut (g). The module activation was tested by PCR and showed clear dosage dependence (Figure 9). Mice with 5 Tamoxifen injections showed the strongest module activation. Mice with three Tamoxifen injections showed a little less activation and mice with only one Tamoxifen injection showed weak bands for all three modules. Both controls showed no activation at all. This excludes spontaneous recombination in the MultiHit construct and leakiness of the RosaCreER^{T2} transgene.

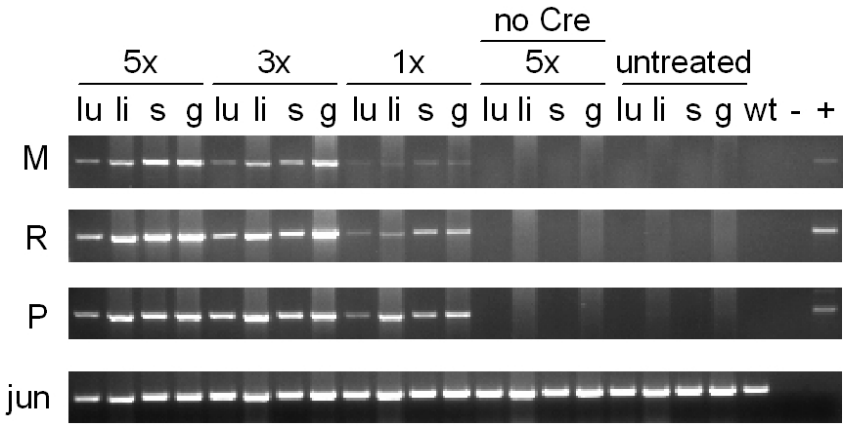


Figure 9: Activation of the MultiHit construct in healthy tissue. PCR analysis for activation of Ras effector mutants was performed with DNA from lung (lu), liver (li), spleen (s) and gut (g) of RasE Rosa26CreER^{T2} double transgenic mice after 5x, 3x or 1x Tamoxifen injection. Mice were killed three days after the last injection. RasE mice treated 5x with Tamoxifen and untreated RasE Rosa26CreER^{T2} mice were used as controls. wt: wild-type; -: no DNA; +: equimolar plasmid positive control; M: MAPK on; R: Ral on; P: PI3K on; PCR for jun was performed as loading control.

6.2. Elevated Ras levels in different tissues

Activation of the three expression modules should lead to a considerable increase of PanRas concentration in different organs. Protein extracts were made from RasE Rosa26CreER^{T2} double transgenic mice injected 5x, 3x and 1x with Tamoxifen. The proteins were separated on an acryl amide gel and blotted on a nylon membrane. The membrane was incubated with an antibody detecting all three Ras protein (HRas, NRas and KRas) (Figure 10).

Lung and liver extracts did not show an increased amount of Ras protein after Tamoxifen injection. This effect may be due to the already high Ras expression levels in these organs. Spleen and gut showed dosage depended increase of Ras expression levels. It is apparent that the 5x control without Cre has the same amount of Ras, as the wild type control.

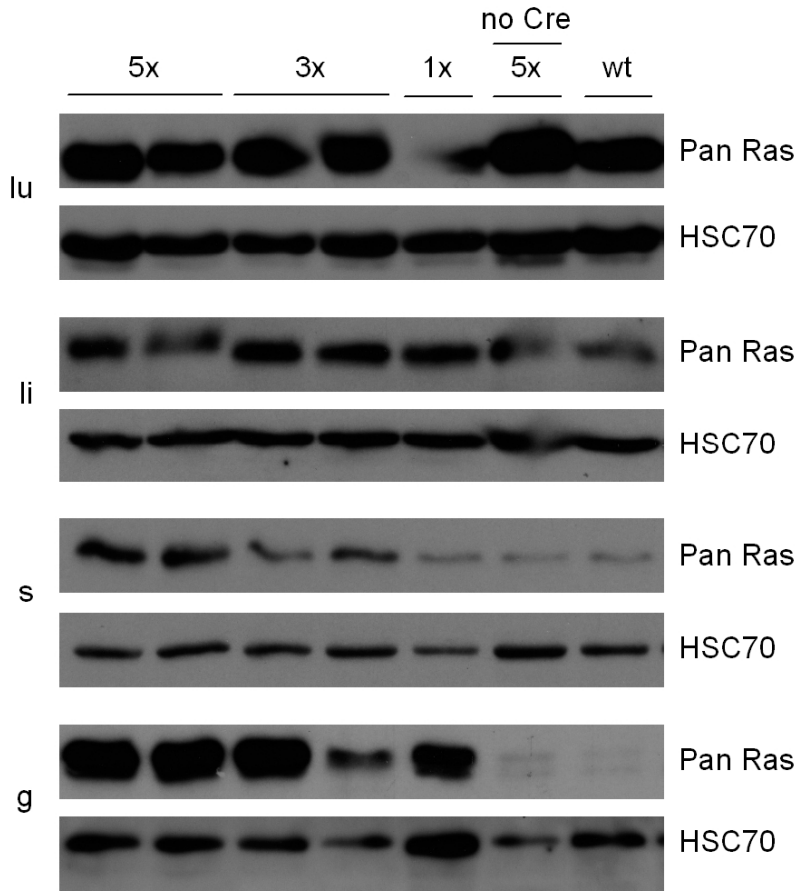


Figure 10: Western blot analysis for activation of Ras effector mutants. Protein extracts from lung (lu), liver (li), spleen (s) and gut (g) of RasE Rosa26CreER^{T2} double transgenic mice after 5x, 3x or 1x Tamoxifen injection were tested for Ras expression levels. RasE mice treated 5x with Tamoxifen and untreated wild-type (wt) mice were used as controls. Protein expression of HSC70 was analysed as loading control.

6.3. Different tumor types develop 10 weeks post Tamoxifen injection

Rosa26CreER^{T2} double transgenic mice were injected intraperitoneally with Tamoxifen on five consecutive days. These mice rapidly lost weight and became sick between 4.5 and 8 weeks after injection. The strong Cre-activation leads to several different tumors and rapid mortality (Figure 11). For the following experiments mice were injected only 3x and 1x with Tamoxifen.

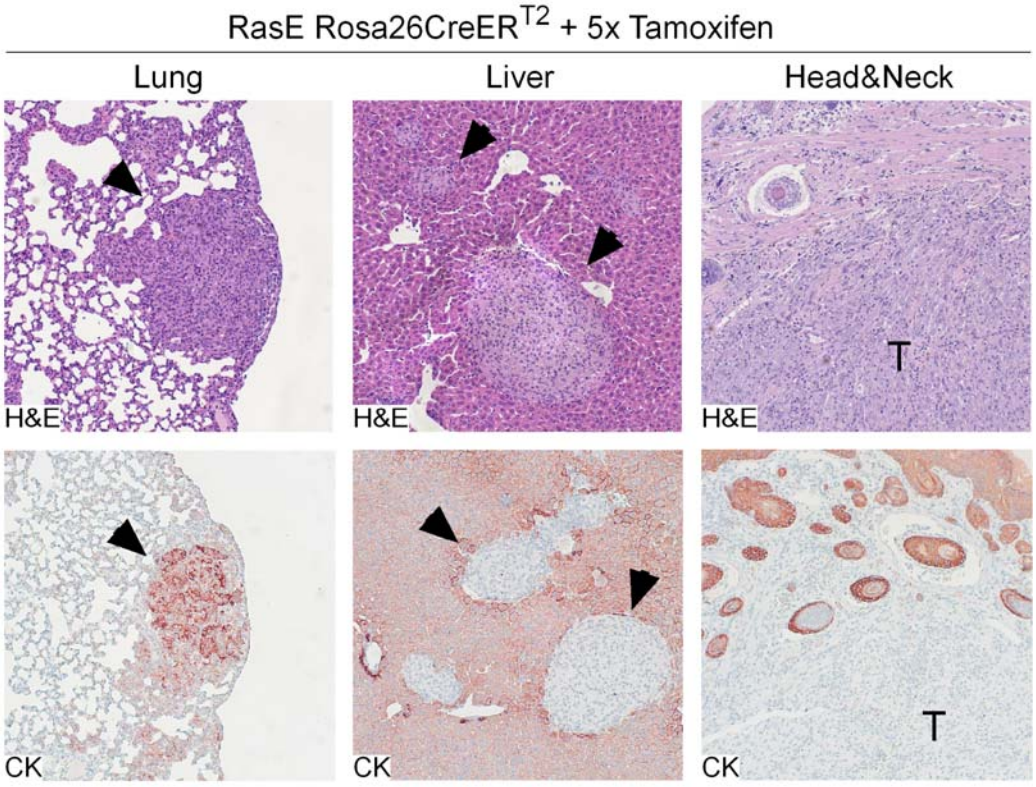


Figure 11: Different tumor types arise upon 5x Tamoxifen injection. The figure shows HE and cytoceratin stainings of a lung, liver and head and neck tumor (not further characterized) that arose six weeks after Cre-activation with five consecutive Tamoxifen injections.

Upon 1x Tamoxifen injection three different tumor types arise (Figure 12). Lung adenocarcinomas and pancreatic ductal adenocarcinomas were observed with 100% penetrance. Skin appendage tumors were observed with about 80% penetrance.

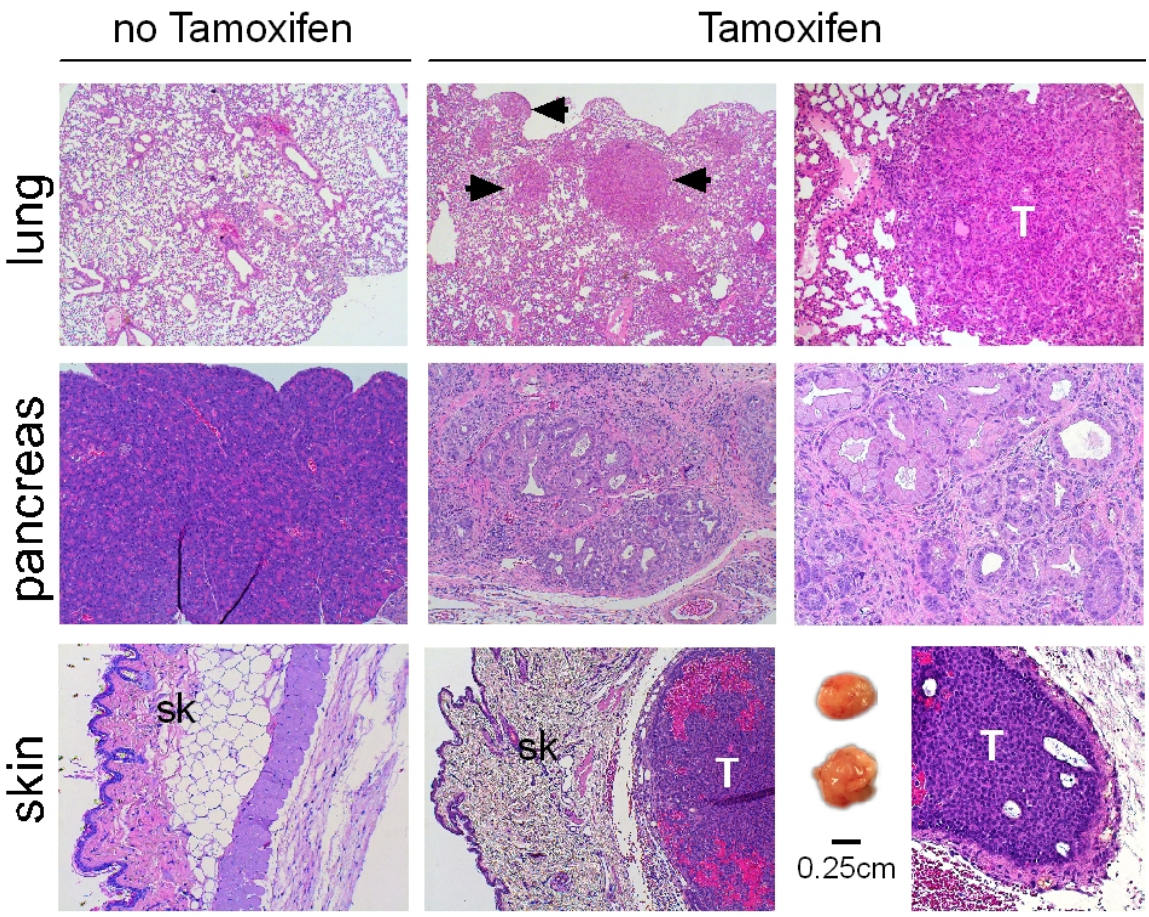


Figure 12: Three different tumor types arise upon 1x Tamoxifen injection in RasE Rosa26CreER^{T2} mice. Lung tumors were visible as focal lesions (arrows in upper middle image) in RasE Rosa26CreER^{T2} mice. Pancreatic tumors (second row, middle and right image) appeared histologically as ductal adenocarcinomas. Moreover, skin appendage tumors developed (lower right image). The macroscopic view of two isolated skin appendage tumors demonstrates that this tumor type grew to a considerable size and was well encapsulated. A higher magnification of all three tumor types is shown in the right images. No tumor formation was observed in untreated age-matched RasE Rosa26CreER^{T2} mice. Lung (upper left), pancreas (middle left) and skin (lower left) are shown. T: tumor; sk: normal skin

Untreated Rosa26CreER^{T2} double transgenic mice develop lung adenocarcinomas and skin appendage tumor after 38 weeks of age. Pancreatic ductal tumors were never observed (Figure 13). This is most likely due to the spars leakiness of the RosaCreER^{T2} system. Untreated RasE mice, without RosaCre, never developed tumors at all.

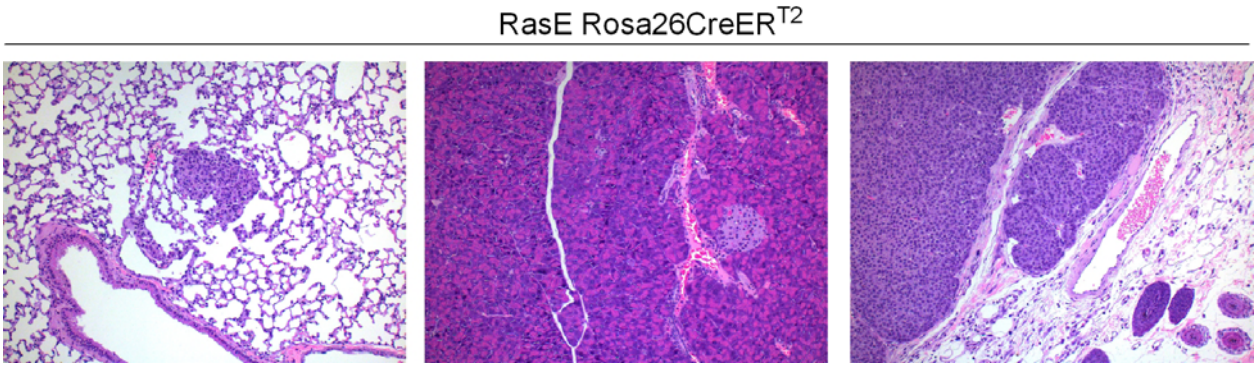


Figure 13: Tumor formation in untreated RasE Rosa26CreER^{T2} mice after > 38 weeks. Most untreated RasE Rosa26CreER^{T2} mice developed lung tumors (left image) and skin appendage tumors (right image) but no pancreatic ductal adenocarcinomas (middle image) after >38 weeks of age.

6.4. Sebaceous gland tumors

A protocol to activate Cre in a skin specific manner was used to investigate the skin appendage tumors in more detail. Hydroxytamoxifen (OH-Tamoxifen) dissolved in ethanol was applied atopically on the shaved back skin of RasE Rosa26CreER^{T2} double transgenic mice. This way of Tamoxifen application induced skin appendage tumors with 100% penetrance about 8 - 10 weeks after the treatment. Figure 14a shows one of this tumors and the immunohistochemical characterization. The upper right image shows that the tumor is negative for high molecular weight cytokeratin (HMW CK) which excludes the possibility that the tumor is derived from hair follicles. Positive hair follicles are marked with arrows. The tumors are also negative for N-Cam. Merkel cell carcinomas are neuroendocrine tumors of the skin[56]. The negative staining for N-Cam excludes that OH-Tamoxifen induced tumors are derived from Merkel cells. The tumors are positive for cytokeratin 7 (CK 7), shown in the lower left image. This suggests that the cells of origin are sebaceous gland cells. The left inset shows a higher magnification of CK7-positive tumor tissue and the right inset shows CK 7 positive cells in normal skin.

The activation of the three modules on DNA level was checked by Southern blot (Figure 14b). Atopic treatment of the skin with OH-Tamoxifen is an inefficient inducer of M, R and P since activation was not detectable in the Southern blot. In contrast, skin tumors displayed a prominent band for M, R and P activation demonstrating that they arose from rare cells after positive selection. Untreated skin and the RasE MultiHit BAC were used as control. The mode of tumor induction does not seem to influence the tumor type. Two individual tumors 8.5 weeks after atopic treatment with OH-Tamoxifen and 10 weeks after 1x

Tamoxifen injection are shown in the Southern blot. No differences in histology or Southern blot could be detected. The tumor tissue used for DNA isolation was dissected carefully to isolate pure tumor tissue. However, the strength of the "off band" depends on the amount of surrounding non tumor tissue (e.g. uninduced skin or connective tissue).

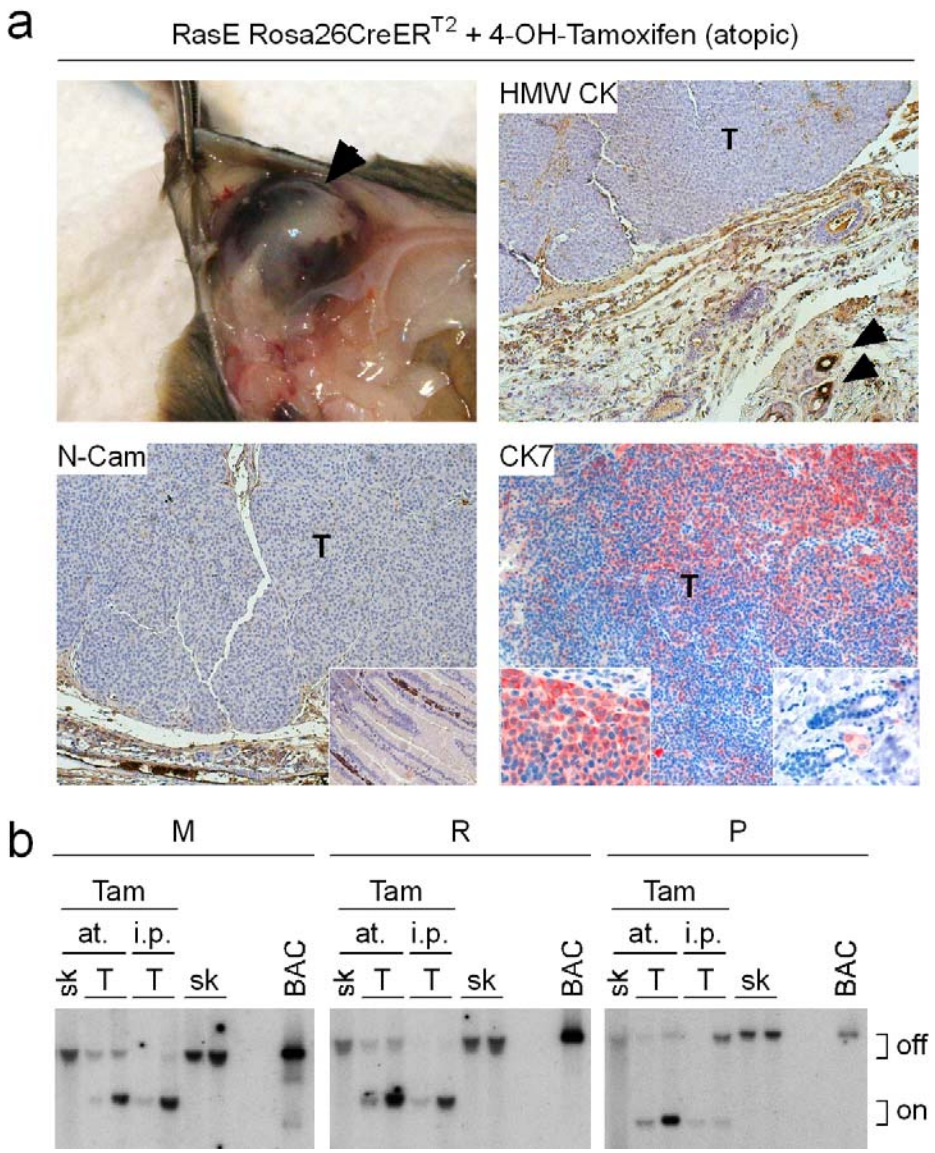


Figure 14: Characterization of sebaceous gland tumors. (a) Macroscopic view of a tumor located underneath the skin (8.5 weeks after treatment). Tumors were negative for high molecular weight cytokeratin (HMW CK; upper right image) as revealed by IHC. HMW CK-positive hair follicles are marked with arrowheads. Tumors were negative for N-Cam. Villi from the gut with N-Cam-positive nerves are shown as positive control (inset). Tumors were positive for Cytokeratin 7 (CK7; lower right image). (b) Southern blot analysis with skin (sk) and tumors (T) from RasE Rosa26CreER^{T2} mice induced by atopic treatment with 4-OH-Tamoxifen (at.) or 1x injection of Tamoxifen (i.p.). T: tumor, sk: skin.

6.5. Pancreatic ductal adenocarcinomas

Pancreatic ductal carcinomas were observed in RasE Rosa26CreER^{T2} double transgenic injected 1x Tamoxifen with 100% penetrance. Figure 15 shows the immunohistochemical characterization of these tumors. The HE staining shows clearly the abnormal appearance of the pancreas. Insulin producing Langerhans islets are highlighted with arrows in the HE and Insulin staining of the untreated tissue. However, the tumors are negative, indicating that they do not derive from insulin producing cells. Hence there are still residual Insulin positive islets visible in the tumor tissue.

The periodic acidic-Schiff (PAS) staining in the untreated tissue shows the positive, mucus producing goblet cells of adjacent gut tissue (arrows). The tumors consist of several PAS positive ducts. Ki67, which is a marker for dividing cells, is much more abundant in tumor tissue than in healthy pancreas where only a few cells are positive.

The tumors are also positive for cytokeratin 7 and 19. Together with the positive PAS staining, this is the best hint to the ductal nature of the tumors[57]. In normal pancreas only the bile ducts are positive.

Because of very high background levels it was not possible to determine the module activation in these tumors.

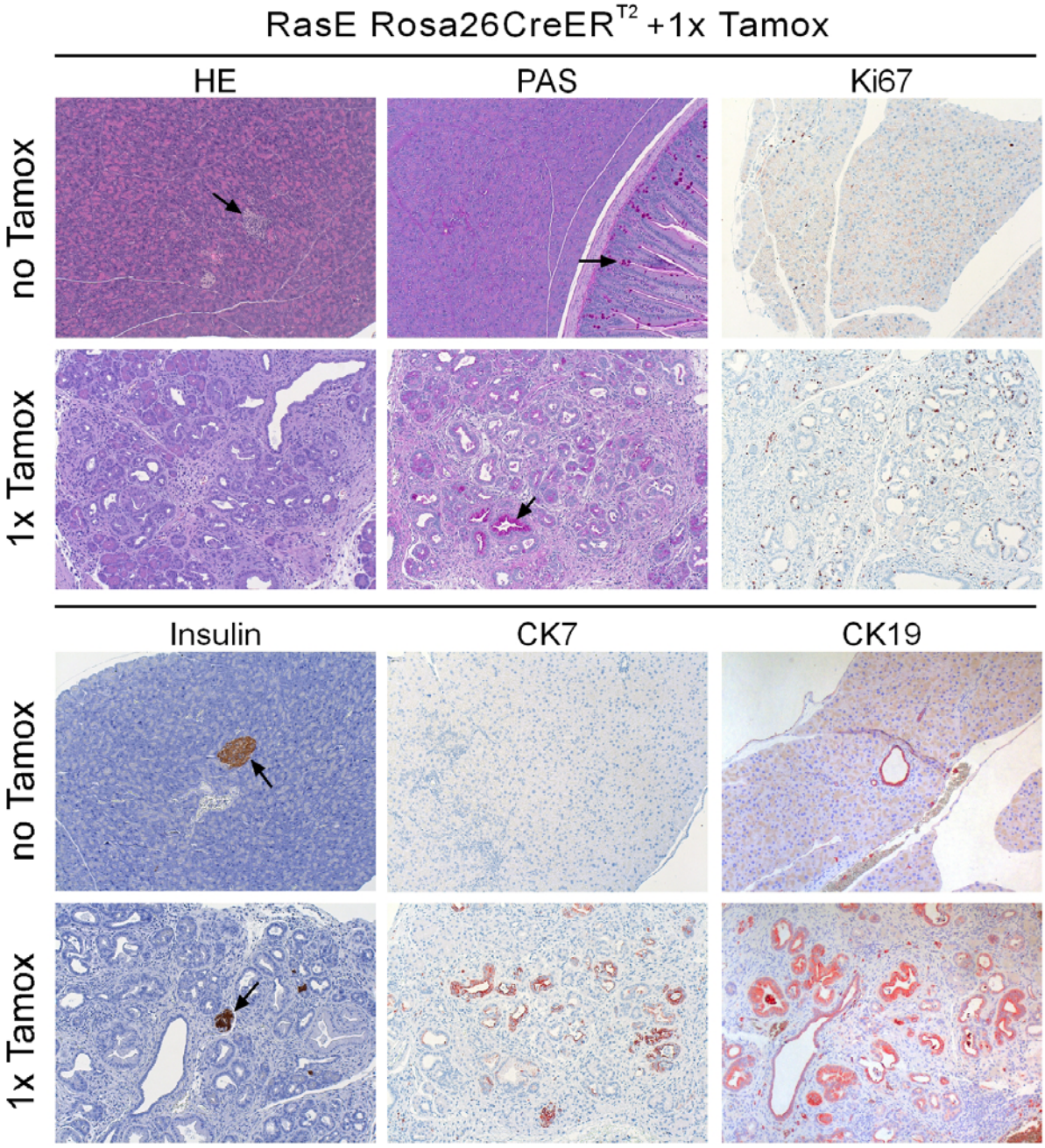


Figure 15: Immunohistochemical characterization of pancreatic ductal adenocarcinomas. Pancreatic tissue of 1x Tamoxifen treated and untreated RasE Rosa26CreER^{T2} mice was stained for several markers of ductal adenocarcinomas.

6.6. Lung specific module activation upon AdenoCre inhalation

To induce the Cre-recombinase in a lung specific manner, an adenoviral inhalation protocol was used. This genetically engineered adenovirus expresses Cre-recombinase upon infection of lung cells. The left panel of Figure 16 shows macroscopically visible lung

tumor 18 weeks after AdenoCre treatment. These tumors appear as focal lesions positive for Pan-Cytokeratin (middle and right panel).

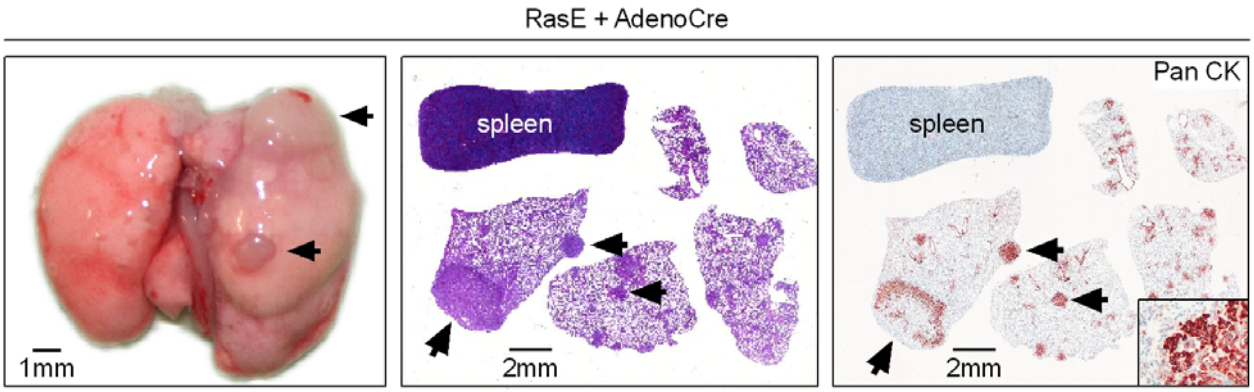


Figure 16: Lung adenocarcinomas were induced in RasE mice by administration of AdenoCre particles. Macroscopic view of a lung 18 weeks after administration of AdenoCre particles (left image). Arrowheads indicate tumors. Gross histology of lung tissue showing lung tumors (arrowheads) as focal lesions (middle image). Tumors (arrowheads) stained positive for Pan-Cytokeratin (right image). The inset shows a Cytokeratin-positive tumor at higher magnification.

To characterize these adenocarcinomas in detail it is important to determine the cell of origin of these tumors. There are several different cell types in the lung. Type I pneumocytes are responsible for the gas exchange in the lung and cover about 95% of the alveolar surface. Type II pneumocytes are producing surfactant to reduce the surface tension in the alveoli[58]. Clara cells are found in the epithelium of the small airways, producing substances that protect the bronchiolar lining[59].

Figure 17 shows consecutive sections of a lung tumor stained for CC10, a marker for Clara cells, and for SP-C, a marker for type-II-pneumocytes. The left image shows the corresponding HE staining of the region. The staining for CC10 shows positive Clara cells in bronchi already infiltrated and closed by the tumor. The tumor itself is negative for CC10 but clearly positive for SP-C. The observed adenocarcinomas are derived from type-II-pneumocytes.

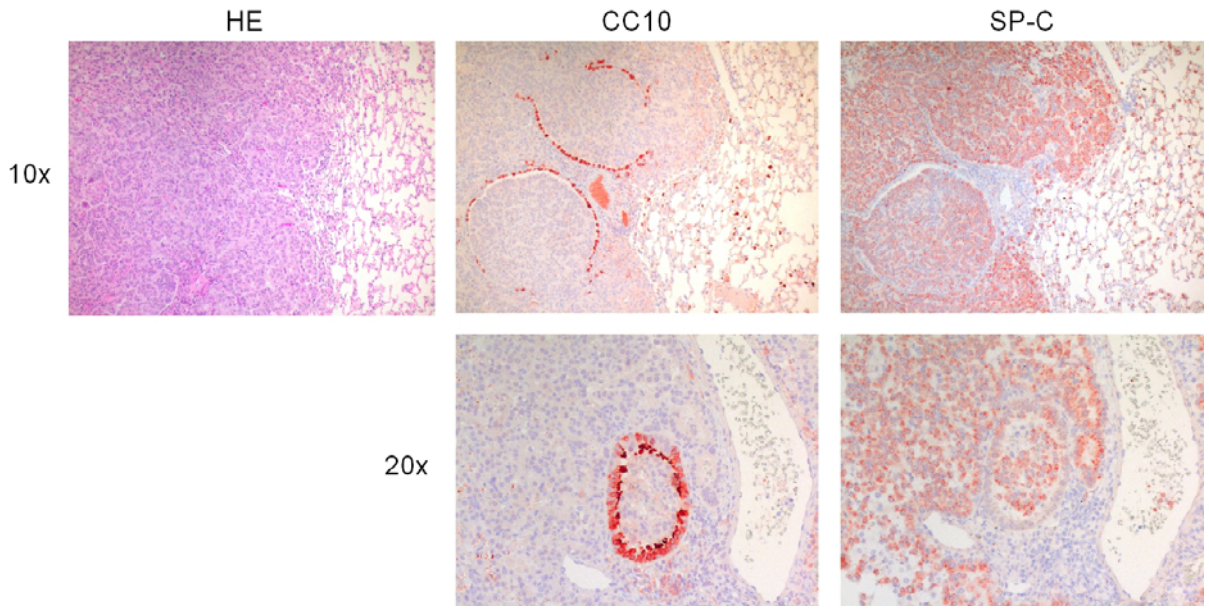


Figure 17: Determination of the cell type of origin for the lung tumors. IHC for the clara cell marker CC10 and the type-II-pneumocyte marker SP-C in two different lung tumors (upper lung tumor at low magnification, lower lung tumor at high magnification) of RasE MultiHit mice. Note that the lung tumors are positive for SP-C but negative for CC10. The upper left image shows the corresponding HE staining.

As a next step the module activation in lung tissue was tested. Lung tissue taken 6 days after AdenoCre inhalation was compared to tissue from mice 12 weeks post infection. DNA was isolated from one short term lung and two lungs with beginning tumor formation and Southern blot analysis for the three modules was performed (Figure 18). Obviously the AdenoCre particles are poor activators of the MultiHit construct as for none of the three modules an "on" band is visible. In tissue harvested 12 weeks after AdenoCre inhalation there is a clear signal for the three "on" bands. This shows that cells with tumor supporting hits are positively selected.

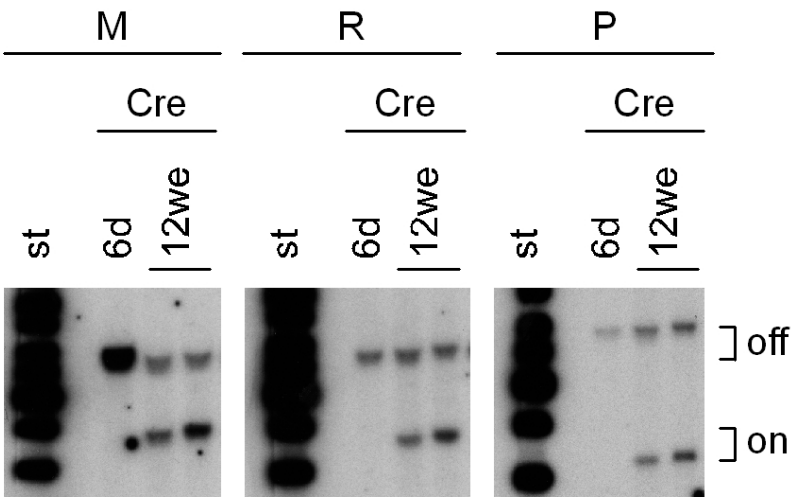


Figure 18: Module activation in lung tissue. Southern blot analysis was performed with lung tissue after short-term treatment with AdenoCre particles (6d) and with lung tissue bearing focal lesions (12wks). Note that short-term AdenoCre treatment is an inefficient inducer of M, R and P since only a few cells are infected by the viral particles. In contrast, a prominent band for M, R and P activation was present tumor-bearing lung tissue reflecting positive selection of Ras-transformed lung cells.

6.7. Expression of all three modules in lung and sebaceous tumors

Another way to check for mRNA levels is real time PCR (rtPCR). RNA was isolated from two tumor bearing lung samples and two skin appendage tumors (Figure 19). The obtained RNA is transcribed with reverse transcriptase and random hexamer primers into cDNA. The real time PCR machine measures the double stranded DNA content after each cycle of amplification. The relative expression can be calculated by comparing the measured values with the expression of a housekeeping gene like GAPDH. The graph shows similar expression of all three modules in the individual samples.

The lower level of MRP expression in lung tissue of MH90 (when compared to MH64) is most likely due to lower tumor load. Similarly, the lower level of MRP expression in T1 (when compared to T2) could be due to the presence of substantial amounts of non-tumorigenic tissue (uninduced skin, stroma or inflammatory cells).

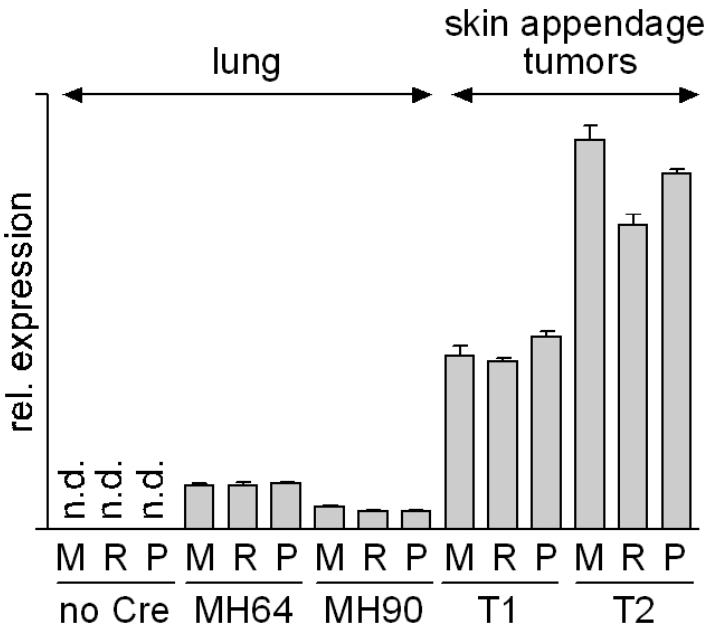


Figure 19: RNA expression analysis of tumor-bearing lung tissue and sebaceous gland tumors in RasE Rosa26CreER^{T2} MultiHit mice with realtime PCR. No expression of M, R and P was detected in the lung of RasE MultiHit mice without Cre (n.d.). Importantly, Cre-induction led to similar expression levels of M, R and P in individual tumor bearing lung tissues and sebaceous gland tumors (T1 and T2).

6.8. Single tumor genotyping with in situ hybridization

In situ hybridization was used to determine the module activation in single tumors. Labeled, antisense RNA probes against the coding sequence of the three reporter genes (EYFP, dsRed and hCD2t) were used to detect mRNA from the expression modules. The antisense probes hybridize to the corresponding mRNA molecules. The RNA probes are DIG labeled and can be detected with an anti-DIG antibody. Sense probes are used as negative controls to rule out unspecific staining.

6.8.1. Single tumor genotyping of lung tumors

Figure 20 shows the genotyping of four single tumors (T1-T4). The left images show the corresponding HE staining for all four tumors. Tumor T1 is positive for M and P but clearly negative for R, whereas tumor T2 is positive for all three modules. Tumor T3 is positive for M only and T4 is again positive for all three modules (lower panel). The lower images in both, the upper and the lower, panel show the staining with the sense probes. No unspecific staining could be detected in any of the slides.

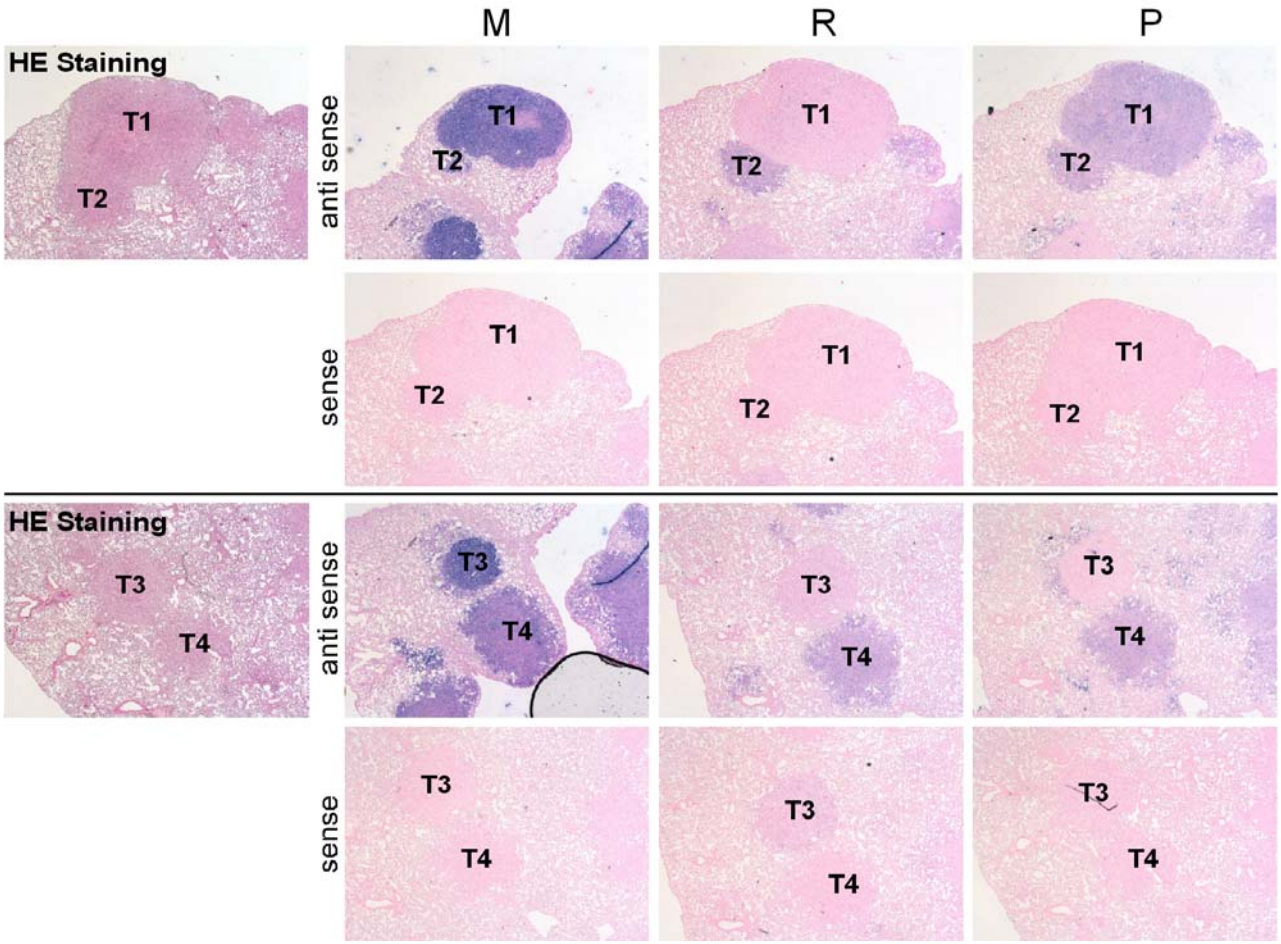


Figure 20: Single tumor genotyping with ISH. Probes detecting the individual reporter sequences EYFP, dsRed and hCD2t were used to detect M, R and P, respectively. ISH analysis of focal lesions demonstrates a differential activation pattern (activation in blue) in individual lung tumors. Four tumors (T1-T4) with different activation patterns are shown. Tumor 1 is positive for M and P, whereas T3 shows activation of M only. Tumors 2 and 4 are positive for all three modules Tissue sections hybridized with corresponding sense probes were used as control and did not give background signals (lower panels).

The appearance of all seven combinations in 190 analyzed tumors is summarized in Figure 21a and b. MRP tumors are by far the most abundant with 85.7%. Tumors induced by P activation alone came up in 6.6% of all cases, followed by MR and MP tumors. M and R tumors came up only once or twice, respectively and the combination RP was never found. Figure 21c shows the comparison of M, R and P activation in non-MRP tumors. From the activated single modules only P is a very good tumor inducer whilst the individual activation of M and R was only found once or twice, respectively.

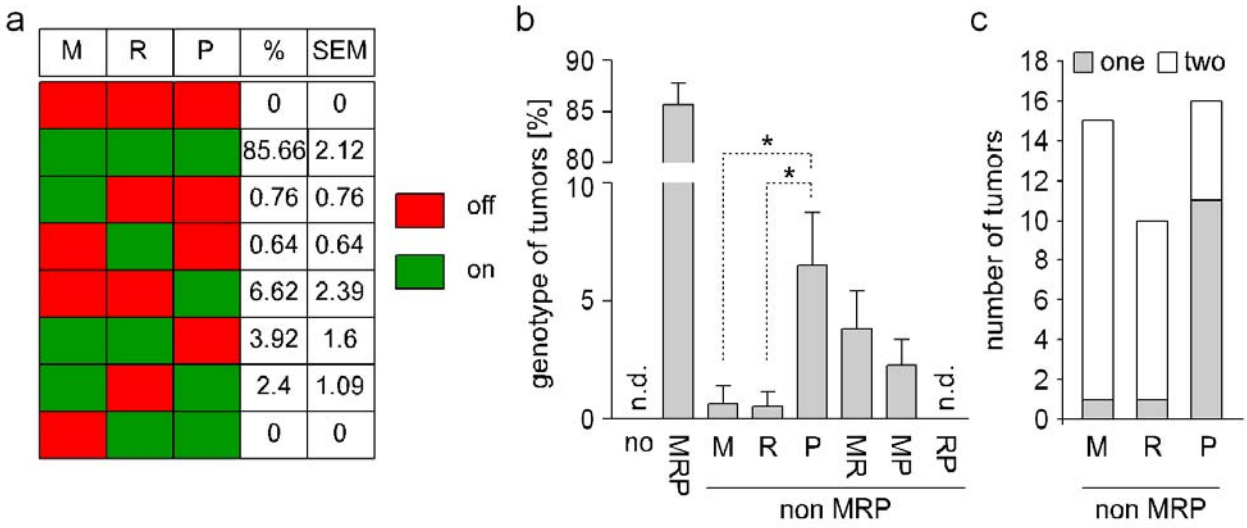


Figure 21: Analysis of module activation in 190 single tumors. (a) "Activation map" of M, R and P in lung tumors of 7 RasE MultiHit mice after AdenoCre treatment. The number and percentage of tumors positive for M, R and P combinations is summarized in the table. Red means module "off", green means module "on". (b) Tumors with one or two activation events are indicated as non MRP tumors. n.d.: not detectable. (c) Comparison of M, R and P activation in non MRP tumors. Gray areas in the bars indicate tumor numbers with single activation events M, R or P. M and R need cooperating activation events (white areas in bars indicate tumor numbers harboring M, R or P plus cooperating activation events).

6.8.2. Single tumors genotyping of sebaceous gland tumors

In situ hybridization was used to check the module activation on RNA level (Figure 22). The vast majority of tumors analyzed showed activation of all three tumors. However, one tumor out of 50 showed only activation of P.

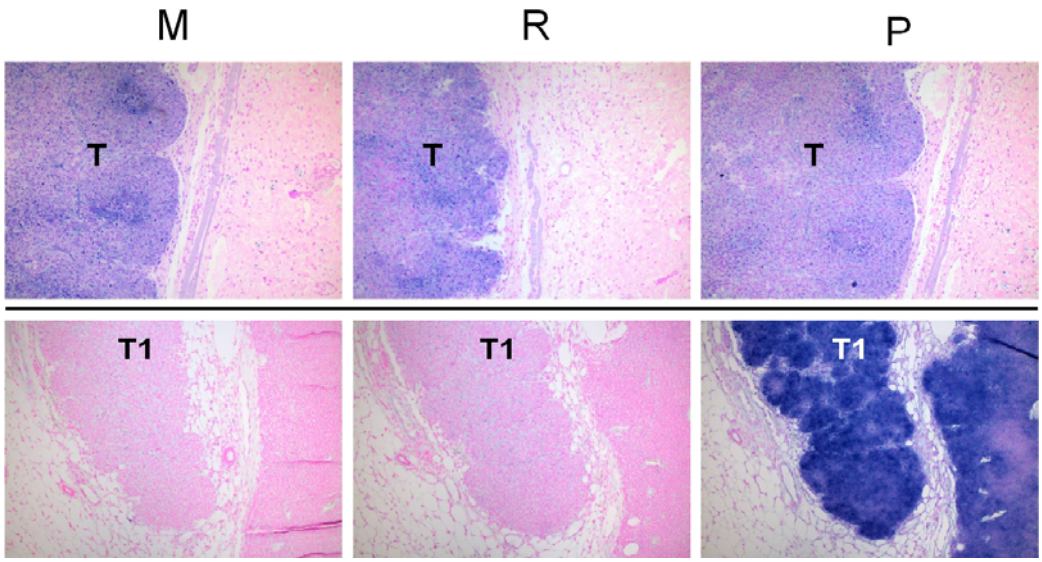


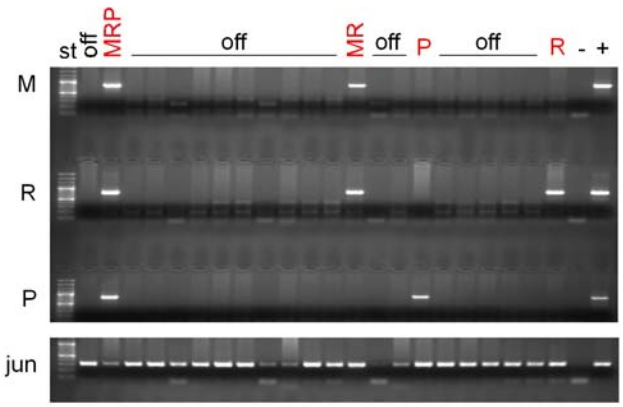
Figure 22: ISH analysis of sebaceous gland tumors. 98% of all sebaceous gland tumors showed activation (blue staining) of all three expression modules (upper row). Only one tumor out of 50 showed a different activation pattern (lower images, just P activated).

6.9. In vitro activation of the three expression modules

To exclude biased Cre-mediated activation effects the MultiHit construct was tested in vitro first. Mouse embryonic fibroblasts (HM1) containing a single copy of the MultiHit BAC were transfected with a Cre-expression plasmid. The cells were plated in low density and single cell clones were tested for module activation with PCR. Figure 23a shows a representative experiment with twenty two tested clones. Primers for Jun were used for the loading control.

Most of the clones have all three modules in off orientation because the transfection efficiency is only 10 - 12%. This is a situation mimicking the low infection efficiency of AdenoCre particles in the lung. The gel shows one MRP, one MR, one P and one R clone. The results of 13 similar experiments are summarized in Figure 23b. It shows clearly that all possible combinations can be found. RP and P clones are a little more frequent than the others. However, the RP combination was never found in vivo which suggests selection of certain combinations. Moreover, there was no overrepresentation of ES subclones with the MRP genotype that was frequently found in lung tumors. These data indicate that all possible Ras effector combinations were present in the lung of RasE mice after AdenoCre treatment but certain combinations are selected and give rise to tumors.

a



b

exp.	off	MRP	M	R	P	MR	MP	RP
1	21					1	1	1
2	20		1		1			
3	18			1	2			1
4	19	1		1				1
5	21	1						
6	20				1			1
7	18	1		1	1	1		
8	21				1			
9	18				2			2
10	20			1				1
11	21				1			
12	20		2					
13	18				1		1	
total	255	3	3	4	10	2	2	7
%	88.5	1.1	1.1	1.4	3.5	0.7	0.7	2.5

Figure 23: In vitro activation of the MultiHit construct in embryonic stem cells (ESC). PCR analysis of ESC clones excluded substantial Cre-biased activation effects. Transgenic embryonic stem (ES) cells with a single integration of the RasE MultiHit BAC were generated, transiently transfected with a Cre-expression plasmid and plated at low density in culture dishes. (a) After colony formation, individual ESC clones were used for DNA isolation and genotyped by PCR (one representative experiment is shown); -: no DNA; +: equimolar plasmid positive control, M: MAPK on; R: Ral on; P: PI3K on;. (b) Summary of 13 PCR experiments with ESC clones.

6.10. Histopathological analysis of lung adenocarcinomas

With support of the lung pathologist Helmuth Popper (Medical University of Graz) lung tumors with different module combinations were analyzed for their histopathological appearance (Figure 24). 29 MRP and 29 non MRP tumors were analyzed. Some MRP tumors are invasive and display an acinar architecture whereas non MRP tumors are never invasive and show no clear acinar architecture. Figures 24c and 24d show invasive MRP tumors. Newly formed stroma was detected by Movat pentachrome-staining[60]. Figure 24e depicts non-invasive P tumors growing into bronchi (upper left and upper right images) but respecting the blood boundary (arrowheads in upper middle image). Mixed solid and acinar tumor architecture of a P tumor is showed in the lower left and middle images of figure 24e. Low and high-grade in-situ carcinomas could be discerned based on the nuclear/cytoplasmic ratio and the presence or absence of large granules within the cytoplasm (lamellar bodies). The lower right picture shows one of the high-grade MP tumors.

Figure 24f summarizes the cooperations found between the three Ras effector pathways. It looks like Ral prevents PI3K-induced lung cancer formation (red) because this combination was not found in one of the lung tumors. PI3K alone has turned out to be the most effective tumor inducer upon the three effectors. To a lower extent cooperation of MAPK/Ral and MAPK/PI3K promotes lung cancer induction (yellow). Cooperation between MAPK, Ral and PI3K not only induces lung tumor formation but also promotes invasiveness (green).

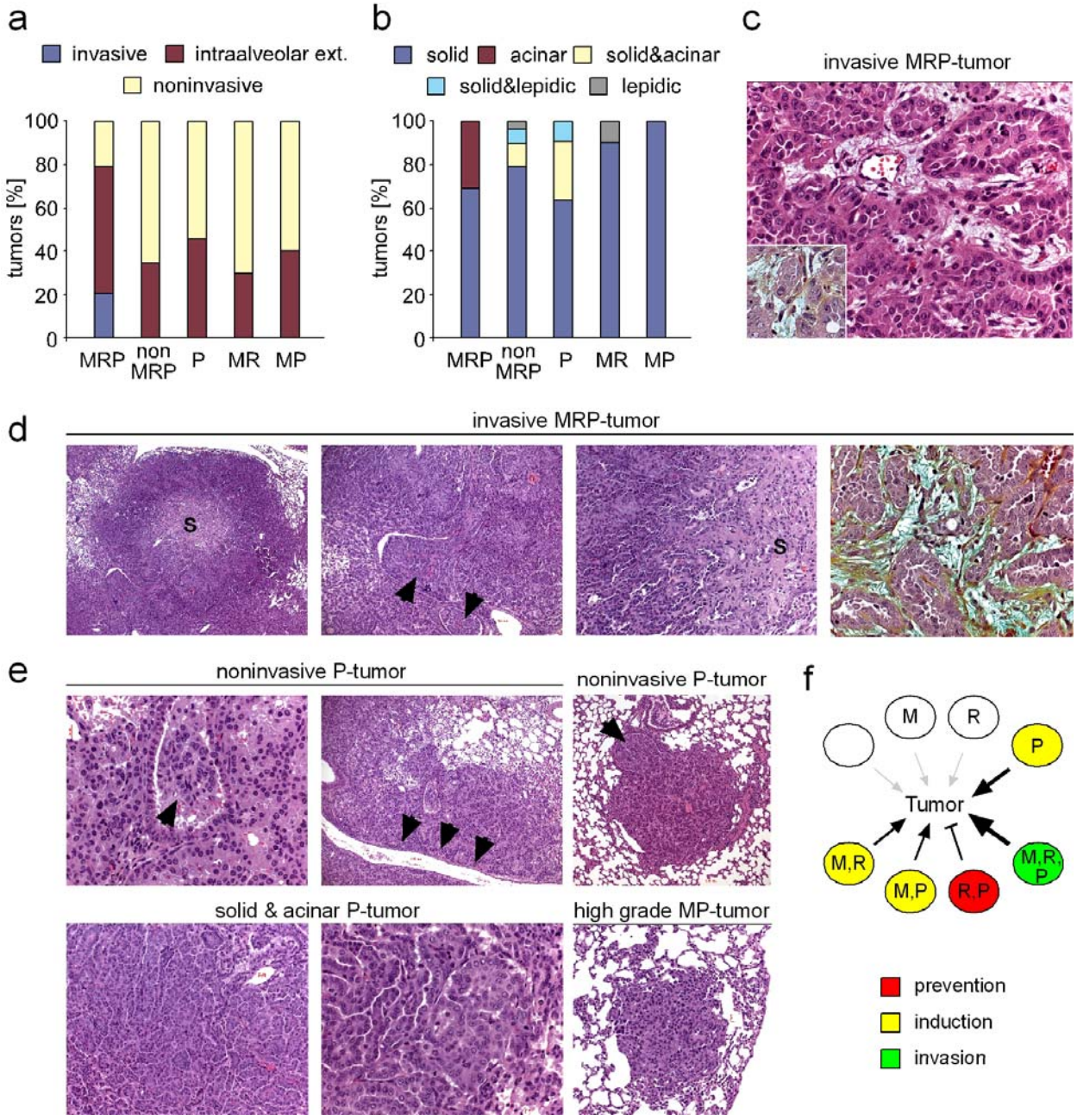


Figure 24: Histopathological evaluation of lung tumors. 29 MRP tumors and 29 non MRP tumors were histopathologically analysed for invasiveness (a) and tumor architecture (b). (c) Invasive acinar MRP tumor with carcinoma cells infiltrating a desmoplastic stroma. The inset represents Movat pentachrome-staining with newly formed tumor stroma in green. (d) Invasive acinar MRP carcinoma with desmoplastic stroma (S) in the center (left image and third image from the left). Presence of newly formed tumor stroma was confirmed by Movat pentachrome-staining (right image; newly formed stroma in green). Invasion occurred in this central area. Higher magnification demonstrated growth into bronchi (arrowheads) and establishment of tumor blood vessels (neoangiogenesis; second image from the left). (e) Examples of non MRP tumors.. (f) Summary of Ras effector pathway cooperations in lung tumorigenesis.

7. Conclusion and Outlook

In summary, the described results prove that the MultiHit construct is working in vitro and, as well, in vivo. The in vitro experiments showed that all eight combinations are technically possible after Cre-activation and none of the eight possible combinations was overrepresented.

We have established a MultiHit mouse model that mimics the positive selection process for cooperative hits in tumor formation. This model can be used in different experimental setups. We employed the MultiHit approach to investigate Ras downstream effector pathways MAPK (M), Ral (R) or PI3K (P) in lung cancer formation of mice. Our data indicate that lung cancer in mice is induced by Ras effector cooperativities with the following potency: MRP > P > MR > MP > M > R > RP.

Being aware of the difficulty to confer mouse data on humans, our analyses suggest that combined inhibition of MAPK and PI3K pathways might be the most powerful treatment for Ras-induced lung cancer. The importance of Ras-mediated PI3K activation in lung cancer and the efficacy of combined MAPK/PI3K inhibition in the KRas mouse model for lung cancer have been demonstrated previously[37,61,62] which positively evaluates our RasE MultiHit mouse model.

Inhibition of Ral might be bypassed by MAPK/PI3K cooperation. Moreover, in the absence of MAPK activation (or after inhibition of MAPK signaling by therapeutic intervention), inhibition of Ral might promote rather than prevent formation of PI3K-driven lung tumors. Our findings further suggest that all three Ras effector pathways are required for development of lung tumor invasiveness in RasE mice. Continuing experiments in the RasE mouse model should elucidate if additional genetic hits (e.g. loss of Pten or p53) change the requirement of individual Ras effector pathways in lung tumorigenesis. These analyses might suggest alternative routes for combinatorial drug treatment of tumors depending on the individual genetic status.

8. Abbreviations

APS	ammonium persulfate
bp	base pairs
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
ECL	enzymatic chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EtBr	etidium bromid
EtOH	ethanol
GAP	GTPase activating proteins
GAPDH	glycerinaldehyde-3-phosphate-dehydrogenase
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GTP	guanosine triphosphate
h	hour
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HISS	heat inactivated sheep serum
IHC	immunohistochemistry
ISH	in situ hybridization
kb	kilo bases
µ	micro (10^{-6})
m	milli (10^{-3})
M	molar
MAPK	mitogen activated protein kinase
min	minute
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase

MPW	MiliPore water
NaN_3	sodium acide
NaOH	sodium hydroxide
NaPi	sodium phosphate buffer
o/n	over night
PBS	phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinases
PCR	polymerase chain reaction
rt	room temperature
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
TE	Tris/EDTA
TEA	Triethanole amine
TEMED	Tetramethylethylenediamine
rpm	rotations per minute
Tris	tris hydroxymethyl aminomethane

9. References

1. Jemal, A., Siegel, R., Xu, J. & Ward, E. Cancer statistics, 2010. *CA Cancer J Clin* **60**, 277-300.
2. Chiang, A.C. & Massague, J. Molecular basis of metastasis. *N Engl J Med* **359**, 2814-23 (2008).
3. Sherr, C.J. Principles of tumor suppression. *Cell* **116**, 235-46 (2004).
4. Croce, C.M. Oncogenes and cancer. *N Engl J Med* **358**, 502-11 (2008).
5. Renan, M.J. How many mutations are required for tumorigenesis? Implications from human cancer data. *Mol Carcinog* **7**, 139-46 (1993).
6. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
7. Drake, J.W., Charlesworth, B., Charlesworth, D. & Crow, J.F. Rates of spontaneous mutation. *Genetics* **148**, 1667-86 (1998).
8. Jackson, A.L. & Loeb, L.A. The mutation rate and cancer. *Genetics* **148**, 1483-90 (1998).
9. Siderovski, D.P. & Willard, F.S. The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. *Int J Biol Sci* **1**, 51-66 (2005).
10. Wennerberg, K., Rossman, K.L. & Der, C.J. The Ras superfamily at a glance. *J Cell Sci* **118**, 843-6 (2005).
11. Reis, K., Fransson, A. & Aspenstrom, P. The Miro GTPases: at the heart of the mitochondrial transport machinery. *FEBS Lett* **583**, 1391-8 (2009).
12. Malumbres, M. & Barbacid, M. RAS oncogenes: the first 30 years. *Nat Rev Cancer* **3**, 459-65 (2003).
13. Drosten, M. et al. Genetic analysis of Ras signalling pathways in cell proliferation, migration and survival. *Embo J* **29**, 1091-104.
14. McCubrey, J.A. et al. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul* **46**, 249-79 (2006).
15. Chardin, P. & Tavitian, A. The ral gene: a new ras related gene isolated by the use of a synthetic probe. *Embo J* **5**, 2203-8 (1986).
16. Feig, L.A. Ral-GTPases: approaching their 15 minutes of fame. *Trends Cell Biol* **13**, 419-25 (2003).

17. Nicholson, K.M. & Anderson, N.G. The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* **14**, 381-95 (2002).
18. Chang, F. et al. Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia* **17**, 590-603 (2003).
19. Joneson, T., White, M.A., Wigler, M.H. & Bar-Sagi, D. Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. *Science* **271**, 810-2 (1996).
20. Camonis, J.H. & White, M.A. Ral GTPases: corrupting the exocyst in cancer cells. *Trends Cell Biol* **15**, 327-32 (2005).
21. Rodriguez-Viciana, P. et al. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* **89**, 457-67 (1997).
22. Travis, W.D., Travis, L.B. & Devesa, S.S. Lung cancer. *Cancer* **75**, 191-202 (1995).
23. van Zandwijk, N., Mooi, W.J. & Rodenhuis, S. Prognostic factors in NSCLC. Recent experiences. *Lung Cancer* **12 Suppl 1**, S27-33 (1995).
24. Schiller, J.H. Current standards of care in small-cell and non-small-cell lung cancer. *Oncology* **61 Suppl 1**, 3-13 (2001).
25. Morandi, U., Casali, C. & Rossi, G. Bronchial typical carcinoid tumors. *Semin Thorac Cardiovasc Surg* **18**, 191-8 (2006).
26. Etienne-Mastroianni, B. et al. Primary sarcomas of the lung: a clinicopathologic study of 12 cases. *Lung Cancer* **38**, 283-9 (2002).
27. Biesalski, H.K. et al. European Consensus Statement on Lung Cancer: risk factors and prevention. Lung Cancer Panel. *CA Cancer J Clin* **48**, 167-76; discussion 164-6 (1998).
28. Hecht, S.S. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. *Nat Rev Cancer* **3**, 733-44 (2003).
29. Villeneuve, P.J. & Mao, Y. Lifetime probability of developing lung cancer, by smoking status, Canada. *Can J Public Health* **85**, 385-8 (1994).
30. Schmid, K., Kuwert, T. & Drexler, H. Radon in indoor spaces: an underestimated risk factor for lung cancer in environmental medicine. *Dtsch Arztebl Int* **107**, 181-6.
31. Darnton, A.J., McElvenny, D.M. & Hodgson, J.T. Estimating the number of asbestos-related lung cancer deaths in Great Britain from 1980 to 2000. *Ann Occup Hyg* **50**, 29-38 (2006).

32. Giuliani, L. et al. Detection of oncogenic viruses SV40, BKV, JCV, HCMV, HPV and p53 codon 72 polymorphism in lung carcinoma. *Lung Cancer* **57**, 273-81 (2007).
33. Shimkin, M.B. & Stoner, G.D. Lung tumors in mice: application to carcinogenesis bioassay. *Adv Cancer Res* **21**, 1-58 (1975).
34. Chen, B., Johanson, L., Wiest, J.S., Anderson, M.W. & You, M. The second intron of the K-ras gene contains regulatory elements associated with mouse lung tumor susceptibility. *Proc Natl Acad Sci U S A* **91**, 1589-93 (1994).
35. Johnson, L. et al. Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. *Nature* **410**, 1111-6 (2001).
36. Dutt, A. & Wong, K.K. Mouse models of lung cancer. *Clin Cancer Res* **12**, 4396s-4402s (2006).
37. Jackson, E.L. et al. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev* **15**, 3243-8 (2001).
38. DuPage, M., Dooley, A.L. & Jacks, T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nat Protoc* **4**, 1064-72 (2009).
39. Guerra, C. et al. Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. *Cancer Cell* **4**, 111-20 (2003).
40. Murray, A.W. & Szostak, J.W. Construction of artificial chromosomes in yeast. *Nature* **305**, 189-93 (1983).
41. Shizuya, H. et al. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. *Proc Natl Acad Sci U S A* **89**, 8794-7 (1992).
42. Copeland, N.G., Jenkins, N.A. & Court, D.L. Recombineering: a powerful new tool for mouse functional genomics. *Nat Rev Genet* **2**, 769-79 (2001).
43. Muyrers, J.P., Zhang, Y., Testa, G. & Stewart, A.F. Rapid modification of bacterial artificial chromosomes by ET-recombination. *Nucleic Acids Res* **27**, 1555-7 (1999).
44. Chappell, S.A., Edelman, G.M. & Mauro, V.P. A 9-nt segment of a cellular mRNA can function as an internal ribosome entry site (IRES) and when present in linked multiple copies greatly enhances IRES activity. *Proc Natl Acad Sci U S A* **97**, 1536-41 (2000).
45. Potts, W., Tucker, D., Wood, H. & Martin, C. Chicken beta-globin 5'HS4 insulators function to reduce variability in transgenic founder mice. *Biochem Biophys Res Commun* **273**, 1015-8 (2000).

46. Walters, M.C. et al. The chicken beta-globin 5'HS4 boundary element blocks enhancer-mediated suppression of silencing. *Mol Cell Biol* **19**, 3714-26 (1999).
47. Blaas, L., Musteanu, M., Zenz, R., Eferl, R. & Casanova, E. PhiC31-mediated cassette exchange into a bacterial artificial chromosome. *Biotechniques* **43**, 659-60, 662, 664 (2007).
48. Zhu, X.D., Pan, G., Luetke, K. & Sadowski, P.D. Homology requirements for ligation and strand exchange by the FLP recombinase. *J Biol Chem* **270**, 11646-53 (1995).
49. Lee, G. & Saito, I. Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination. *Gene* **216**, 55-65 (1998).
50. Branda, C.S. & Dymecki, S.M. Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. *Dev Cell* **6**, 7-28 (2004).
51. Hameyer, D. et al. Toxicity of ligand-dependent Cre recombinases and generation of a conditional Cre deleter mouse allowing mosaic recombination in peripheral tissues. *Physiol Genomics* **31**, 32-41 (2007).
52. Kuhn, R., Schwenk, F., Aguet, M. & Rajewsky, K. Inducible gene targeting in mice. *Science* **269**, 1427-9 (1995).
53. Harvey, M. et al. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. *Nat Genet* **5**, 225-9 (1993).
54. Suzuki, A. et al. T cell-specific loss of Pten leads to defects in central and peripheral tolerance. *Immunity* **14**, 523-34 (2001).
55. Vasioukhin, V., Degenstein, L., Wise, B. & Fuchs, E. The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. *Proc Natl Acad Sci U S A* **96**, 8551-6 (1999).
56. Hitchcock, C.L. et al. Neuroendocrine (Merkel cell) carcinoma of the skin. Its natural history, diagnosis, and treatment. *Ann Surg* **207**, 201-7 (1988).
57. Guerra, C. et al. Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. *Cancer Cell* **11**, 291-302 (2007).
58. Bishop, A.E. Pulmonary epithelial stem cells. *Cell Prolif* **37**, 89-96 (2004).
59. Atkinson, J.J., Adair-Kirk, T.L., Kelley, D.G., Demello, D. & Senior, R.M. Clara cell adhesion and migration to extracellular matrix. *Respir Res* **9**, 1 (2008).
60. Movat, H.Z. Demonstration of all connective tissue elements in a single section; pentachrome stains. *AMA Arch Pathol* **60**, 289-95 (1955).

61. Engelman, J.A. et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. *Nat Med* **14**, 1351-6 (2008).

62. Gupta, S. et al. Binding of ras to phosphoinositide 3-kinase p110alpha is required for ras-driven tumorigenesis in mice. *Cell* **129**, 957-68 (2007).

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