Genetic predictors for the response to therapy in colorectal cancer cells

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Genetic predictors for the response to therapy in colorectal cancer cells

Genetische voorspellers voor de respons op therapie in colorectale tumorcellen

(met een samenvatting in het Nederlands)

Proefschrift

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Aan mijn ouders

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Chapter 1

Introduction

The first description of gastrointestinal cancer goes back to Hippocrates (460-377 b.c.) (1;2). Until the 19th century no effective treatment for colorectal cancer had been described when in 1829 the Parisian surgeon Jacques Lisfranc performed the first transanal rectal cancer excision in a 45 year old patient, with full recovery (3). The non-surgical treatment made its first breakthrough in 1957, with the discovery of fluorouracil (4). Earlier treatments with mustard gas (5) and the intravenous administration of lead (6) had shown no effect.

Fluorouracil in combination with the platinum compound oxaliplatin, which was added in the 90's, has now become part of the standard chemotherapeutic treatment for metastasized colorectal cancer. Nonetheless, response rates to currently used therapeutics are limited to 40-50% (7;8), and drug resistance is thought to cause treatment failure in over 90% of the initial responders (9). These data show that at least half of the patients will not show a treatment response. Identification of patients for whom current treatment will be beneficial is crucial, considering therapy associated morbidity, mortality and costs.

While the term "colorectal cancer" suggests a single disease, the genetic causes of this disease are extremely heterogeneous. This genetic heterogeneity is related to a widely diverse response to therapy. This makes it difficult to treat patients effectively with universally applied therapeutics as is mostly done today. Finding genes or combinations of genes, with predictive value could therefore improve treatment standards.

CHEMOTHERAPY INDUCED CELL DEATH

5-fluorouracil

5-fluorouracil (5-FU), inhibits thymidylate synthase (TS) and functions as a pyrimidine analogue. By inhibiting TS, 5-FU blocks the synthesis of the pyrimidine thymidine which is a nucleotide required for DNA synthesis. TS methylates deoxyuridine monophosphate (dUMP) into thymidine monophosphate (dTMP). Facing a scarcity of dTMP, rapidly dividing cancerous cells undergo thymineless death (10). As a pyrimidine analogue, 5-FU is transformed inside the cell into different cytotoxic metabolites which are then incorporated into DNA and RNA. Both mechanisms are thought to cause DNA damage. If DNA damage accumulates to unacceptably high levels, the cell will initiate a suicide program (apoptosis). The tumor suppressor gene *TP53* is an important initiator of apoptosis following activation of the DNA damage response. Following DNA damage, p53 is stabilized and translocates to the nucleus. p53 acts as a transcription factor. Its target genes include genes that promote DNA repair, cell cycle arrest, senescence and apoptosis (11;12). Indeed, 5-FU causes accumulation of p53 and activation of p53 response genes (13). In addition, colorectal cancer cells without wild type p53 are less sensitive to 5-FU treatment (14).

Oxaliplatin

Oxaliplatin is a platinum compound which binds cellular DNA. Oxaliplatin adducts mainly form links within single DNA strands (intrastrand links) but, to a lesser extent , also between the two strands of a DNA molecule (interstrand links) and even between protein molecules and DNA (15). Crosslinks are formed between two guanine residues or a guanine and an adenine. This disrupts DNA replication and transcription and causes DNA damage, resulting in activation of the DNA damage response and p53 stabilization (16). Targeted inactivation of p53 also increases oxaliplatin resistance in colorectal cancer cells (17).

APOPTOSIS AND CHEMO-RESISTANCE

The intrinsic and extrinsic apoptotic pathway

Programmed cell death, or apoptosis, can be caused by both the intrinsic and extrinsic pathways (18). The intrinsic, or mitochondrial apoptosis pathway, is characterized by a cascade of signaling events which is initiated by DNA damage, growth-factor deprivation, oncogene activation, hypoxia and microtubule disruption. As a result of these stress signals, p53 is activated. p53 in turn, induces expression of the "BH3-only" proteins Noxa and Puma. BH3-only proteins are pro-apoptotic proteins which are members of the family of Bcl-2 proteins. This family consists of both pro-apoptotic proteins (Noxa, Puma, Bad, Bmf, tBid, Hrk, Bim and Bik), and pro-survival proteins (Bcl-2, Bcl-x_L, Bcl-w, Mcl1 and A1). BH3-only proteins induce apoptosis by neutralizing the pro-survival proteins through direct binding (see Figure 1). This enables the pro-apoptotic Bcl-2 effector modules, Bax and Bak, to form oligomers in the outer mitochondrial membrane. Subsequently, the integrity of the mitochondrial membrane is compromised (mitochondrial outer membrane permeabilization, MOMP) which leads to release of cytochrome c into the cytosol. Cytochrome c is essential for the formation of the 'apoptosome', a multiprotein complex that binds and activates caspase 9. Caspase 9 in turn activates the executioner caspase 3, which subsequently breaks down essential cellular structures (19;20).

How stress signals stimulate expression of BH3-only proteins is partly explained by stabilization of p53, but is still incompletely understood. Of the BH3-only proteins, Noxa and Puma are two classical p53 target genes. BH3-only proteins can be activated by increased transcription, protein stabilization and by post-translational modification (21-24). The pro-apoptotic proteins bind to their pro-survival relatives and, in doing so, neutralize their activity. As a result Bcl-2 can no longer neutralize Bak and Bax, which leads to activation of Bax or Bak and execution of the death program. Bim and Bid are reported to be direct activators of Bax and Bak, independently of their ability to bind Bcl-2. In addition, recent reports indicate that other BH3-only proteins can also directly activate Bax and Bak (25).

The extrinsic apoptotic pathway is triggered by the activation of death receptors on the cell membrane by their ligands, Fas (CD95) ligand and TRAIL. These ligands are members of the tumor necrosis factor (TNF) super family of cytokines. Binding of the ligand to its death receptor induces oligomerization of the receptors which initiates the formation of the death-inducing signaling complex (DISC). The DISC is formed by the recruitment of the adaptor protein FADD which recruits pro-caspase 8 and caspase 10 which are activated in the DISC.

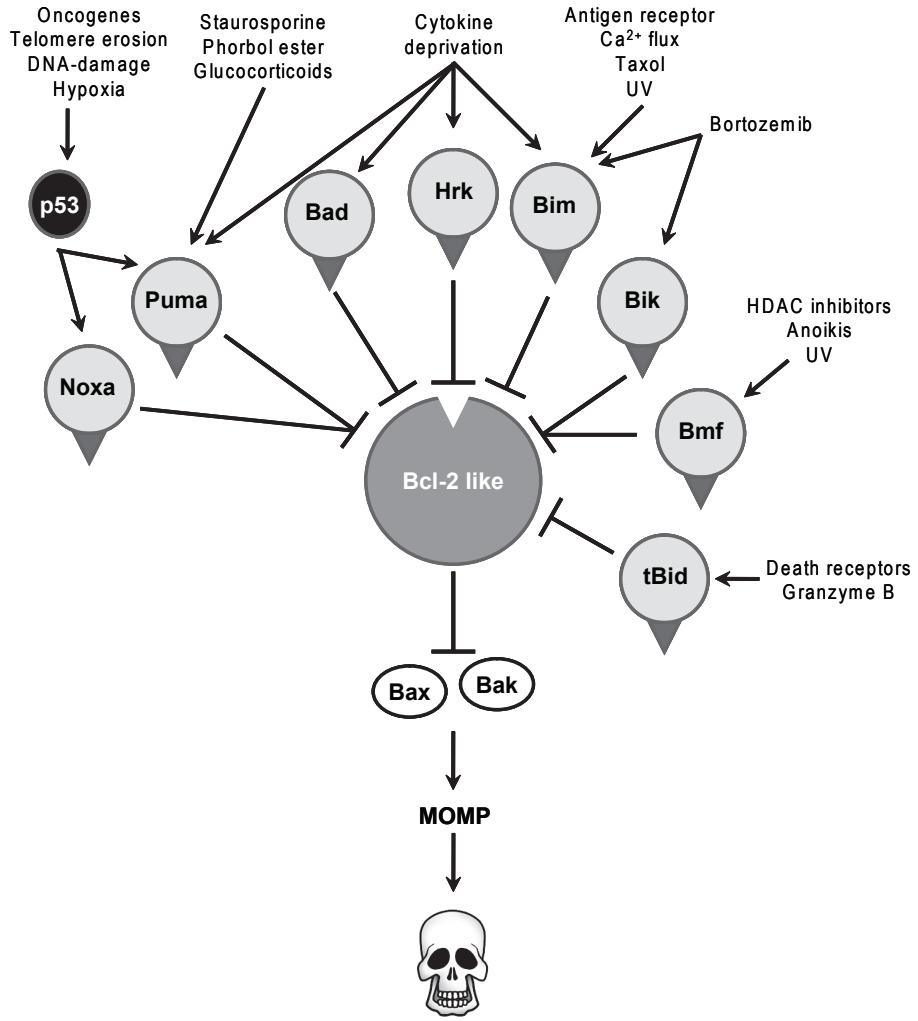

Figure 1. BH3-only proteins neutralize the Bcl-2 pro-survival proteins, which leads to Bax and Bak activation and subsequent mitochondrial outer membrane permeabilization (MOMP) and cell death. Adapted from Willis et al, Current Opinion in Cell Biology, 2005.

The initiator caspases 8/10 in the DISC then activate the downstream effector caspases 3, 6 and 7, which mediate cell destruction (26). In some cells (called type 1) the extrinsic pathway generates a signal that is strong enough to initiate apoptosis by itself. In most cells however, the death signal generated by the extrinsic pathway needs to be amplified (type 2 cells) to induce apoptosis. This amplification can occur through cross-talk between the extrinsic and the intrinsic pathway, caused by caspase 8 mediated truncation of the BH3-only protein Bid. Truncated Bid translocates to the mitochondria and neutralizes the Bcl-2 pro-survival proteins, and thus activates the intrinsic route (27).

The intrinsic and extrinsic pathways have been implicated in the tumor cell response to 5-FU and oxaliplatin. Both therapeutics induce apoptosis through the intrinsic pathway, by activation of p53 (28;29). However, the extrinsic pathway may also be involved, as both oxaliplatin and 5-FU mediate cell death through upregulation of the Fas-receptor and suppression of c-Flip, an inhibitor of the DISC complex (29-32).

Bcl-2 proteins in colorectal cancer

Expression of Bcl-2 is associated with resistance to 5-FU, irinotecan and cisplatin in various cancer model systems (33-36) and in patients with colorectal cancer (37). No clear correlation was found between the expression of Bcl-x_L, Bcl-w, Mcl-1, Bax or Bak and patient survival, or chemotherapy response (38-41). However, high levels of the BH3-only proteins Bad and Bid showed a correlation with longer progression free and overall survival in colorectal cancer patients receiving 5-FU based therapy (42).

Shifting the balance from pro-survival to pro-apoptotic protein expression and activity as a treatment strategy is gaining more and more interest. Tumor cells experience increased stress levels, caused by oncogenes, DNA-damage and an insufficient supply of oxygen and nutrients as a result of their rapid expansion. Overexpression of Bcl-2 is a mechanism by which tumor cells can withstand these stress levels (43). Specific therapeutic targeting of the pro-survival members of the Bcl-2 family of proteins could therefore increase apoptosis in cancer cells. Indeed, a small molecule inhibitor called ABT-737 has been developed by Abbott Laboratories. ABT-737 inhibits Bcl-2, Bcl-x_L and Bcl-w, but not Mcl-1 or A1, and has shown anti-tumor efficacy *in vitro* and in xenograft models (44;45). Tumor cells expressing increased levels of Mcl-1, however, show resistance to ABT-737 (46), most likely because Mcl-1 provides sufficient Bak/Bax-neutralizing capacity. Induction of Puma, Bim and tBid, which inhibit all Bcl-2 like proteins, results in a strong apoptotic response. In contrast, expression of the other BH3-proteins result in a weak apoptotic response, and a strategy is favored in which all Bcl-2 like proteins are neutralized (see Figure 2) (47). Indeed, ABT-737 is most efficient as an apoptosis inducer in tumor cells with high levels of the Mcl-1-neutralizer Noxa. Whether ABT-737 could have an application in the treatment of colorectal cancer is unknown.

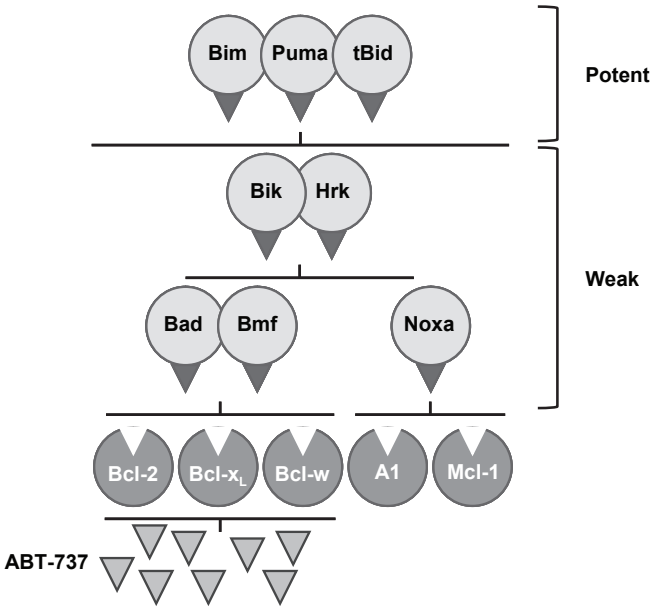

Figure 2. Pro-apoptotic proteins and their and anti-apoptotic binding partners. ABT-737 inhibits three members of the anti-apoptotic proteins. Adapted from Willis et al, Current Opinion in Cell Biology, 2005.

KRAS AND *TP53* AS PREDICTIVE BIOMARKERS IN COLORECTAL CANCER

While prognostic biomarkers indicate the expected natural course of tumor progression, predictive biomarkers indicate the expected response to a specific treatment. To identify predictive biomarkers, one can make a rough subdivision in systemic biomarkers (e.g. circulating tumor cells and serum protein levels) and tumor related biomarkers (e.g. (epi) genetic alterations, protein expression levels and histopathological appearance (TNM or Dukes classifications)). Systemic predictive biomarkers are currently not in routine clinical use. Although histopathological tumor classification can separate patients into groups with distinct outcomes, it offers little information about response to treatment in individual patients.

The commonly mutated *KRAS* oncogene and the *TP53* tumor suppressor gene have frequently been studied as potential prognostic and predictive biomarkers for treatment response in colorectal cancer

The KRAS oncogene and the MAPK signaling pathway

KRAS mutations are present in ~40% of all colorectal carcinomas and are associated with a worse prognosis (48;49). A number of studies have investigated KRAS mutations as a predictor for response to chemotherapy in colorectal cancer. When cetuximab (a monoclonal antibody against the EGF-receptor) is added to the conventional

chemotherapy, KRAS mutations are associated with a lack of response (50-52). Preclinical data suggests that the response to 5-FU, camptothecin (the active metabolite of irinotecan) (53) and oxaliplatin (54) is increased in cells with a KRAS mutation. However, although retrospective and small in patient number, clinical studies have not been able to show that KRAS status has value in predicting response to these chemotherapeutics (55-59).

Activating mutations in the *KRAS* oncogene are confined to codons 12, 13 and 61 and are acquired early in the adenoma-carcinoma sequence. However, mutations in additional genes (i.e. in the tumor suppressor gene APC)) are required for tumor development (60). The KRAS gene is a member of the RAS family, and encodes a small membrane-bound and growth factor-activated GTPase. The GTPase controls a "switch" to transmit growth factor signals from the receptor on the cell membrane into the cell. The RAS proteins activate different signaling pathways (RAF/MEK/ERK, PI3K/Akt, RalGDS, Rac/Rho, PLC/PKC), and mutations cause constitutive activation of these signal transduction pathways (61). This leads to uncontrolled growth, migration, invasion and apoptosis resistance (62). The oncogene itself has been thoroughly explored as a drugable target, however, without success (61;62). Components of the RAF/MEK/ERK pathway, which is associated with cell growth, proliferation and survival, are also investigated as therapeutic targets, most notably, RAF and MEK. The assumption is that constitutive activation of the MEK/ERK pathway in tumors with mutant KRAS is essential for tumor cell growth. Specific inhibitors of RAF and MEK1/2 have been developed to target this pathway. While such compounds could effectively block ERK phosphorylation in patient tumors, the anti-tumor responses were disappointing. This could be due to the fact that colorectal tumors do not rely on this pathway for their growth or that alternative compensatory pathways are activated during treatment (63;64).

***TP53* mutation status**

The *TP53* tumor suppressor gene is mutated in ~50% of all colorectal carcinomas (65) and is thought to play an important role in the progression of colorectal cancer. p53 activation can result in cell cycle arrest, accelerated DNA repair, apoptosis, senescence and/or differentiation (66). *TP53* becomes activated when cells are exposed to a variety of stress conditions, which are associated with cancer initiation and progression. These conditions include direct DNA damage, chromosomal aberrations, activation of oncogenes, hypoxia and telomere shortening. p53 is a sequence specific transcription factor, activating a number of specific target genes. One of the p53 target genes is CDKN1, encoding the cell cycle inhibitor p21. p21 binds and inactivates the cyclin-dependent kinase-2 which is essential for the G1-S transition and initiation of DNA synthesis. p53 also stimulates DNA damage repair by regulation of nucleotide excision repair and base excision repair, both through transcriptional activation and direct protein-protein interactions (67). Lastly, p53 may eliminate cells from the replicative pool either by promoting cell senescence, a state of irreversible cell cycle arrest, or by stimulating apoptosis (reviewed in (66)).

In doing so, *TP53* prevents the expansion of damaged cells into a large population of malignant offspring. Oncogene activation frequently leads to activation of p53, which explains the need to lose functional p53 for cancer cells to thrive. This probably explains why *TP53* is so frequently mutated in colorectal cancer and other cancers.

In contrast to the *KRAS* oncogene, mutations in *TP53* (mostly missense) are not localized to true hotspots, but occur throughout the gene. The phenotypic effects of *TP53* mutations can be classified into three non-mutually exclusive groups (68;69). First, mutations can abrogate or attenuate the binding of p53 to its consensus DNA-sequence and, consequently, impede the transcriptional activation of p53 target genes (70). Second, many mutations produce a highly expressed full length mutant p53 that inhibits the function of the wild type protein encoded by the second allele. The inhibition is caused by the fact that p53 is a tetrameric transcription factor, where the highly expressed inactive mutant causes formation of mixed tetramers that are incapable of DNA binding and transactivation (71). Lastly, several mutations endow mutant p53 with novel functions that are independent of wild type p53 and that stimulate rather than prevent tumorigenesis. Most gain of function properties are believed to stem either from direct transcriptional regulation and inactivation of the p53-related proteins p63 and p73, typically by blocking activation of their target genes, or by protein-protein interactions with numerous transcription factors and regulating their transcriptional output. Both positive and negative regulation occurs, by recruiting co-repressors or co-activators. What dictates whether mutant p53 recruits co-repressors or co-activators is currently unknown (72).

Most studies addressing the potential value of *TP53* as a predictor of response to therapy have simply categorized tumors as either mutant or wildtype. However, the highly divergent mutation-specific phenotypes suggest that different mutations in p53 may have a radically different impact on therapy response. This may explain why attempts to determine a possible predictive role in chemotherapy for mutant *TP53* have been unsuccessful so far (73).

THESIS OUTLINE

The general goal of this thesis is to create a better insight into the genes and signaling proteins that influence the response of colorectal cancer cells to MEK-targeted therapy and chemotherapy (5-FU and oxaliplatin).

In **chapter 2** of this thesis we set out to explore the mechanisms of resistance to inhibition of the MEK/ERK pathway. We found that mutant KRAS allows colorectal cancer cells to compensate for the loss of ERK activity by activating an alternative signaling pathway.

Chemotherapeutics induce DNA-damage by various mechanisms, which prompts the tumor suppressor gene *TP53* to halt the cell cycle and either repair the damage or induce

programmed cell death. In order to recommence the cell cycle following DNA repair, cells have to remain 'competent' for continued cell cycle progression. In **chapter 3** we show that the wild type p53-induced phosphatase (Wip1) enables cells to resume the cell cycle after DNA repair has been completed.

We and others previously observed an increased sensitivity of KRAS mutated colorectal cancer cells to 5-FU and oxaliplatin (53;54). This suggested that mutant KRAS could be of predictive value in the context of conventional chemotherapy treatment.
In **chapter 4** we elucidated the mechanism underlying this observation. The pro-apoptotic protein Noxa (Latin for damage) was a crucial factor in sensitizing tumor cells to apoptosis. We also show that both mutant KRAS and wild type p53 are required for Noxa induction and for sensitization to apoptosis.
In **chapter 5** we studied whether the combination of wildtype p53 and oncogenic KRAS was associated with increased response rates to oxaliplatin and fluoropyrimidine based chemotherapy in a cohort of colorectal cancer patients. We performed mutation analysis for both *KRAS* and *TP53* in patients and correlated them with response to chemotherapy.

After our discovery that upregulation of the pro-apoptotic gene Noxa (and hence neutralization of Mcl-1 and A1) was essential for oxaliplatin-induced apoptosis (chapter 4), we hypothesized that neutralization of the other Bcl-2 pro-survival proteins could synergize with oxaliplatin in inducing tumor cell apoptosis. Indeed in **chapter 6**, we show that the small molecule Bcl-2/Bcl-w/Blc-x_L-inhibitor ABT-737 and oxaliplatin induce apoptosis of colorectal tumor cells in a synergistic fashion, both in vitro and in tumor xenografts.

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Chapter 2

Oncogenic KRAS activates p38 to maintain colorectal cancer cell proliferation during MEK inhibition

ABSTRACT

Background

Colon carcinomas frequently contain activating mutations in the *KRAS* proto-oncogene. KRAS itself is a poor drug target and drug development efforts have mostly focused on components of the classical Ras-activated MEK/ERK pathway. Here we have studied whether endogenous oncogenic KRAS/K-ras affects the dependency of colorectal tumor cells on MEK/ERK signaling.

Methods

KRAS/K-ras mutant colorectal tumor cell lines C26, HCT116 and L169 were used. K-ras or components of the MEK/ERK and p38 pathway were suppressed by RNA interference (RNAi). MEK was inhibited by U0126. p38 was inhibited by SB203850.

Results

MEK inhibition, or suppression of MEK1/2 or ERK1/2 by RNA interference, reduced the proliferation rate of all colorectal cancer cell lines. However, cell proliferation returned to normal after two weeks of chronic inhibition, despite the continued suppression of MEK or ERK. In contrast, K-ras-suppressed tumor cells entered an irreversible senescent-like state following ERK pathway inhibition. MEK inhibition or ERK1/2 suppression caused activation of p38α in a KRAS/K-ras-dependent manner. Inhibition or suppression of p38α prevented the recovery of KRAS/K-ras mutant tumor cells during prolonged MEK inhibition.

Conclusion

Oncogenic KRAS/K-ras activates p38α to maintain cell proliferation during MEK inhibition. MEK-targeting therapeutics can create an acquired tumor cell dependency on p38α.

INTRODUCTION

Activating mutations in the *KRAS/K-ras* proto-oncogene are found in approximately 40% of colorectal tumors. Deletion or suppression of endogenous oncogenic *Ras* alleles from human and mouse colon tumor cells strongly reduces their tumorigenic potential (1-4). Efforts to generate effective Ras oncoprotein inhibitors have so far remained unsuccessful (5;6). Ras-activated signaling intermediates may serve as alternative targets for therapy. Indeed, components of the classical Ras-activated MEK/ERK pathway, in particular RAF and MEK, have served as targets for the development of novel anti-cancer drugs (7-9). MEK inhibitors are especially effective in tumor cells with activating mutations in the BRAF oncogene (10). However, tumor cell lines with activating mutations in *KRAS/K-ras* display a highly variable response to MEK inhibitors (11-15). Furthermore, the levels of steady state ERK phosphorylation in these cell lines also vary extensively and do not predict response to MEK inhibition (16). In mice, intestine-specific expression of K-ras^{D12} induces hyperplasia which depends on activation of the ERK pathway (17;18). However, the ERK pathway is no longer activated in intestinal tumors generated by oncogenic K-ras in cooperation with mutant APC and these tumors fail to respond to MEK inhibitors (19;20). In colon cancer patients, the majority of tumors display elevated levels of phosphorylated ERK and MEK when compared to normal mucosa (21;22). Although MEK inhibitors can effectively suppress ERK phosphorylation in human tumors, this does not correlate with robust anti-tumor responses (23;24). This demonstrates the existence of resistance mechanisms also in human tumors (25;26). The mechanisms underlying resistance to MEK-targeted therapy are incompletely understood. Recent work has shown that activation of the PI(3)-kinase pathway, by activating mutations in *PIK3CA* or inactivating mutations in PTEN, is a major cause of tumor cell resistance to MEK inhibitors (27).

The aim of this study was to assess whether tumor cells that are dependent on endogenous KRAS/K-ras, also depend on the MEK/ERK pathway. We show that oncogenic Ras does not cause addiction to this pathway, but allows tumor cells to recover from its inhibition by activating p38α. The finding that ERK pathway inhibition can create an acquired dependency on p38α may have implications for the use of MEK and p38-targeted therapeutics.

MATERIALS AND METHODS

Cell lines

The colorectal cancer cell lines C26 (K-ras^{G12D}) and HCT116 (KRAS^{G13D}) were obtained from ATCC. L145 and L169 were freshly isolated from colorectal liver metastases, and were established as spheroid cultures. Both spheroid populations contain a mutant KRAS^{G12D} allele. We previously established C26 cell lines in which the endogenous K-ras^{D12} allele is stably suppressed by mutant specific RNA interference, using a lentiviral vector (C26-KrasKD) (28). Control C26 cells were transduced with a lentiviral shRNA construct

targeting luciferase (see below). C26, all its derivatives, and HCT116 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine, 0,1 mg/ml streptomycin, and 100 U/ml penicillin. Human intestinal epithelial cells (HIEC) were kindly provided by Prof JF Beaulieu.

Human colorectal tumor specimens were obtained in accordance with the ethical standards of the institutional committee on human experimentation from patients undergoing a colon or liver resection for metastatic adenocarcinoma. Informed consent was obtained from both patients. The spheroid cells were cultured in advanced DMEM/F12 (Gibco) supplemented with 0,6% glucose (BDH Lab. Supplies), 2 mM L-glutamine (Biowhittaker), 9.6 µg/ml putrescin (Sigma), 6.3 ng/ml progesterone (Sigma), 5.2 ng/ml sodium selenite (Sigma), 25 µg/ml insulin (Sigma), 100 µg/ml apotransferrin (Sigma), 5 mM hepes (Gibco), 0,005 µg/ml trace element A (Cellgro), 0,01 µg/ml trace element B (Cellgro), 0,01 µg/ml trace element C (Cellgro), 100 µM β-mercapto ethanol (Merck), 10 ml antibiotic-antimycotic (Gibco), 4 µg/ml gentamicine (Invitrogen), 0.002% lipid mixture (Sigma), 5 µg/ml glutathione (Roche) and 4 µg/ml Heparin (Sigma). Growth factors (20 ng/ml EGF (Invitrogen) and 10 ng/ml b-FGF (Abcam)) were added to the cell culture medium freshly each week. All cell culture was carried out in non-tissue culture treated flasks (BD Falcon) at 37° C in a 5% CO2 humidified incubator. Spheroid cultures were maintained in low-adhesion flasks in stem cell medium without serum. Two-monthly mycoplasm tests confirmed that all experiments were performed in mycoplasm-free cell cultures.

Antibodies and Inhibitors

The following antibodies were obtained from Cell Signaling Technology Inc, Danvers, MA, USA: rabbit anti-pERK p44/42 (thr 202/tyr 204), rabbit anti-pAKT (ser 473), rabbit anti-pMEK1/2 (#9121) and the secondary antibody peroxidase conjugated anti-rabbit IgG. The following antibodies were all obtained from Santa Cruz biotechnology, Heidelberg, Germany: rabbit anti-MEK1 (sc219), rabbit anti-MEK2 (sc524), rabbit anti-p21 (sc397), mouse anti-cyclin D1 (sc450), goat anti-phospho-pRb (sc12901), mouse anti-p53 (sc126), rabbit anti-p16 (sc468), and goat anti-AKT1 (sc1618). Anti-p38a (#9218) and phospho-p38 (T180/T182) (#9211) were from Cell Signalling. Anti-phospho-MPM2 was from Millipore (#05-368). Anti-p27 (#554069) was from BD Biosciences (Alphen aan den Rijn, The Netherlands) and anti-actin (NB 600501) was from Novus Biological (Littleton, CO, USA.) The MEK inhibitor U0126 was from Promega, Madison, WI, USA and the PI3K inhibitor LY 294002 and the p38α,β inhibitor SB203850 were from Sigma, Saint Louis, MO, USA.

Lentiviral Constructs

To stably knock down ERK1, ERK2, MEK1 and MEK2 we used short hairpin RNAs (shRNAs) expressed by lentiviral vectors. The ERK1 and ERK2 constructs were kindly provided by Dr. Brambilla and were previously described (29). For knockdown of MEK1 and MEK2, we obtained lentiviral constructs from the TRC-library (Open Biosystems, Huntsville,

AL, USA). For MEK1 and MEK2 we used the target sets NM_008927 and NM_023138. TRCN0000025214 and TRCN0000055063 produced the most effective MEK1 and MEK2 knock down respectively and were used for all subsequent experiments. For p38 we used the target set NM_ 011951 of which TRCN0000055223 produced the most effective knock down. The lentiviral construct targeting luciferase (control) harbored the targeting sequence TGACCAGGCATTCACAGAAAT. Lentivirus production was performed as described before (30). Lentiviral infection was performed according to the Open Biosystems protocol.

Western Blotting

Western blotting was performed as described in (31), using 50 µg of cell lysate.

Proliferation and Population Doubling Assays

Cells were plated at a density of 5000 cells/well in 96-well plates. The relative number of viable cells in each well was then analyzed for 3-6 consecutive days by standard 3- (4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazoleumbromide (MTT) assays (Roche Diagnostics) according to the manufacturer's instructions. All proliferation asays were performed at least two times in triplicate. For population doubling assays the cells were seeded at a density of 50.000 cells/well on 6 wells plates and each well was passed at confluence in a 1:4 dilution (2 population doublings). The inhibitors U0126, LY 294002 and SB were used at a concentration of 10µM, unless stated otherwise. All population doubling experiments were performed at least twice. For measuring growth rates the [# population doublings / # days] was determined for all control and inhibitor-treated cell populations. The mean growth rate in control-treated cell populations was set to 100% and was used to calculate the relative growth rate in inhibitor-treated cell populations.

Statistical Analysis

Differences between the distinct treatment groups were evaluated using the student's t-test. Asterisks indicate statistical significance, based on two-tailed analyses of the data sets. Differences with p-values <0.05 were considered statistically significant.

RESULTS

Suppression of oncogenic K-ras sensitizes C26 colon tumor cells to MEK/ERK inhibition

First, we tested whether K-ras^{D12}-dependent C26 colon tumor cells (32) are also dependent on MEK/ERK pathway activity. Pharmacologic inhibition of MEK by U0126 reduced the rate of cell proliferation and caused extensive cell flattening (Figure 1A and B left upper and middle panel). U0126-treated C26 cells displayed strongly reduced levels of phosphorylated ERK1 and ERK2 as expected (Figure 1C), and accumulated in G1 with a concomitant reduction in S and M-phase cells (Figure 1D and E). Cell viability was not affected by U0126 treatment (Figure 1B and D).

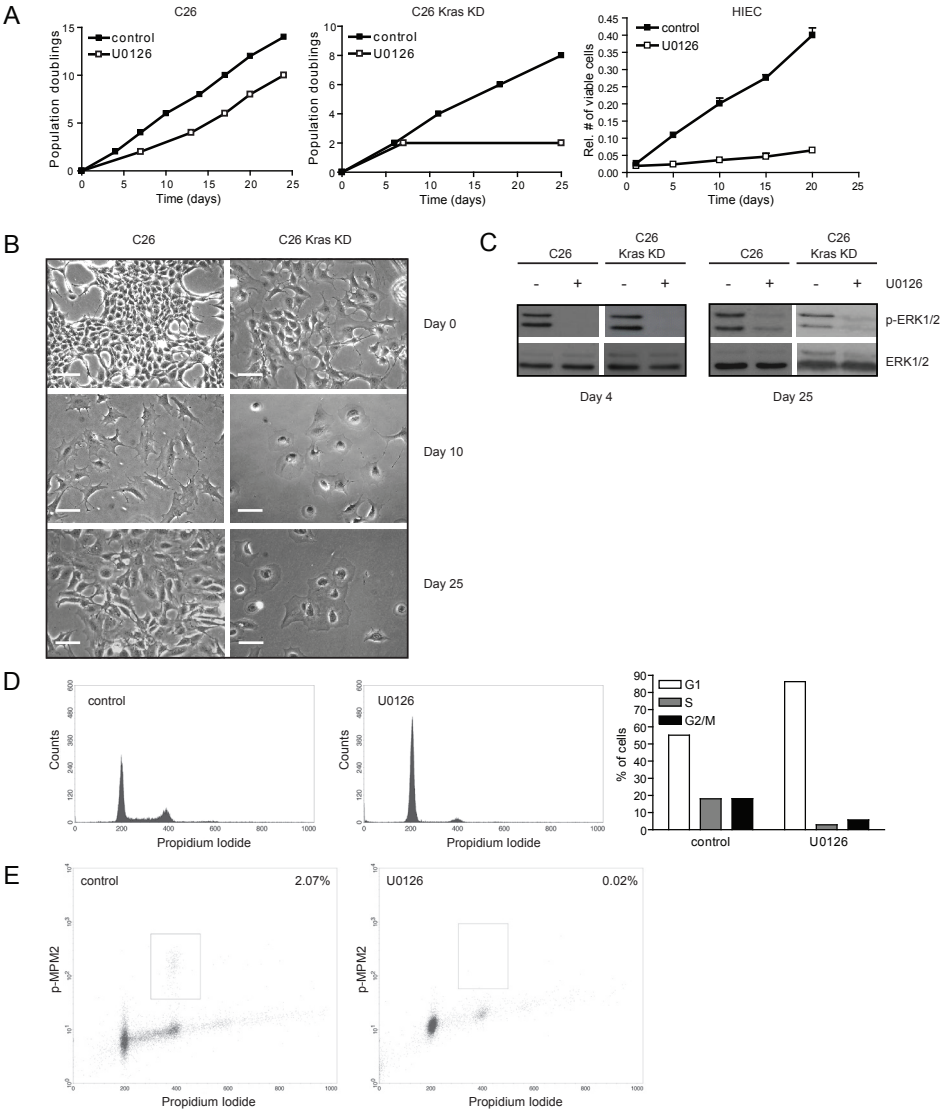
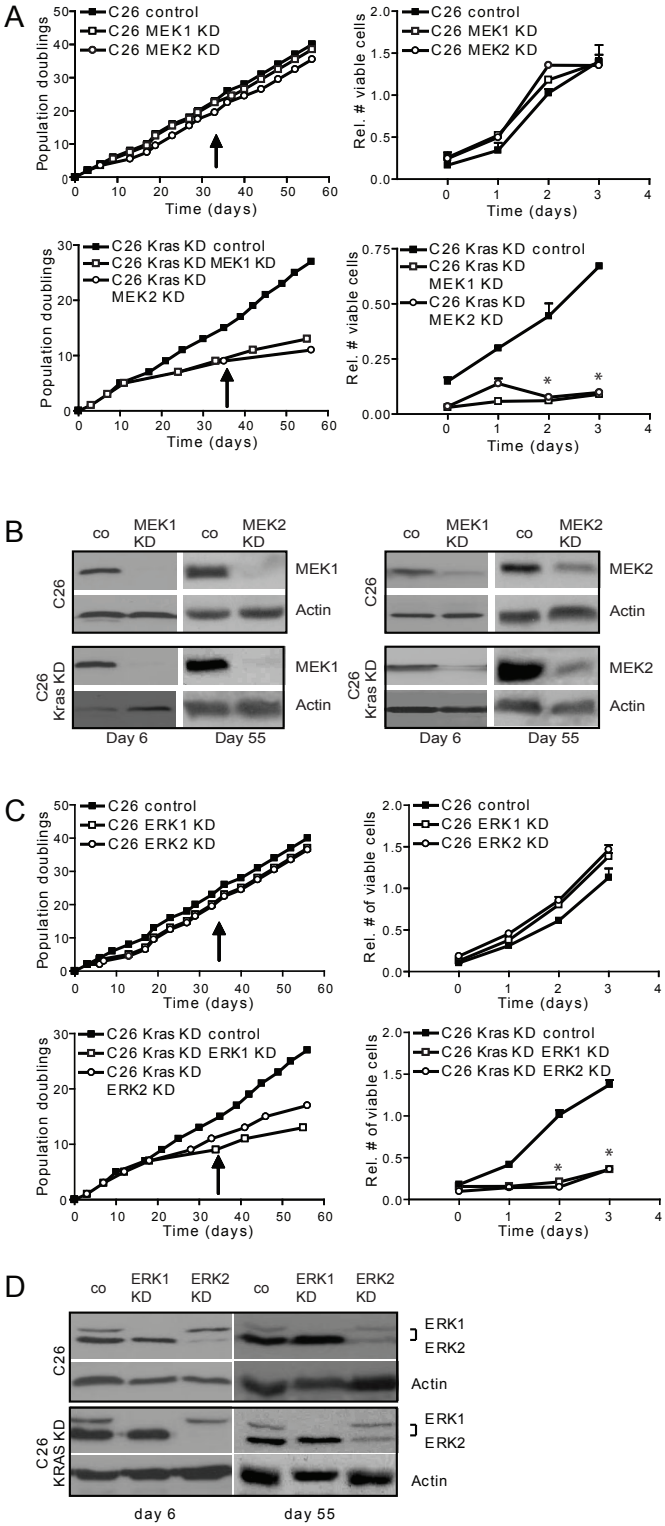


Figure 1. Oncogenic K-ras reduces C26 tumor cell dependency on MEK activity

(**A**) C26 and C26-KrasKD cells were treated with 10μM U0126, which was refreshed every 3 days. Cells were passed 1:4 at confluence so that each passage correlates with 2 population doublings. The graphs show population doublings of control and U0126 treated cells over time. The selective recovery of C26 cells from U0126-mediated growth arrest was observed in four independent experiments. A representative experiment is shown. (**B**) Photomicrographs showing C26 and C26-KrasKD cells (left and right panels) treated with 10μM U0126 for three days (middle panels) and 25 days (lower panels). (**C**) C26 and C26-KrasKD cells were treated as in A and the phosphorylation of ERK1 and ERK2 was assessed by anti-phospho-ERK1/2 Western blot analysis. (**D**) C26 cells were treated with 10μM U0126 for 2 days and the cell cycle profile was assessed by FACS analysis of propidium-iodide-stained cells. U0126-induced accumulation of C26 cells in G1 was observed in four independent experiments. A representative experiment is shown. (**E**) Cells were treated as in D, and the number of mitotic cells was assessed by FACS analysis for phospho-MPM2.


A
C26 control
C26 MEK1 KD
C26 MEK2 KD
Population doublings
Time (days)
Rel. # viable cells
C26 Kras KD control
C26 Kras KD MEK1 KD
C26 Kras KD MEK2 KD
B
co
MEK1 KD
MEK2 KD
C26
C26 Kras KD
MEK1
MEK2
Actin
Day 6
Day 55
C
C26 ERK1 KD
C26 ERK2 KD
C26 Kras KD ERK1 KD
C26 Kras KD ERK2 KD
Rel. # of viable cells
D
ERK1 KD
ERK2 KD
ERK1
ERK2
C26 KRAS KD
day 6
day 55

After approximately 2 weeks of chronic MEK inhibition C26 cell proliferation returned to normal and this was accompanied by a partial restoration of the original cell morphology, despite continued suppression of ERK1/2 phosphorylation (Figure 1A-C). Strikingly, U0126 treatment of C26 cells in which oncogenic K-ras is suppressed (C26-KrasKD (33)) caused extensive cell flattening and a complete loss of cell proliferation from which the cells did not recover (Figure 1A-C). Similarly, U0126 treatment of primary human intestinal epithelial cells caused a lasting growth inhibition from which cells were unable to recover (Supplementary Figure 1).

Next we tested whether K-ras suppression would similarly sensitize cells to inhibition of other Ras-effector pathways. To this end, C26 and C26-KrasKD cells were treated with the PI(3)K inhibitor LY294002 and cell proliferation was followed over time. LY294002 reduced the rate of cell proliferation in both cell types to a similar extent (by approximately 30%), without inducing gross alterations in cell morphology and without affecting cell viability (Supplementary Figure 2A and B). The levels of pAKT were stably suppressed in both cell types during the course of the experiment (Supplementary Figure 2C). These results show that endogenous oncogenic K-ras provides resistance to MEK inhibition, but not to PI3K inhibition.

Oncogenic K-ras reduces tumor cell dependency on MEK and ERK

We next assessed the relative importance of MEK1 and MEK2 in maintaining proliferation of tumor cells in the presence or absence of K-ras^{D12}. To this end, expression of either MEK1 or MEK2 was suppressed by using lentiviral RNA interference (RNAi) vectors. Stable knockdown of either MEK1 or MEK2 had no effect on the proliferation of C26 cells (Figure 2A and B). In contrast, knockdown of either MEK1 or MEK2 had a profound and lasting inhibitory effect on the proliferation of K-ras^{D12}- suppressed cells (Figure 2A and B), although the effect was less dramatic than that observed following U0126 treatment (Figure 1A). In both cell types MEK1 and MEK2 expression were successfully and stably suppressed (Figure 2B).

Figure 2. Oncogenic K-ras reduces C26 tumor cell dependency on MEK1&2 and ERK1&2

(**A**) C26 and C26-KrasKD cells were transduced with lentiviral shRNA constructs targeting MEK1, MEK2 or firefly luciferase (control). After puromycin selection population doubling assays were performed. The selective proliferation-suppressing effect of MEK1 and MEK2 knockdown in the C26-KrasKD cells was observed in four independent experiments. A representative experiment is shown. Short-term mitochondrial activity assays (MTT) were carried out 35 days post-selection in triplicate (indicated by the arrow). *indicates statistically significant differences ($p<0.01$). (**B**) At the indicated times following puromycin selection cell lysates were prepared and expression of MEK1 and MEK2 were determined by Western blotting. (**C**) C26 and C26-KrasKD cells were transduced with lentiviral shRNA constructs targeting ERK1, ERK2 or firefly luciferase (control). After puromycin selection population doubling assays were performed. The selective proliferation-suppressing effect of ERK1 and ERK2 knockdown in the C26-KrasKD cells was observed in four independent experiments. A representative experiment is shown. Short-term mitochondrial activity assays (MTT) were carried out 35 days post-selection in triplicate (indicated by the arrow). *indicates statistically significant differences ($p<0.01$). (**D**) At the indicated times following puromycin selection cell lysates were prepared and expression of ERK1 and ERK2 were determined by Western blotting.

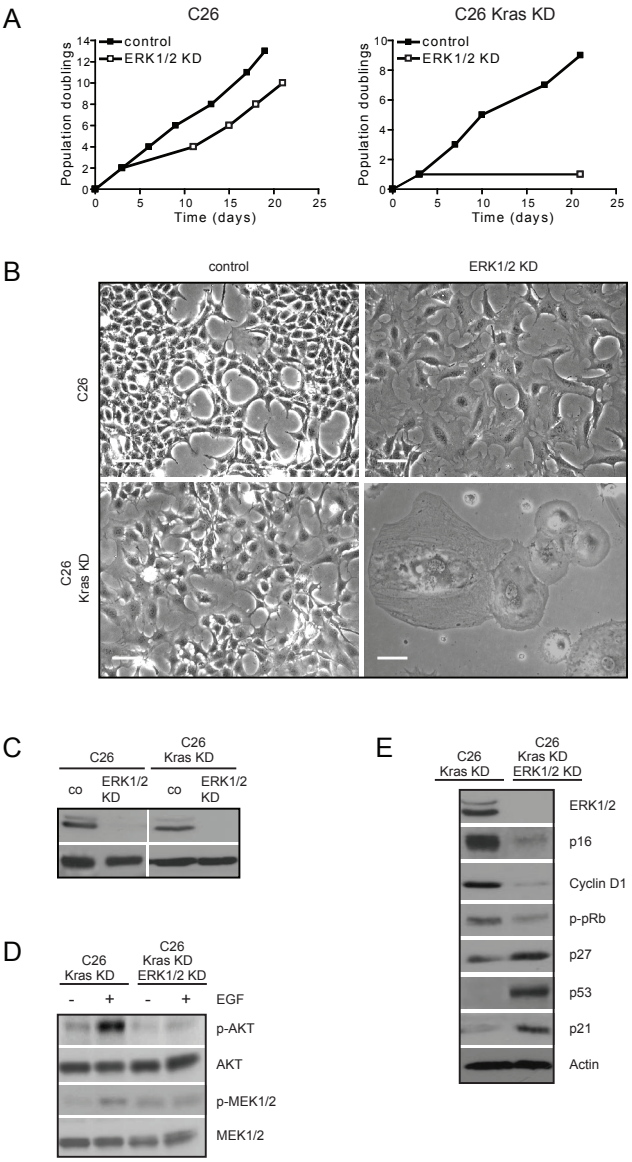


Figure 3. Oncogenic K-ras prevents senescence induction following ERK silencing

(**A**) C26 and C26-KrasKD cells were transduced with lentiviral shRNA constructs targeting ERK1 plus ERK2 (ERK1/2 KD) or targeting firefly luciferase (control). After puromycin selection population doubling assays were performed. The proliferation of ERK1/2-suppressed C26-KrasKD cells was completely abrogated after a single passage three days after initiation of selection. The selective proliferation-suppressing effect of ERK1/2 knockdown in the C26-KrasKD cells was observed in four independent experiments. A representative experiment is shown. (**B**) Photomicrographs of C26 and C26-KrasKD cells fourteen days after targeting luciferase (control) or ERK1/2. Bars 50µm. (**C**) The cells in B were lysed and expression of ERK and ERK2 was assessed by Western blotting using a polyclonal anti-ERK1/ERK2 antibody. (b) Fourteen days after transduction of the control (luciferase) and ERK1/2 targeting shRNA vectors, the cells were stimulated with EGF (20 ng/ml, 3 minutes) and analyzed for AKT and MEK phosphorylation by Western blotting using phospho-specific antibodies. (**E**) C26-KrasKD control and senescent-like cells were lysed and analyzed for expression of the indicated markers of cell cycle arrest and senescence by Western blotting.

The classical MEK targets are ERK1 and ERK2. Suppression of either kinase by RNA interference had a transient but reproducible inhibitory effect on the proliferation of C26 cells, but cell proliferation returned to normal after approximately two weeks of cell culture (Figure 2C and D). However, ERK1 or ERK2 knockdown had a far more dramatic effect on K-ras^{D12}-suppressed cells, causing profound and long-term inhibition of cell proliferation (Figure 2C and D). Taken together, the results show that endogenous K-ras^{D12} reduces the dependency of C26 cells on MEK and ERK.

Oncogenic K-ras prevents senescence induction following ERK silencing

The inhibition of MEK in cells lacking K-ras^{D12} resulted in complete cessation of cell proliferation, but did not induce cell death (Figure 1A). This prompted us to investigate whether the suppression of ERK1 and ERK2 in the absence of K-ras^{D12} would be sufficient to induce senescence. To this end, C26 cells and C26-KrasKD cells were transduced with a combination of the ERK1 and ERK2-targeting RNAi vectors. Combined knockdown of ERK1 and ERK2 induced cell flattening and reduced cell proliferation in C26 cells (Figure 3A-C), similar to U0126 treatment (Figure 1A). Cell proliferation returned to normal after approximately two weeks of cell culture similar to what was observed following treatment with U0126, or after single ERK1 or ERK2 knockdown. However, the combined knockdown of ERK1 and ERK2 in C26-KrasKD cells caused a complete cessation of cell proliferation after a single passage three days after initiation of selection (Figure 3A-C). The cells did not die but obtained a morphology that was reminiscent of the morphology of senescent cells (i.e. large flattened pancake-shaped cells; Figure 3B). An important hallmark of senescent cells is their inability to respond to growth factors. Indeed, EGF-stimulated MEK and AKT phosphorylation in control C26-KrasKD cells, but failed to do so in the senescent-like ERK1/2 knockdown cells (Figure 3D).

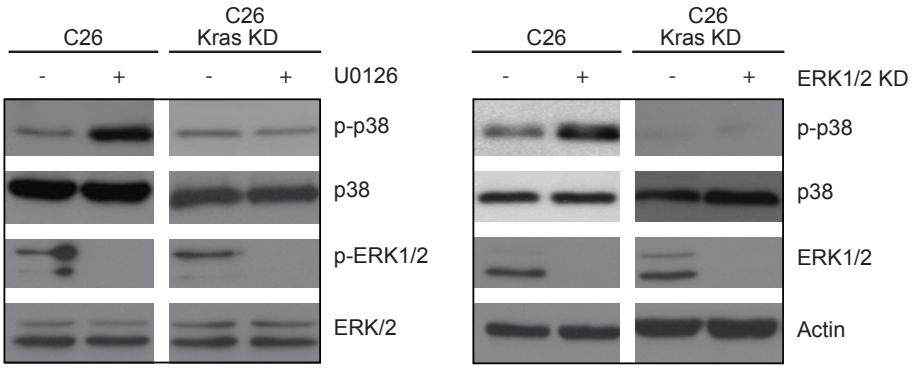
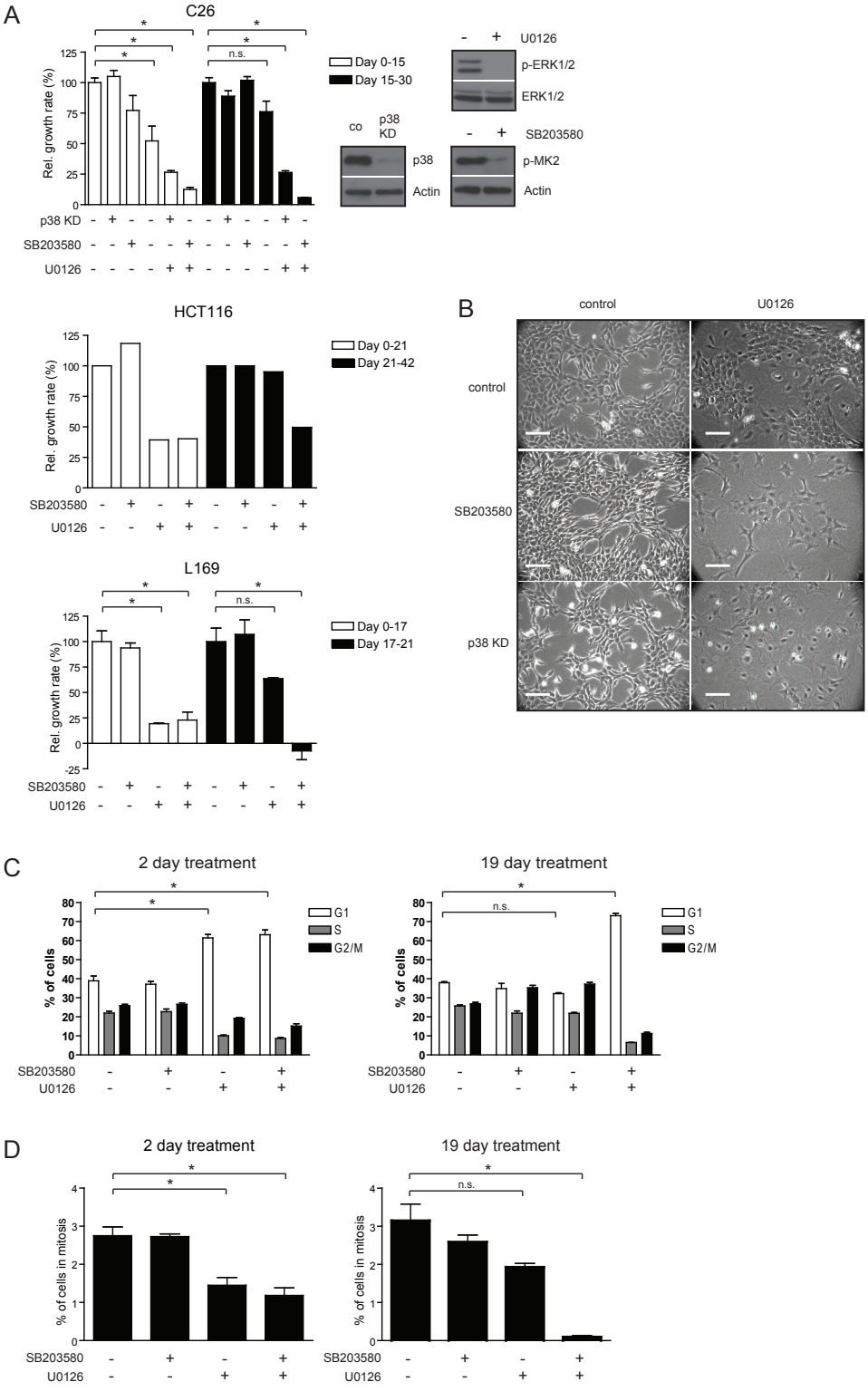


Figure 4. MEK/ERK inhibition causes K-ras-dependent p38α phosphorylation

C26, C26-KrasKD and HCT116 cells were treated overnight with U0126 as indicated. Alternatively, C26 and C26-KrasKD cells were transduced with shRNA vectors targeting luciferase (control) or ERK1 and ERK2 (ERK1/2) as indicated. After puromycin selection, cells were lysed and analyzed for the levels of total and phosphorylated p38α, ERK1 and ERK2. Both experiments were performed three times with similar results.


A
C26
Rel. growth rate (%)
Day 0-15
Day 15-30
n.s.
p38 KD
SB203580
U0126
co
p38 KD
p38
Actin
U0126
p-ERK1/2
ERK1/2
SB203580
p-MK2
Actin
HCT116
Day 0-21
Day 21-42
L169
Day 0-17
Day 17-21
B
control
U0126
control
SB203580
p38 KD
C
2 day treatment
19 day treatment
% of cells
G1
S
G2/M
D
2 day treatment
19 day treatment
% of cells in mitosis

In addition, the senescence-like cells also displayed loss of cyclin D1 expression and reduced phosphorylation of the retinoblastoma tumor suppressor protein pRb (Figure 3E). Although the cyclinD/cdk4 inhibitor p16 is usually strongly expressed in senescent cells its expression was lost in ERK1/2-suppressed cells, which further implicates ERK1/2 signaling in the control of p16 expression (34). The senescence-like cells also displayed activation of the p53 tumor suppressor and its target p21, and of the related cell cycle inhibitor p27 (Figure 3E). Taken together the data show that oncogenic K-ras prevents senescence induction as a result of ERK silencing.

Ras-dependent activation of p38α allows cell proliferation during ERK suppression

We next tested whether recovery of C26 cells from the U0126-imposed cell cycle arrest was correlated with activation of other MAP kinase pathways. Indeed, we found that MEK inhibition by U0126 or ERK1/2 suppression increased the phosphorylation of p38α in a K-ras^{D12}-dependent manner, both in C26 cells and in HCT116 cells (Figure 4).

To assess whether p38α activation was involved in mediating recovery from MEK/ERK inhibition we generated stable p38α knockdown cells and made use of the p38 inhibitor SB203580. Knockdown of p38α or treatment with SB203580 had no discernable effect on cell proliferation or on the cell cycle profile of C26 cells (Figure 5A-C). In addition, SB203580 had no effect on long-term proliferation of two additional human colorectal cancer cell lines expressing endogenous oncogenic KRAS (HCT116 and L169) (Figure 5A). U0126 treatment caused a temporary decrease in the growth rate of all three colorectal cancer cell lines (C26, HCT116, L145) (Figure 5A). However, all three cell types recovered from growth inhibition during chronic exposure to U0126 (Figure 5A). Strikingly, combination treatment with U0126 and the p38α,β inhibitor SB203580 completely prevented the recovery from U0126-induced growth inhibition in all three cell types (Figure 5A-C).

Figure 5. p38α mediates Ras-dependent tumor cell recovery from MEK inhibition

(**A**) C26 cells were transduced with lentiviral shRNA vectors targeting luciferase (control) or p38α. A western blot showing successful p38α knockdown is shown on the right. Alternatively, cells were treated with the p38-inhibitor SB203580 (10μM). Successful p38 inhibition was demonstrated by Western blot analysis of the phosphorylation state of the p38 substrate MK2. C26 cells in which p38 was either suppressed inhibited were then treated with U0126 (10μM) for 30 days. Medium (+/- inhibitors) was refreshed every three days. The growth rate of all treated cell populations relative to control cell populations was measured over time by population doubling assays before and after recovery, as indicated. Means and SEM of three independent experiments are shown. *denotes statically significant differences ($p<0.05$). ns: not significant. Similarly, two human colorectal cancer cell lines (HCT116 and L169) were treated with U0126 in the presence or absence of SB203580. The rate of cell proliferation before and after recovery from U0126 was measured as above. (**B**) C26 cells were treated with U0126 (10μM) for 21 days in the presence or absence of SB203580 (10μM). In addition, C26-p38KD cells were treated with U0126 (10μM) alone. The figure shows photomicrographs of C26 control and U0126-recovered cells (top panels), and of non-recovered cells in which p38 was either inhibited (middle panels) or suppressed (lower panels). Bars 50μm. (**C**) C26 cells were treated with U0126 and/or SB203580 as indicated for 2 or for 19 days. Cells were then fixed and stained with propidium iodide and the cell cycle profile was determined by FACS analysis. Means and SEM of three independent experiments are shown. *denotes statically significant differences ($p<0.05$). ns: not significant. (**D**) C26 cells were treated as in C. Inhibitor- and control-treated cell populations were fixed and the percentage of mitotic cells (phospho-MPM2-positive) was determined by FACS analysis. *denotes statically significant differences ($p<0.05$). ns: not significant.

In addition, specific knockdown of p38α completely prevented recovery from U0126-induced growth inhibition (Figure 5A and B), similar to p38 inhibition by SB203580.
FACS analysis of cell cycle profiles showed that U0126 caused a G1 arrest in C26 cells from which they recovered within 3 weeks (Figure 5C). Recovery was accompanied by the re-appearance of mitotic (p-MPM2-positive) cells in U0126-treated cultures (Figure 5D). Co-treatment of tumor cells with U0126 and SB203580 prevented cell cycle normalization and the appearance of mitotic cells (Figure 5C and 5D). Together, these studies indicate that p38α plays an important role in the recovery of colorectal cancer cells from U0126-imposed cell cycle arrest.

DISCUSSION

Our results show that oncogenic K-ras reduces the dependency of colon tumor cells on ERK pathway activity and that it prevents senescence induction as a result of MEK inhibition. The results from clinical studies so far indicate that treatment of cancer patients with MEK inhibitors can effectively lower the levels of pERK1/2 in different tumor types, including those of colorectal origin, but that this is not clearly correlated with changes in tumor cell proliferation or tumor progression (35;36). Tumor cells with oncogenic *Ras* depend on the continued presence of this oncogene for maintaining tumorigenic potential (37-40). However, its presence does not make tumor cells dependent on MEK/ERK pathway activity, but can even cause resistance to inhibition of this pathway (41;42). Identification of the K-ras-activated pathway(s) that desensitize(s) colon tumor cells to ERK pathway inhibition may therefore be the key to effective MEK-targeted therapy. A recent study showed that suppression of the PI(3)K pathway is an important determinant of colorectal tumor cell sensitivity to MEK inhibitors and that its inhibition greatly sensitizes tumor cells with oncogenic K-ras to MEK inhibition (43). Our study identifies the p38α pathway as a second K-ras-activated resistance pathway to MEK inhibitors. p38α was activated as a result of MEK inhibition in a Ras-dependent manner. Furthermore, this was required for recovery from cell cycle arrest and restoration of proliferative capacity in three independent colorectal cancer cell lines. The effect of p38α on cell cycle inhibition or progression is highly context-dependent (44). Most studies indicate that p38α primarily acts as a tumor suppressor (reviewed in (45)). The mechanisms of tumor suppression by p38α may vary under different conditions and in different cell types (46-48) and include suppression of EGFR signaling (49;50), JNK/c-Jun signaling (51;52) and activation of p53 (46;53). Indeed, p38α-suppression caused increased JNK phosphorylation and EGFR signaling in our cells (MdB, unpublished results) but this had no discernable stimulatory effect on tumor cell proliferation. p38α also plays a role in the G2/M checkpoint that halts cell cycle progression after DNA damage (54;55). The latter function of p38 may be less relevant in the context of MEK/ERK suppression which causes accumulation of cells in G1. Furthermore, we did not observe an increased number of cells entering mitosis following inhibition or suppression of p38α.

In addition to its tumor-suppressive activity, p38α can also stimulate cell proliferation. This is mostly observed in established tumor cell lines, for instance by contributing to cyclin D1 and cyclin E expression (56-62). In colorectal HT29 cells p38α contributes to cell proliferation and cyclin E expression (63). These authors also demonstrated that p38α inhibition ultimately caused autophagic cell death in colorectal cancer cell lines and suggested that p38α could serve as a target for therapy in colorectal cancer (64). In addition to p38α, p38γ may also promote colorectal tumor cell proliferation, as its inhibition reduced cell proliferation in HCT116 cells (65). However, p38γ is insensitive to SB203580 and its expression requires ERK pathway activity (66), suggesting that it does not play a role during recovery form MEK inhibition.

Future research should elucidate under which (stressed) circumstances colorectal tumor cells come to rely on p38α. The results presented here suggest that ERK inhibition can induce an acquired dependency on p38α, which offers a possibility for therapeutic exploitation.

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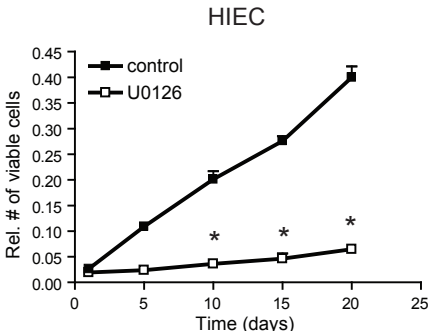
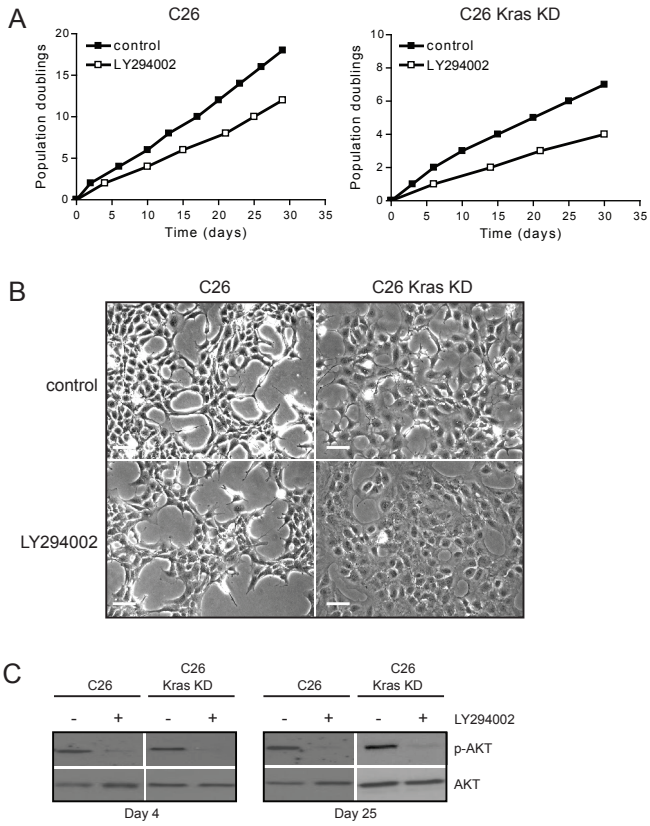
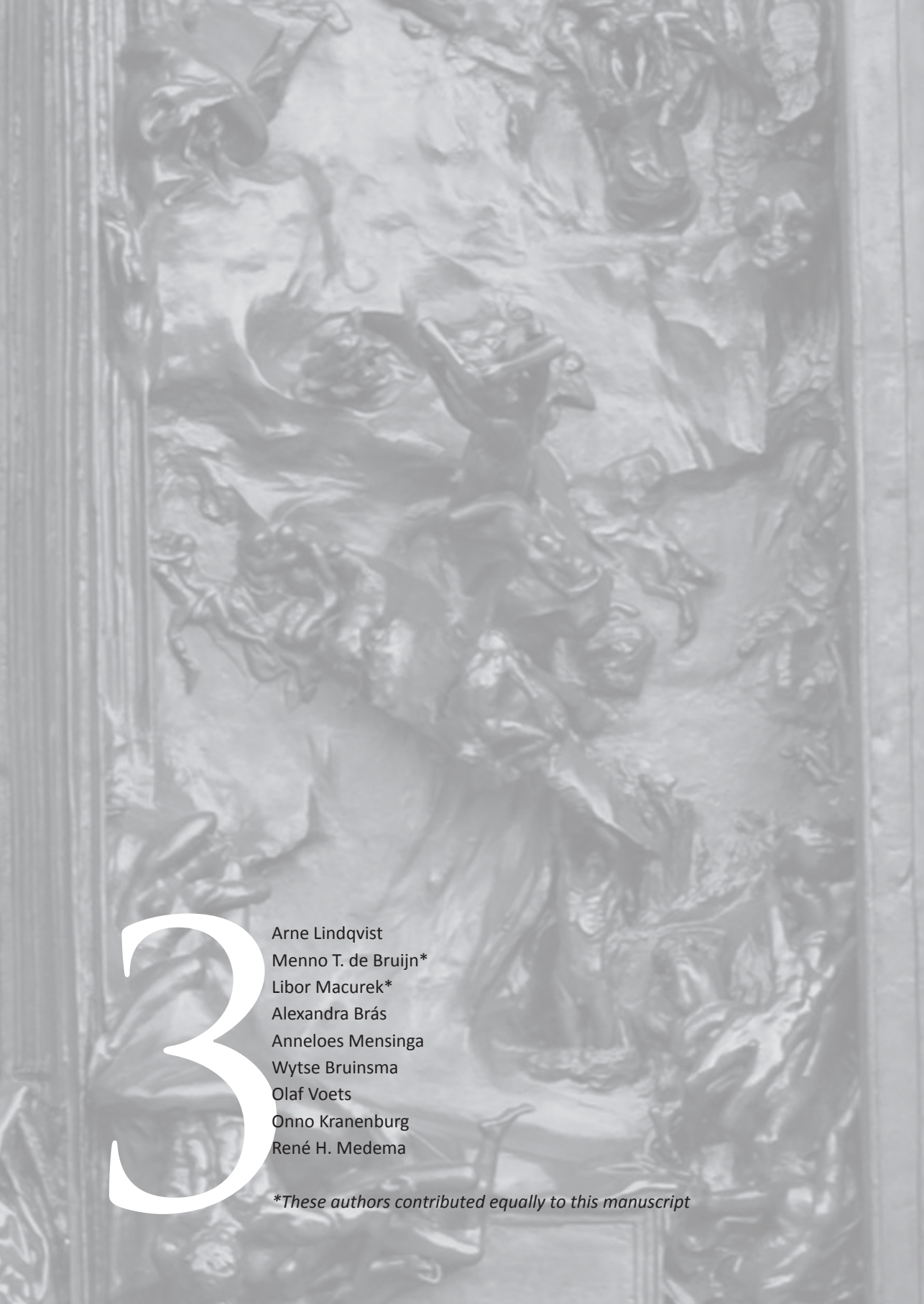
SUPPLEMENTARY FIGURES



Supplementary figure 1. Human intestinal epithelial cells do not overcome MEK inhibition
Cells were exposed to U0126 (5 μM) and were cultured for 20 days. At the indicated time points mitochondrial activity was analyzed by MTT assays, as a measure for the relative number of viable cells. The graph shows means and SEM of triplicate analyses. * denotes statistical significance (p<0.01). Apoptosis was not observed in these cultures.



Supplementary figure 2. Oncogenic Ras does not confer resistance to the PI(3)-kinase inhibitor LY294002
(**A**) C26 and C26-KrasKD cells were treated with 10μM LY294002. Cell proliferation was then followed over time by population doubling assays. (**B**), Photomicrographs showing control cells and cells treated with 10μM LY294002 for three weeks. Bars 50μm. (**C**), After four and twenty-five days all cells were lysed and analyzed for the presence of phosphorylated AKT by Western blotting.



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Chapter 3

Wip1 confers G2 checkpoint recovery competence by counteracting p53-dependent transcriptional repression

ABSTRACT

Activation of the DNA damage checkpoint causes a cell cycle arrest via inhibition of cyclin-dependent kinases (cdks). In order to successfully recover from the arrest, a cell should somehow be maintained in its proper cell cycle phase. This problem is particularly eminent when a cell arrests in G2, as cdk-activity is important to establish a G2 state. Here we identify the phosphatase Wip1 (PPM1D) as a factor that maintains a cell competent for cell cycle re-entry during an ongoing DNA damage response in G2. We show that Wip1 function is required throughout the arrest, and that Wip1 acts by antagonizing p53-dependent repression of crucial mitotic inducers, such as Cyclin B and Plk1. Our data show that the primary function of Wip1 is to retain cellular competence to divide, rather than to silence the checkpoint to promote recovery. Our findings uncover Wip1 as a first in class recovery competence gene, and suggest that the principal function of Wip1 in cellular transformation is to retain proliferative capacity in the face of oncogene-induced stress.

INTRODUCTION

Cyclin-Cdk activity is low in G1 cells and gradually increases until it culminates in mitosis, constituting the basis of a eukaryotic cell-cycle oscillator. The gradual rise in Cyclin-Cdk activity promotes cell-cycle progression by ensuring sequential phosphorylation of targets that enable events such as DNA replication and entry into mitosis. The Cyclin-Cdk complexes themselves largely create this gradual rise in Cyclin-Cdk activity, as Cyclin-Cdk-dependent feedback and feedforward loops ensure production and activation of more and different Cyclin-Cdk complexes (1;2). In this sense, rising Cyclin-Cdk activity not only promotes cell-cycle progression, but also defines the biochemical cell cycle state. Once a Cyclin-Cdk has been activated the positive feedback loops enforce its activity, ensuring that the cell cycle state is maintained. For example in G2 phase, Cyclin A-Cdk activity ensures transcription of targets that promote activation of Cyclin B-Cdk1, but also of targets promoting and maintaining Cyclin A-Cdk activity, thereby defining and maintaining a G2 state (3;4).

Activation of the DNA damage checkpoint in G2 by ATM and ATR kinases culminates in activation of the tumor suppressor protein p53 and inhibition of the Cyclin-Cdk complexes that are essential for mitotic entry (5). As a consequence, cells arrest in G2, with high levels of p53 and inactive Cyclin B-Cdk1 and Cyclin A-Cdk1/2 complexes (6-8). In addition, the checkpoint can reduce the transcription of a large number of G2 genes through the actions of p53 and Chk1 (9-13). Thus, the DNA damage checkpoint counteracts the cell-cycle oscillator through direct Cyclin-Cdk inhibition, but also by counteracting the transcriptional program that defines and maintains the G2-state (8). The question therefore arises how a cell can maintain a G2-state during an ongoing checkpoint arrest and retain competence to resume further activation of the cell cycle oscillator to complete cell division once the checkpoint is switched off.

When DNA double-stranded breaks (DSBs) occur, the checkpoint kinase ATM (ataxia-telangiectasia mutated protein) phosphorylates both p53 and Mdm2 (14-16), resulting in stabilization of p53. Interestingly, p53 levels were shown to oscillate in response to DSB formation (17;18), and these pulses were recently shown to depend on the presence of the phosphatase Wip1 (PPM1D) (19). Wip1 is a transcriptional target of p53 (20) that can antagonize ATM-dependent phosphorylation of p53 (21) and Mdm2 (22). In addition, Wip1 can dephosphorylate ATM (23), as well as other components of the DNA damage checkpoint, such as p38 (24), Chk2 (25;26), Uracil DNA-glycosylase (27) and Chk1 (21). In fact, the Wip1 and the ATM consensus sequences appear to be similar, indicating that Wip1 may dephosphorylate additional proteins involved in checkpoint signalling (28). This suggests that the activation of Wip1 creates an important negative feedback mechanism that controls reversion of the DNA damage-induced arrest when the DNA damage-induced signaling cascades have to be inactivated. However, the importance of p53 pulses is currently unclear. Also, it is not known if the crucial function of Wip1

during a DNA damage response is executed during termination of the response, i.e. by inactivating its various targets in the DNA damage-induced signaling cascade, or rather during the ongoing arrest, by controlling p53 oscillations.

Here we show that Wip1 is required throughout a DNA damage response in G2 to allow for eventual checkpoint recovery. Contrary to our expectations, Wip1 does not promote recovery by mere dephosphorylation of checkpoint kinases when the checkpoint is silenced, but rather exerts its effect on recovery already at earlier stages of the checkpoint response. We find that Wip1 counteracts p53-dependent repression of mitotic regulators throughout the DNA damage response in G2. This action of Wip1 is essential to retain the identity of a G2 cell and maintain its competence for further proliferation.

MATERIALS AND METHODS

Plasmids and antibodies

pRETROSUPER plasmids were targeting GACTCCAGTGGTAATCTAC (nt 775-793), GCAATGGATGATTTGATGC (nt 115-133) and CTACATGTGTAACAGTTCC (nt 705-723) in p53, and GGATGACTTTGTCAGAGCT (nt 612-630) and GTACATGGAGGACGTTACT (nt 57-75) in Wip1. Non-targetable Wip1 was constructed by introducing the silent mutations T615C and C618T in FLAG-Wip1 (Lu et al., 2005) cDNA, and phosphatase dead Wip1 (24) was constructed by introducing the D314A mutations using Quikchange site-directed mutagenesis (Stratagene). Both Wip1 and Wip1 D314A were subcloned to pCDN4TO plasmids (invitrogen). YFP-Cdc25B3 S323G was from (29). PS10 Histone H3, pS139 H2AX, H2AX and Plk1 antibodies were from Upstate, pT68 Chk2, pS317 Chk1, pS392 p53, pS20 p53 and pS15 p53 antibodies were from Cell Signalling, Wip1 antibodies were from R&D systems and Santa Cruz. Actin, p53, p21 and Cyclin B1 antibodies were from Santa Cruz. BrdU and PPP4C antibodies were from Abcam and FLAG antibodies were from Sigma.

Cell culture, transfection and microinjection

U2OS and HCT116 cells were kept in DMEM supplemented with 6% foetal calf serum and antibiotics. Monoclonal U2TR cell lines, containing tetracycline inducible Wip1 or Wip1 D314A were constructed as in (30), and kept in DMEM supplemented with 10% foetal calf serum and antibiotics. Thymidine, Caffeine, Puromycin, Paclitaxel, Nocodazole and Chk2 inhibitor II were from Sigma and used at 2.5 mM, 5 mM, 2 µg/ml, 1 µM, 250 ng/ml and 10 µM, respectively. SB202190 and SB218078 were from Calbiochem and used at 6 µM and 2.5 µM, respectively. UCN01 was a kind gift from Floris Foijer and used at 0.3 µM. Transfection with calcium phosphate was performed using the standard calcium phosphate technique. Cells were microinjected in HEPES-buffered medium with 0.1 µg/µl expression plasmid together with 0.01 µg/µl pEGFP-C1 (clontech) using an Eppendorf Micromanipulator 5171 coupled to a Transjector 5246.

Lentiviral transduction

HCT116 cells were transduced either by control lentivirus targeting luciferase, TGACCAGGCATTCACAGAAAT or by lentivirus targeting Wip1 GTGGACAATCAGGGAAACTTT (nt1135-1155) or CGAGAGAATGTCCAAGGTGTA (nt 1372-1392). Lentiviral particles were generated in COS7 cells by cotransfection (using fuGENE 6 Transfection Reagent; Roche Diagnostics, Mannheim, Germany) of plasmids encoding the lentiviral gag and pol elements (pMDLgpRRE), the rev protein (pRSV Rev), the viral envelope (pMD2G; all kindly provided by D. Trono). After 72h, the supernatant was collected, and cells were transduced for 48h in the presence of hexadimethrine bromide (4 µg/ml; Aldrich, Steinheim, Germany). Stable knock down was performed by puromycin selection.

FACS, RT-PCR and microscopy

FACS was performed as in (Smits et al., 2000). For double BrdU and MPM2 staining, cells were harvested in 70% ethanol, incubated in 2M HCl, 0.1% Triton X-100, and neutralized with 0.1M Borate buffer pH 8.5. The percentage of MPM2-positive cells was assessed on the BrdU-positive population. For RT-PCR, total RNA was extracted using Trizol and converted to cDNA using SuperscriptII (Invitrogen). Standard PCR were run at several dilutions to ensure detection below saturation. Immunofluoresence was performed as described (Lindqvist et al., 2007). Images were acquired on a Zeiss LSM510 META microscope, or a Deltavision imaging system using NA 1.4 objectives. For time-lapse microscopy, cells were imaged with DIC on a Zeiss Axiovert 200M using NA 0.75 objectives.

Luciferase assay and Western Blot

Luciferase activity was determined 48 h after transfection, using the Dual luciferase kit (Promega) according to the manufacturer's instructions. Relative luciferase activity was expressed as a ratio of firefly luciferase activity to control Renilla luciferase activity. Plk1-luciferase reporter (31) and Cyclin B1-Luciferase reporter (32) were described previously. Western blots were either performed on a whole cell lysate (U2OS), or on cells solubilized with 1% NP-40.

siRNA transfection and automated image analysis

For siRNA experiments (supplementary figure 5), cells were grown in 96-well plates (Viewplate-96, Perkin Elmer) and transfected with 20-30 nM siRNA using HiPerFect (Qiagen) according to manufacturers recommendations. ON-Targetplus SMARTpools of 4 siRNAs targeting Wip1 (J-004554) or PPP4C (J-008486) were from Dharmacon. Cells were fixed by addition of equal volume of an 8% formaldehyde solution to the medium to prevent loss of mitotic cells, permeabilized with methanol and stained with DAPI and Cyclin B1 and pHistone H3 antibodies. Image acquisition was performed using a Cellomics ArrayScan VTI (Thermo Scientific) using a 20x 0.40 NA objective. Image analysis was performed using Cellomics ArrayScan HCS Reader (Thermo Scienctific). In short, cells were identified based on DAPI staining and they were scored as mitotic if the intensity

of pHistoneH3 staining reached a pre-set threshold. Cyclin B intensity was measured by quantifying the average fluorescence of a 5-pixel wide region surrounding the DAPI staining. All images and automated image quantifications were subsequently checked manually. Image analysis was performed on at least 500 cells per condition.

RESULTS

Wip1 is required for checkpoint recovery

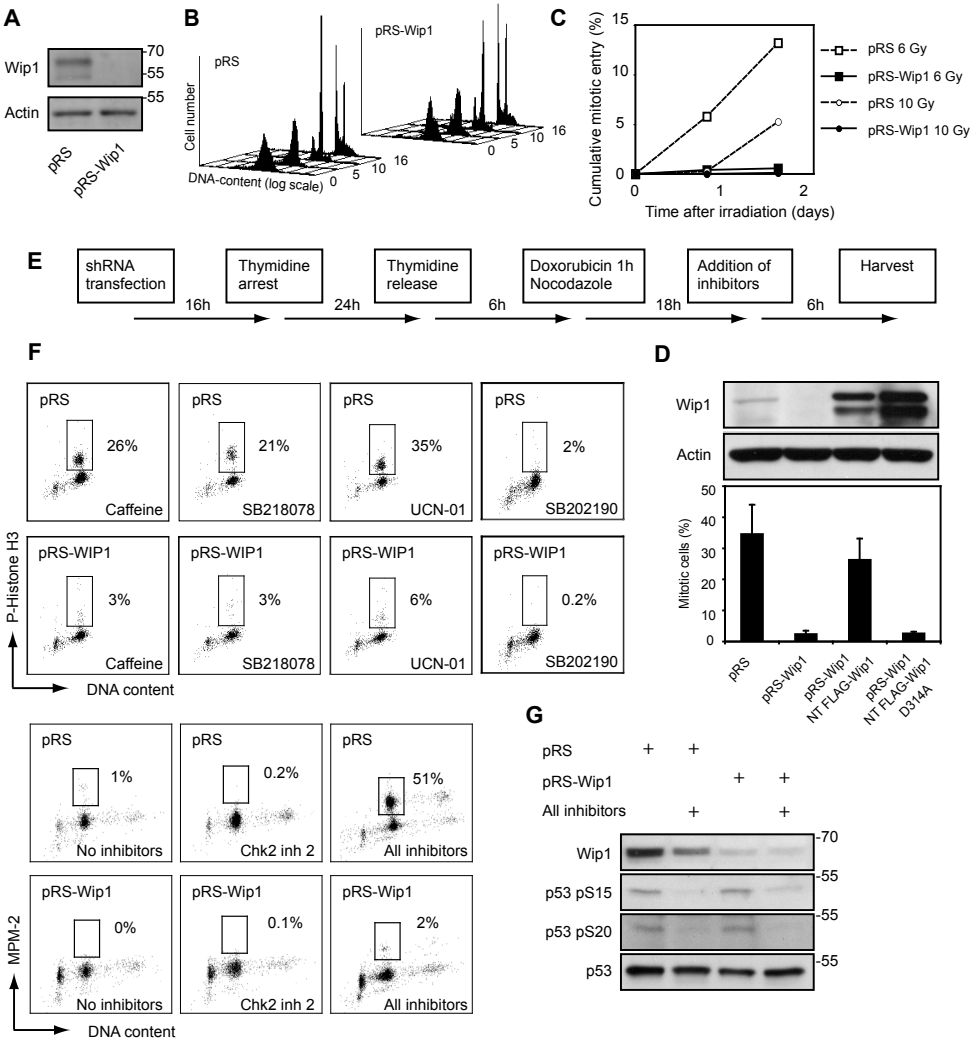
To test if Wip1 regulates recovery from a DNA damage-induced arrest in G2 we depleted endogenous Wip1 by shRNA (Figure 1A). shRNA-mediated depletion of Wip1 only marginally affected normal kinetics of cell division (Figure 1B), indicating that Wip1 is not essential for progression through an unperturbed cell cycle. However, when Wip1-depleted cells were arrested in G2 by γ-irradiation, checkpoint recovery was strongly inhibited compared to control cells (Figure 1C). Checkpoint recovery was also inhibited when Wip1-depleted G2 cells were arrested with the DNA damaging agent doxorubicin, and subsequently stimulated to re-enter the cell cycle by treatment with caffeine, an inhibitor of ATM/ATR (Figure 1D). Similar results were obtained with another shRNA sequence (data not shown). Importantly, this phenotype could be reverted by reconstituting the cells with RNAi-insensitive FLAG-Wip1 (Figure 1D), indicating that the recovery defect is due to specific depletion of the Wip1 protein. However, rendering the RNAi-insensitive Wip1 phosphatase-deficient by mutation of D314 to alanine (24) resulted in a failure to revert the Wip1 RNAi phenotype, indicating that phosphatase activity of Wip1 is important for recovery from a DNA damage checkpoint (Figure 1D).

Wip1 has been reported to dephosphorylate several checkpoint kinases, suggesting that it could function in recovery by removing activating phosphosites from checkpoint kinases to turn off the checkpoint signal (21-27). Therefore we treated G2 DNA damaged cells with inhibitors to these kinases and monitored if cells could enter mitosis in the absence of Wip1 (Figure 1E,F). Treatment with caffeine (ATM/ATR inhibitor), UCN-01 (Chk1 and MAPKAP2 inhibitor), or SB218078 (Chk1 inhibitor) could all mediate checkpoint recovery in control cells, whereas treatment with SB202190 (p38 inhibitor) or Chk2-inhibitor2 (Chk2 inhibitor) only marginally affected checkpoint recovery. However, none of these inhibitors could promote checkpoint recovery after Wip1 RNAi. Importantly, whereas combination of these inhibitors efficiently mediated checkpoint recovery in control cells, they did not promote checkpoint recovery in Wip1-depleted cells, indicating that the checkpoint recovery defect after Wip1 RNAi does not merely depend on sustained activation of these kinases (Figure 1F,G).

Wip1 controls recovery competence, rather than recovery itself

We next created stable cell lines where we could induce the expression of RNAi-insensitive Wip1 or a phosphatase-inactive version of Wip1 (D314A). Induction of Wip1, but not Wip1 D314A, after doxorubicin treatment in G2 promoted mitotic entry (Figure 2A).

This observation raises an apparent paradox, as it demonstrates that Wip1, like caffeine and other checkpoint kinase inhibitors, acts to shut down the checkpoint, while enforced silencing of the checkpoint in Wip1-depleted cells fails to promote recovery. A possible explanation for this discrepancy could be that Wip1 needs to be present throughout the checkpoint response, and has an additional function that precedes its role during the recovery phase. To test whether Wip1 is required early during the DNA damage response, before actual silencing of the checkpoint, we reconstituted Wip1-depleted cells with RNAi-resistant Wip1 shortly after the DNA damage checkpoint was activated, or 18 hours after initial checkpoint activation and monitored whether these cells could enter mitosis after silencing of ATM/ATR with caffeine (Figure 2B). Importantly, when reconstituted late, Wip1-depleted cells did not recover from the checkpoint, indicating



that Wip1 needs to be present throughout the DNA damage checkpoint arrest to mediate checkpoint recovery. In contrast, when Wip1-expression was induced early after checkpoint activation, cells could enter mitosis after caffeine treatment, albeit not as efficiently as when Wip1 was already present during the DNA damage treatment (Figure 1D, 2B). Similar results were seen when microinjecting RNAi-resistant Wip1 early or late after DNA damage into Wip1-depleted cells (Supplementary Figure 1). Nonetheless, Wip1 was functional both when reconstituted early and late after DNA damage, since p53 S15, Chk1 S317, Chk2 T68 and H2AX S139 were dephosphorylated to similar extent, indicating that large parts of the checkpoint signalling was silenced (Figure 2C). Moreover, p21 levels were similarly reduced, indicating that p53 activity was inhibited both after early and late Wip1-reconstitution. This shows that although Wip1 can deactivate major parts of the checkpoint signalling, Wip1 needs to be present throughout the DNA damage checkpoint to mediate full checkpoint recovery. Thus, Wip1 confers checkpoint recovery competence during a DNA damage arrest.

Wip1 controls recovery competence through p53

Since deactivation of the DNA damage checkpoint through inhibition of checkpoint kinases could not rescue the checkpoint defect of Wip1-depleted cells, we sought to identify which proteins mediate the loss of recovery competence. A candidate target that is activated by DNA damage signalling is p53. Although p53 is not essential for induction of the G2 DNA damage checkpoint, p53 has been described to be required to sustain a long-term arrest in G2 (33).

Figure 1. Wip1 is essential for checkpoint recovery. (**A**) Wip1 RNAi efficiently reduces the levels of endogenous Wip1. U2OS cells, transfected with empty pRS or pRS-Wip1, were selected with puromycin and subjected to immunoblotting with the indicated antibodies. (**B**) Wip1 RNAi cells progress through S, G2 and M-phases in the absence of DNA-damage. U2OS cells co-transfected with GFP-Spectrin and pRS-Wip1 or empty pRS were synchronized in S-phase with a single 24 hour thymidine block. The cell-cycle profile of GFP-expressing cells was assessed by FACS at different time-points after release. Y-axis: number of cells, x-axis: DNA-content (logarithmic scale), z-axis: time after thymidine release (hours). (**C**) Wip1 RNAi inhibits checkpoint recovery. Cells were treated as in B, but subjected to 6 or 10 Gy γ-irradiation seven hours after release from the thymidine block. Immediately after irradiation or after 24 hours incubation, taxol was added for 24h before harvesting. The amount of mitotic GFP-positive cells was assessed by MPM-2 positivity using FACS. (**D**) Wip1 RNAi inhibits caffeine-induced checkpoint recovery. U2OS cells were co-transfected with GFP-spectrin and pRS, pRS-Wip1, pRS-Wip1 + RNAi insensitive FLAG-Wip1, or pRS-Wip1 + RNAi insensitive phosphatase dead FLAG-Wip1 (D314A) and synchronized by a single 24h thymidine block. Six hours after thymidine synchronization, cells were treated with the topoisomeraseII inhibitor doxorubicin, and nocodazole was added to trap cells entering mitosis. 15h later, cells were treated for 6h with caffeine (inhibitor of ATM/ATR) and scored for phosphorylated Histone H3 by FACS. Graph denotes average of three independent experiments, error bars represent standard error. Western Blot shows endogenous and reconstituted Wip1 levels from a single experiment. (**E**) Outline of experimental setup used in F,G and Fig 3A. (**F**) A range of kinase inhibitors does not promote checkpoint recovery after Wip1-depletion. Cells were treated as in B, but treated with a 1h pulse of doxorubicin six hours after release from thymidine block. 18h later, cells were treated for 6h with caffeine (inhibitor of ATM/ATR), SB218078 (inhibitor of Chk1), UCN-01 (inhibitor of Chk1 and MK-2), SB202190 (inhibitor of p38), Chk2 inhibitor-2 (Chk2 inhibitor), or a combination of these inhibitors. The percentage of mitotic cells was assessed by scoring GFP positive cells for P-Histone H3 or MPM2 positivity by FACS. (**G**) P53 is dephosphorylated on the ATM/ATR-site S15 and the Chk1/Chk2 site S20 after combined inhibitor treatment (53;54). Cells treated or not with all inhibitors as in Figure 1F were subjected to Western Blot with the indicated antibodies.

In addition, there is considerable evidence that p53 is a major target of Wip1. Not only is p53 a direct target of Wip1 (21), but Wip1 can also dephosphorylate ATM, Chk1, Chk2, p38, and Mdm2, all direct regulators of p53 (21-27). Moreover, Wip1 inhibition or RNAi decreases the proliferation of p53-positive, but not p53-negative cancer cell-lines (34;35), and Wip1 is required for APC(Min)-induced colon tumour formation and intestinal stem cell maintenance through counteracting p53 (36). As expected, depletion of p53 by shRNA did not interfere with the ability of cells to arrest in G2 in response to doxorubicin (33) (Figure 3A).

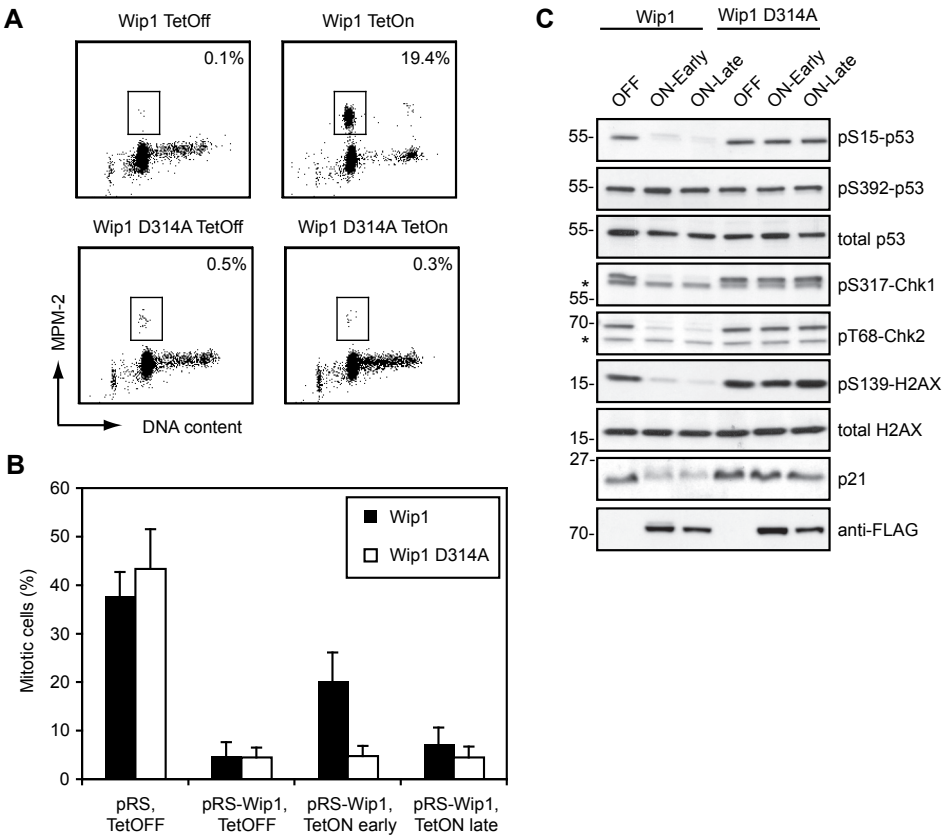


Figure 2. Wip1 is required throughout the checkpoint arrest to mediate recovery. (**A**) Forced expression of Wip1 can induce checkpoint recovery. U2TR cells stably expressing inducible wt or phosphatase-deficient Wip1 (D314A) were synchronized by thymidine and 6 hours after release treated with doxorubicin for 1h, after which the cells were treated for 24 hours with Tetracyclin and taxol. The percentage of mitotic cells was assessed by scoring MPM2 positivity by FACS. (**B**) Wip1 is required during a G2 checkpoint arrest. U2TR cells stably expressing inducible RNAi-resistent wt or phosphatase-deficient Wip1 (D314A) were co-transfected with GFP-Spectrin and pRS or pRS-Wip1. Cells were synchronized with thymidine and 6 hours after release treated with doxorubicin for 1h, followed by taxol addition. Tetracyclin was added immediately (early) or 18 hours after (late) after doxorubicin wash-out, after which caffeine was added for 8 hours. The percentage of mitotic cells was assessed by scoring GFP positive cells for MPM2 positivity by FACS. (**C**) Checkpoint signaling is inhibited both after early and late Wip1-induction. Cells were treated as in B, but selected with puromycin and without caffeine addition, harvested as in B and subjected to immunoblotting with the indicated antibodies.

Importantly, co-depletion of p53 restored the ability of Wip1-depleted cells to recover after addition of caffeine, UCN01 or SB218078, indicating that the primary function of Wip1 in checkpoint recovery is to counteract the function of p53 during the ongoing arrest (Figure 3A).

To validate whether Wip1 can mediate checkpoint recovery through p53 in an independent system, we next infected lentiviral Wip1 shRNA vectors in HCT116 and HCT116 p53-/- colon carcinoma cells. Two Wip1 shRNAs, independent from the shRNAs used in U2OS cells, mediated efficient knockdown in both cell lines (Figure 3B). Similar to U2OS cells, only a fraction of the Wip1-depleted HCT116 cells treated with doxorubicin entered mitosis after caffeine addition, whereas HCT116 p53-/- cells could recover in the absence of Wip1, indicating that Wip1 promotes checkpoint recovery by counteracting p53 also in HCT116 cells (Figure 3C). To see if Wip1 is also important for spontaneous checkpoint recovery, when the checkpoint is not switched off by the addition of kinase inhibitors, we next filmed HCT116 cells after treatment with doxorubicin and monitored mitotic entry (Figure 3D,E). Importantly, whereas HCT116 p53-/- cells depleted of Wip1 entered mitosis, parental HCT116 cells depleted of Wip1 did not enter mitosis, showing that Wip1 is required also for spontaneous checkpoint recovery by counteracting p53. Using one of the Wip1 shRNAs, we reproducibly observed a slight delay in mitotic entry of HCT116 p53-/- cells, suggesting that Wip1 may have additional targets besides p53 that affect checkpoint recovery. Next, we used a BrdU-pulse to monitor whether cells that were in late S-phase or G2 during either doxorubicin addition or γ-irradiation could eventually recover from the DNA damage checkpoint and enter mitosis (Figure 3F,G). HCT116 p53-/- cells entered mitosis more efficiently than HCT116 cells, showing that p53 indeed participates in the G2 DNA damage checkpoint (33). However, whereas Wip1-depleted HCT116 p53-/- cells recovered from both γ-irradiation and doxorubicin treatment as control-depleted cells, the checkpoint recovery of HCT116 cells depleted of Wip1 was impaired (Figure 3F,G). Thus, Wip1 counteracts p53 to confer checkpoint recovery competence to G2 cells.

Wip1 antagonizes p53-mediated repression of mitotic regulators

So why does Wip1 need to counteract p53, a non-essential component of the G2 DNA damage checkpoint, to mediate checkpoint recovery competence? Upon DNA damage, p53 can induce multiple target genes, including the Cdk-inhibitor p21. However, p21 is reduced to similar levels after early or late reconstitution of Wip1, whereas only cells reconstituted early could recover, arguing that p21 accumulation can not explain the Wip1 phenotype (Figure 2C). p53 has also been described to reduce the transcription of a large number of G2 genes in response to DNA damage (9;11-13). Therefore we analysed the levels of Cyclin B1, a key mitotic inducer, and Plk1, which is required for mitotic entry after recovery from DNA damage (37). The mRNA levels of Plk1 and Cyclin B1 in DNA-damaged U2OS cells were reduced compared to control G2 cells, indicating that Plk1 and Cyclin B1 are indeed targets of transcriptional repression (Supplementary Figure 2). Despite that the majority of HCT116 cells arrest in G2 after doxorubicin

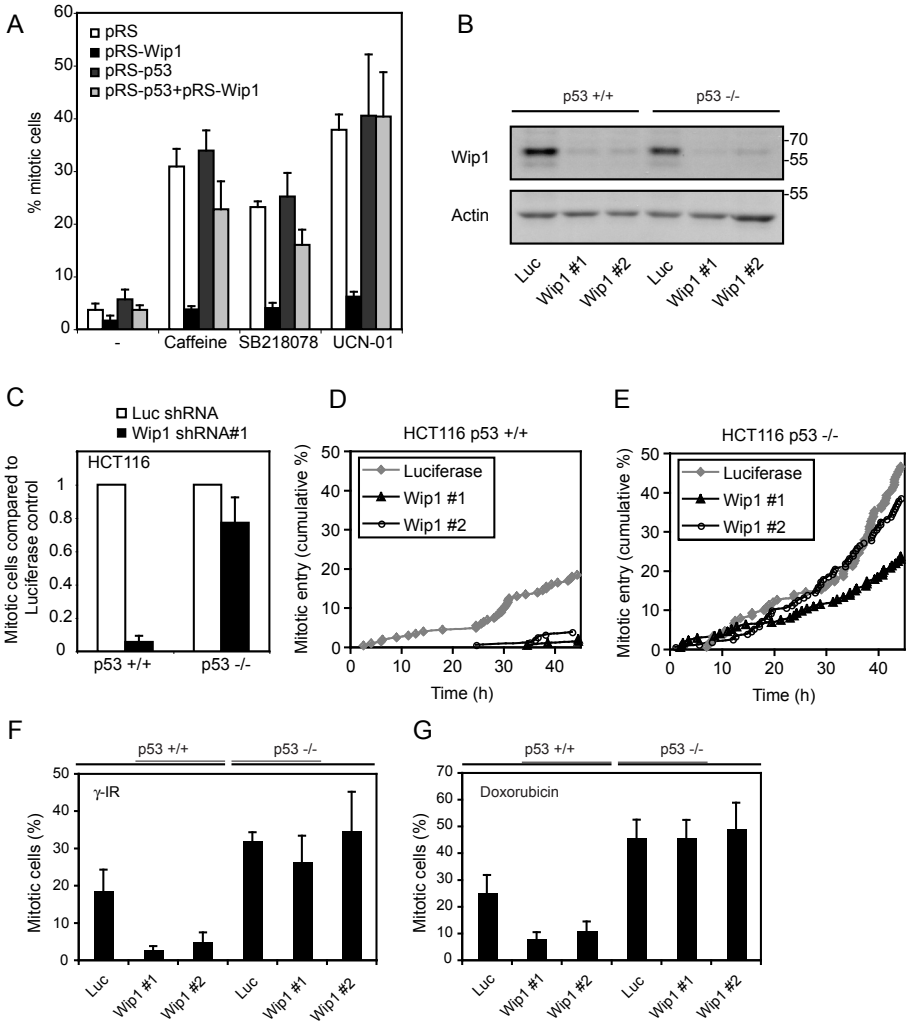


Figure 3. Wip1 is essential for checkpoint recovery by antagonizing p53. (**A**) p53 RNAi reverses Wip1 phenotype. U2OS cells were transfected with empty pRS, pRS + pRS-Wip1, pRS + pRS-p53 or pRS-Wip1 + pRS-p53. All samples were co-transfected with GFP-Spectrin. Checkpoint recovery was assessed as in Fig 1E and F. Graph denotes percentage of mitotic cells and error bars represent standard error of three independent experiments. (**B**) Knockdown of Wip1 in HCT116 cells. HCT116 p53+/+ or p53-/- cells were infected with lentiviral shRNA constructs targeting luciferase or Wip1. After selection with puromycin, harvested cells were subjected to immunoblotting with the indicated antibodies. (**C**) Wip1 is necessary for caffeine-induced checkpoint recovery in HCT116 cells, but not in HCT116 p53-/- cells. HCT116 and HCT116 p53-/- cells were infected with lentivirus expressing Wip1 shRNA#1 or Luciferase shRNA, selected with puromycin and treated with doxorubicin for 16h. Caffeine and taxol, to induce checkpoint recovery and to trap cells in mitosis, respectively, were added for 12h after the doxorubicin treatment. Graph shows the ratio of mitotic Wip1 RNAi cells in relation to the amount of mitotic Luciferase RNAi cells. (**D**),(**E**) Wip1 is necessary for checkpoint recovery in HCT116 cells, but not in HCT116 p53-/- cells. HCT116 and HCT116 p53-/- cells were infected with lentivirus expressing Wip1 shRNA or Luciferase shRNA and selected with puromycin. After treatment with 0.75 μM doxorubicin for 2h, cells were followed by time-lapse microscopy and scored for entry into mitosis. (**F**),(**G**) Wip1 is necessary for checkpoint recovery from late S/G2 in HCT116 cells, but not in HCT116 p53-/- cells. HCT116 and HCT116 p53-/- cells were infected with lentivirus expressing Wip1 shRNA or Luciferase shRNA and selected with puromycin. Four hours after a BrdU-pulse, cells were treated with 6 Gy γ-irradiation or 1h of 0.5 μM doxorubicin. Taxol was added immediately after DNA-damage, and cells were harvested 24 hours later. The percentage of recovering cells was assessed by scoring MPM2 positivity on the BrdU positive population by FACS.

treatment, only a marginal increase in the expression of Cyclin B1 and a minor decrease in Plk1 levels compared to asynchronic cells was observed (Figure 4A, lane 1 and 7). Importantly, both Plk1 and Cyclin B1 levels were dramatically reduced after DNA damage in Wip1-depleted HCT116 cells, whereas only limited effects were seen in HCT116 p53-/- cells, likely depending on DNA damage-induced degradation of Plk1 (6) (Figure 4A). This shows that Wip1 can counteract p53-mediated repression of Cyclin B1 and Plk1 after DNA damage. A reduction in Plk1 levels was also observed in Wip1-depleted untreated HCT116 cells, probably a consequence of low levels of intrinsic DNA damage occurring in these cells (Figure 4A, Supplementary Figure 3). A similar pattern was seen in U2OS cells, where Wip1-depleted DNA-damaged G2 cells contained both less protein and mRNA of Plk1 and Cyclin B1 (Figure 4B-D). However, both the protein and mRNA levels of Cyclin B1 and Plk1 were restored after co-depletion of p53, indicating that Wip1 counteracts a p53-mediated reduction of Cyclin B1 and Plk1 mRNA during a DNA damage response (Figure 4B-D).

Since p53 can act as a transcriptional repressor of G2 genes (9;11-13) and p53-depletion could rescue the effect of Wip1-depletion (Figure 3), we next wondered if the reduced Cyclin B1 and Plk1 mRNA levels after Wip1-depletion depended on p53-mediated repression of Cyclin B1 and Plk1 transcription. Therefore we expressed transcriptional reporters driven by Cyclin B1 or Plk1 promoters in G2-arrested DNA-damaged cells. Forced expression of Wip1, but not of phosphatase dead Wip1, in DNA-damaged G2 cells elevated the expression of Plk1 and Cyclin B transcriptional reporters, indicating that Wip1 can counteract transcriptional repression in G2 (Supplementary Figure 4). Moreover, the expression of both Plk1 and Cyclin B1-transcriptional reporters were reduced after Wip1 RNAi and restored after p53 and Wip1 double RNAi, indicating that Wip1 can indeed counteract p53-dependent repression of Plk1 and Cyclin B1 transcription after DNA damage (Figure 4E,F). Taken together, these data suggest that Wip1 acts in a negative feedback loop that is activated in response to DNA damage to limit the suppressive effect of p53 on the expression of mitotic regulators during a checkpoint arrest.

Cyclin B1 becomes a rate-limiting factor for recovery in Wip1-depleted cells

Our data show that Wip1 can antagonize p53-dependent expression of Cyclin B1 and Plk1. If true, then the recovery defect of Wip1-depleted cells should be due to a deficiency of mitotic inducers. Indeed, when re-examining the samples from early and late Wip1-reconstitution, where no difference in phosphorylation or levels of several checkpoint components was observed, we noted that both Cyclin B1 and Plk1 levels were elevated after early Wip1-reconstitution (Figures 2C, 5A). However, late Wip1-reconstitution could not rescue the very low Cyclin B1 and Plk1 levels, indicating that the levels of mitotic inducers may determine if cells are competent for checkpoint recovery. To test if the recovery deficiency in Wip1-depleted cells is due to a lack of mitotic inducers, we microinjected GFP-Cdk1 AF, a form of Cdk1 that lacks inhibitory phosphorylation sites and efficiently induces premature mitotic entry in the presence of cyclin B (38). Whereas injection of GFP-CDK1 AF rapidly promoted mitotic entry in DNA

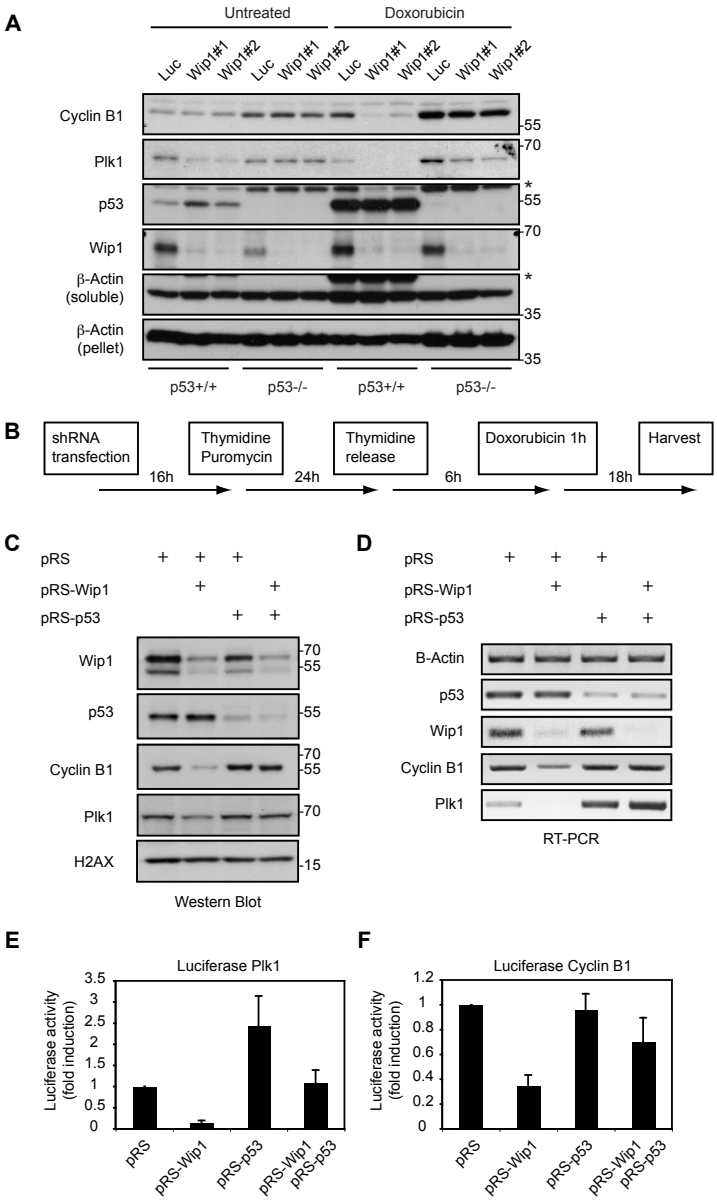


Figure 4. Wip1 counteracts p53-mediated transcriptional repression. (**A**) Wip1 antagonizes p53-mediated downregulation of Plk1 and Cyclin B1 in HCT116 cells. HCT116 and HCT116 p53-/- cells were infected with lentivirus expressing Wip1 shRNA or a Luciferase shRNA, selected with puromycin, treated with doxorubicin for 16h and harvested for Western Blot. Whereas Wip1 was detected in the unsoluble fraction (loading control, β-Actin pellet), p53, Cyclin B1 and Plk1 were detected in the soluble fraction. (**B**) Schematic outline of experiments in C-F. (**C**),(**D**) Wip1 antagonizes p53-mediated downregulation of Plk1 and Cyclin B1. U2OS cells were transfected as in figure 3A, and selected by puromycin addition during a 24 h thymidine block. 6 hours after release from thymidine, cells were treated for 1h with doxorubicin. 18 hours later, at the time corresponding to addition of inhibitors in figure 3A, cells were harvested for Western Blot (C) or RT-PCR (D). (**E**),(**F**) Wip1 counteracts p53-mediated transcriptional repression of Plk1 and Cyclin B1. U2OS cells were transfected as in figure 3A, with the addition of Luciferase-based transcriptional reporters for Plk1 (E) and Cyclin B1 (F), and synchronized as shown in figure 4B. Graphs show the relative Luciferase expression 18h after doxorubicin treatment.

damage-arrested control cells, only a fraction of Wip1-depleted cells entered mitosis, indicating that Wip1 deficient cells do not contain sufficient levels of Cyclin B to promote mitotic entry (Figure 5B). Microinjection of Plk1 did not induce mitotic entry in DNA-damaged control or Wip1-depleted cells, indicating that although Plk1 is necessary, it is not sufficient to promote checkpoint recovery (data not shown) (37).

To test if the levels of the key mitotic inducer Cyclin B1 was a limiting factor for checkpoint recovery in Wip1-depleted cells, we microinjected YFP-Cdc25B3 S323G, a potent activator of Cyclin B1-Cdk1 even in the presence of an active DNA damage checkpoint (39). Whereas injection of YFP-Cdc25B3 S323G resulted in efficient mitotic entry in DNA-damaged control cells, it failed to promote mitosis in Wip1-depleted cells. Importantly, co-injection of Cyclin B1 and YFP-Cdc25B3 S323G forced DNA damaged Wip1 shRNA transfected cells to enter mitosis, demonstrating that the levels of Cyclin B1 are indeed limiting for mitotic entry in DNA-damaged Wip1-depleted cells (Figure 5c). Thus, artificial activation of the remaining Cyclin B-Cdk1 failed to promote mitotic entry in Wip1-deficient DNA-damaged cells, indicating that Cyclin B-Cdk1 levels had fallen below the relatively low threshold required for mitotic entry (40).

Taken together, our data show that Wip1 acts as a checkpoint recovery competence gene by counteracting p53-mediated transcriptional repression of mitotic inducers. To test whether checkpoint recovery competence is a general consequence of loss of any phosphatase that counteracts checkpoint signalling, we transfected siRNA to PPP4C (also known as PP4), a phosphatase proposed to regulate checkpoint recovery through dephosphorylation of γH2AX (41;42). PPP4C-depletion impaired recovery from a DNA damage checkpoint in G2 to some extent compared to control transfected cells (Supplementary Figure 5A,B).

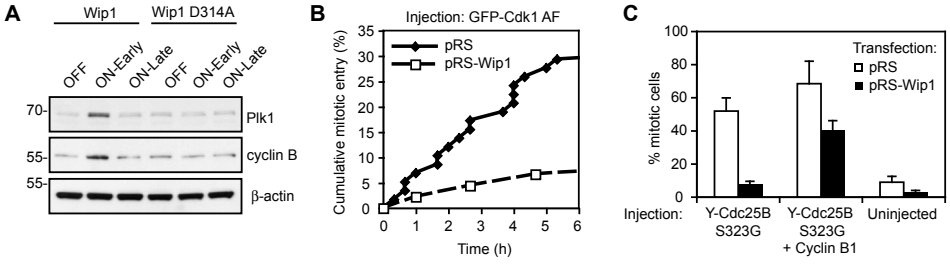


Figure 5. Cyclin B1 is rate-limiting for recovery in Wip1-depleted cells. (**A**) Plk1 and Cyclin B1 levels are low if Wip1 is absent during the DNA damage arrest. The samples used in fig. 2C were subjected to immunoblot for Plk1 and Cyclin B1. (**B**) Injection of GFP-Cdk1 AF fails to induce mitotic entry in DNA-damaged Wip1-depleted cells. pRS or pRS-Wip1 transfected cells were selected with puromycin and six hours after release from a thymidine block exposed for 1h to 0.5 μM doxorubicin. 18 hours after doxorubicin treatment GFP-Cdk1 AF was injected and cells were followed by time-lapse microscopy. Graph denotes cumulative mitotic entry of injected cells (**C**) Injection of YFP-Cdc25B3 S323G fails to induce mitotic entry in DNA-damaged Wip1 RNAi cells, unless co-injected with Cyclin B1. pRS or pRS-Wip1 transfected cells were selected with puromycin and six hours after release from a thymidine block exposed for 1h to 0.5 μM doxorubicin. 18 hours after doxorubicin treatment YFP-Cdc25B3 S323G or YFP-Cdc25B3 S323G and Cyclin B1 were injected to cells in a single dish and nocodazole was added to trap cells entering mitosis. Graph denotes average of four independent experiments where 23-178 cells where counted for each injected construct, error bars represent standard error.

Importantly however, PPP4C-depleted cells arrested in G2 contained high levels of Cyclin B1, indicating that PPP4C is not required to retain sufficient levels of mitotic inducers to allow checkpoint recovery (Supplementary Figure 5C). Thus, not every phosphatase that counteracts checkpoint signalling mediates checkpoint recovery competence.

DISCUSSION

Our data show that unlimited checkpoint activation during a DNA damage-induced arrest in G2 can decrease the levels of mitotic inducers to such extent that a cell cannot enter mitosis, despite inactivation of the checkpoint. Since the DNA damage checkpoint functions by suppressing Cdk activity, which in turn is necessary for stimulating the transcriptional program in G2, our data also suggest that a cell must retain sufficient levels of Cdk activity during an arrest to maintain its G2 state. A cell that has lost its G2 state will not express pro-mitotic genes, and has therefore lost its competence to recover from a G2 DNA damage checkpoint. Thus, a cell needs to actively balance the checkpoint at all stages of the DNA damage response to remain competent for eventual recovery.

Here we show that Wip1 is the first in a novel class of proteins that is required to balance the checkpoint throughout a DNA-damage response. Rather than deactivating the G2-checkpoint to promote recovery, Wip1 counteracts p53-dependent downregulation of pro-mitotic genes during the arrest to confer checkpoint recovery competence. We could show that the defect in checkpoint recovery observed in Wip1-depleted cells, could be reverted by introduction of Cyclin B1 in combination with a checkpoint-insensitive mutant of Cdc25B. While this experiment demonstrates that depletion of Wip1 causes Cyclin B1 to drop below the critical level needed to initiate mitosis, it doesn't rule out the possibility that additional components of the checkpoint recovery pathway are also reduced below their minimal threshold. In fact, hundreds of proteins are transcriptionally upregulated during G2, and their combined status will determine the threshold of Cyclin B-Cdk1 activity needed for mitotic entry (43;44). Indeed, here we show that the expression of Plk1, a central player in the network of proteins that regulate Cyclin B-Cdk1 activity during checkpoint recovery, is also dramatically reduced in a p53-dependent manner in Wip1-depleted cells (37;43).

In addition to p53, DNA damage-induced release of the Wip1 target Chk1 was recently identified to mediate transcriptional repression of mitotic inducers (10). Although our data clearly show that Wip1 mediates checkpoint recovery by counteracting p53, they do not exclude that Wip1 plays an additional role by modulating Chk1-dependent transcriptional repression.

Combining modelling and experimental work, Batchelor and co-workers recently showed that Wip1 is required to control the shape of oscillations in the ATM-p53 signalling cascade, thereby ensuring that the checkpoint reaches points of very low activity during the arrest (19). Our data suggest that these breathing-pauses during the checkpoint could allow for short pulses of transcription that prevent a cell from loosing its G2 identity.

Several studies have linked Wip1 to tumour formation (36;45-49). Here, we show that Wip1 limits the suppression of mitotic inducers during a prolonged checkpoint arrest to remain cells competent for eventual checkpoint recovery. Interestingly, both early during tumour-development and after genotoxic anti-cancer treatment a DNA damage checkpoint is activated, which cells must overcome to continue to proliferate (50;51). Based on our observations we propose that Wip1's function in tumourigenesis is to retain the competence for cell division during a DNA damage response.

Interestingly, loss of Wip1 was recently shown to induce a p53-dependent G2 arrest in neural stem/progenitor cells (52). It therefore remains a possibility that retaining checkpoint recovery competence is required also to maintain a neural stem cell population.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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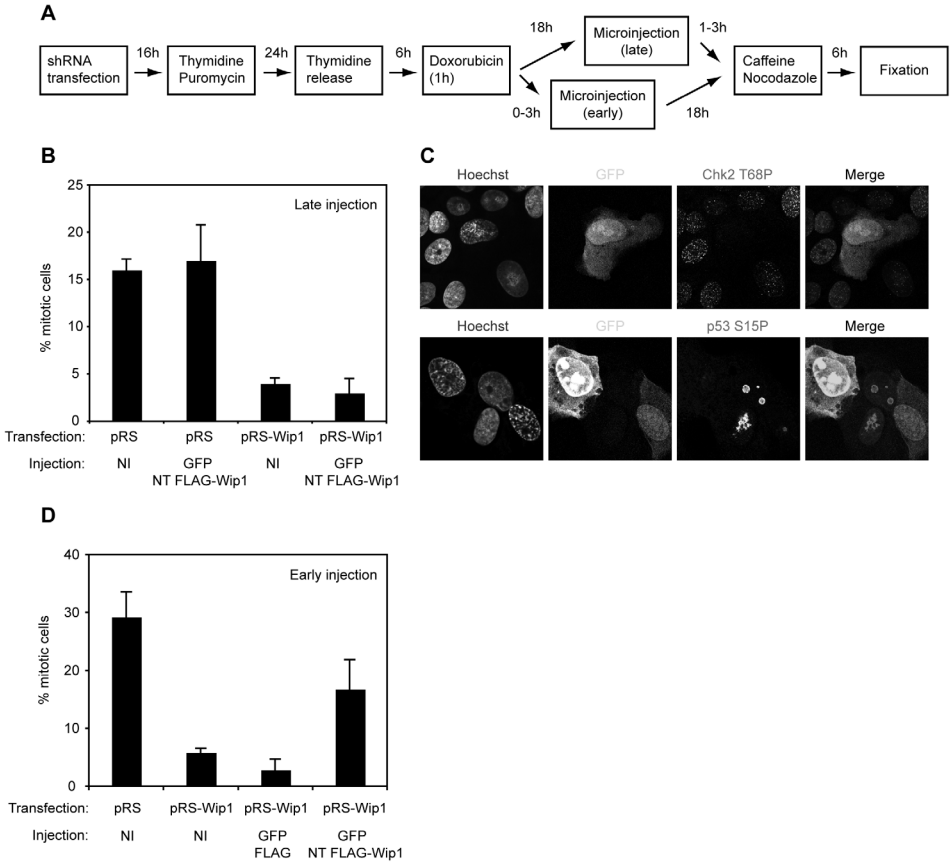
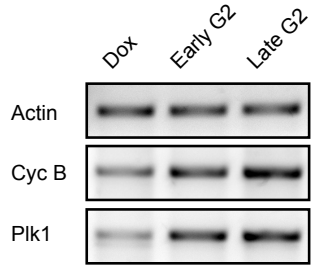
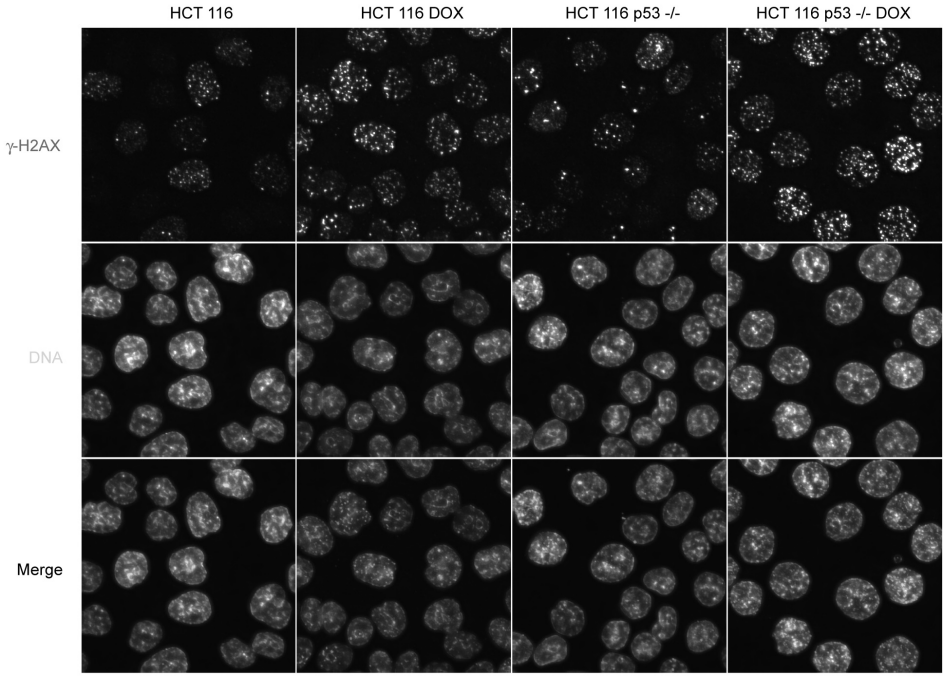
SUPPLEMENTARY FIGURES



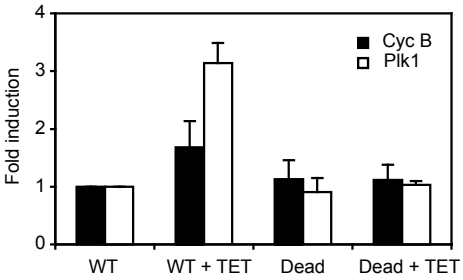
Supplementary Figure 1. Late reconstitution with Wip1 cannot rescue checkpoint recovery. (**A**) Schematic outline of experiments in B-D. (**B**) Late injection of non-targetable FLAG-Wip1 cannot rescue Wip1 RNAi recovery deficient phenotype. pRS or pRS-Wip1 transfected U2OS cells were selected with puromycin, exposed to doxorubicin, 18 hours later co-injected with GFP (to mark successfully injected cells) and non-targetable FLAG-Wip1 and treated with caffeine for 6h, as outlined in A. NI, not injected cells. 57-144 cells were counted for each injected construct in each of three independent experiments. Error bars represent standard error. (**C**) Late injected Wip1 is active since Chk2 T68 (top panels) and p53 S15 (bottom panels) are dephosphorylated in the injected, but not in the surrounding cells. Images show pRS-Wip1 transfected cells co-injected with GFP and non-targetable FLAG-Wip1, as outlined in A. (**D**) Early injection of non-targetable FLAG-Wip1 partially rescues Wip1 RNAi recovery deficient phenotype. Experiments were performed as in B, but injections were performed immediately after doxorubicin wash-out, followed by caffeine addition 18 hours later. 46-135 cells were counted for each injected construct in each of three independent experiments. Error bars represent standard error. See page 132 for a full-color representation of this figure.



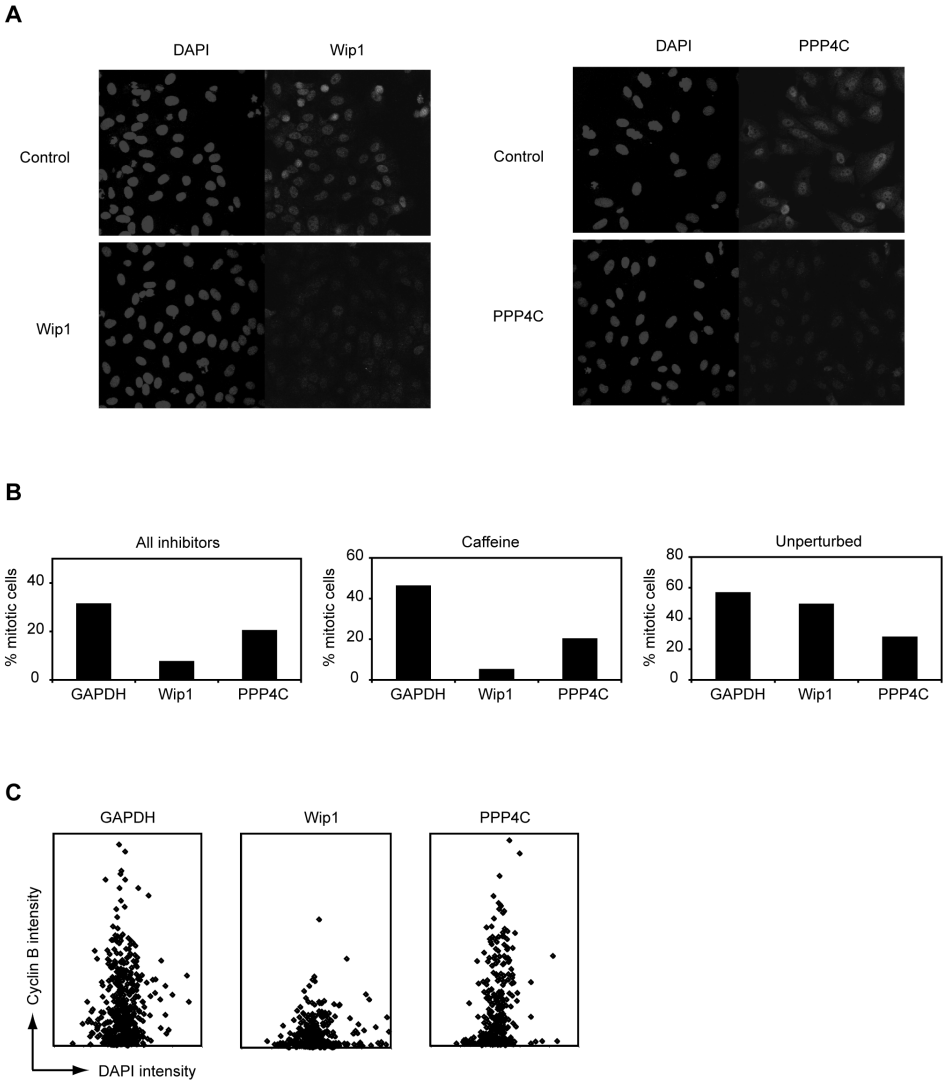
Supplementary Figure 2. mRNA levels of Plk1 and Cyclin B are reduced after DNA-damage in G2. U2OS cells were synchronized in G2 by release for 7h from a double thymidine block and exposed to doxorubicin for 1 hour and harvested 18h later (Dox). Alternatively, cells were harvested 7 (early G2) or 11 hours (late G2) after release from a double thymidine block. Images show RT-PCR using primers for the indicated genes.



Supplementary Figure 3. A subset of untreated HCT116 cells contain γH2AX foci. HCT116 and HCT116 p53-/- cells were left untreated or treated with 0.5 µM doxorubicin for 18 hours, fixed and stained for γH2AX. See page 133 for a full-color representation of this figure.



Supplementary Figure 4. Forced expression of Wip1 enhances expression of Plk1 and Cyclin B transcriptional reporters in DNA-damaged G2 cells. U2TR cells stably expressing inducible RNAi-resistent wt or phosphatase-deficient Wip1 (D314A) were co-transfected with transcriptional reporters for Plk1 or Cyclin B. Cells were synchronized with thymidine and 6 hours after release treated with doxorubicin for 1h. Tetracycline was added 18 hours after doxorubicin wash-out and cells were harvested 8 hours later. Graph shows normalized Luciferase activity from three independent experiments.



Supplementary Figure 5. Not all phosphatases counteracting checkpoint kinases confer checkpoint recovery competence. (**A**) U2OS cells were transfected with siRNAs targeting GAPDH, Wip1 or PPP4C. Images show immunofluorescence stainings using Wip1 or PPP4C antibodies. (**B**) U2OS cells were transfected with siRNAs targeting GAPDH, Wip1 or PPP4C, and treated as in figure 1F (all inhibitors and caffeine). Alternatively, cells were synchronized by a thymidine block and fixed after 20h release into taxol-containing medium (unperturbed). The amount of mitotic cells was assessed by counting MPM2-positive cells using automated image analysis (materials and methods). (**C**) U2OS cells were treated as in B, but in the absence of checkpoint kinase inhibitors. Cells were stained with DAPI and Cyclin B antibodies and the fluorescence intensities of 500 cells per sample were analyzed using automated image analysis (materials and methods). Graphs show the cytoplasmic Cyclin B intensities plotted versus the nuclear DAPI intensities. See page 134 for a full-color representation of this figure.

4

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Chapter 4

Oncogenic KRAS sensitizes colorectal tumor cells to chemotherapy via p53-dependent induction of Noxa

ABSTRACT

Background

Oxaliplatin and 5-fluorouracil (5-FU) currently form the backbone of conservative treatment in patients with metastatic colorectal cancer. Tumor responses to these agents are highly variable, but the underlying mechanisms are poorly understood. Our previous results have indicated that oncogenic KRAS in colorectal tumor cells sensitizes these cells to chemotherapy.

Methods

FACS analysis was used to determine cell cycle distribution and the percentage of apoptotic and mitotic cells. A multiplexed RT-PCR assay was used to identify KRAS-controlled apoptosis regulators following exposure to 5-FU or oxaliplatin. Lentiviral expression of short-hairpin RNA's was used to suppress p53 or Noxa.

Results

Oncogenic KRAS sensitized colorectal tumor cells to oxaliplatin and 5-FU in a p53-dependent manner and promoted p53 phosphorylation at Ser37 and Ser392, without affecting p53 stabilization, p21 induction, or cell cycle arrest. Chemotherapy-induced expression of the p53 target gene Noxa was selectively enhanced by oncogenic KRAS. Suppression of Noxa did not affect p21 induction or cell cycle arrest, but reduced KRAS/p53-dependent apoptosis following exposure to chemotherapy *in vitro* and in tumor xenografts. Noxa-suppression did not affect tumor growth *per se*, but strongly reduced the response of these tumors to chemotherapy.

Conclusion

Oncogenic KRAS determines the cellular response to p53 activation by oxaliplatin or 5-FU, by facilitating apoptosis induction through Noxa.

INTRODUCTION

Chemotherapy of colorectal cancer mainly involves the fluoropyrimidine drug 5-fluorouracil (5-FU) and the platinum drug oxaliplatin (1). The response of colorectal tumors to these compounds is determined both by the genetic background of the tumor cells as well as by environmental cues. Colorectal tumors frequently harbor activating mutations in the *KRAS* proto-oncogene, which drives tumor progression (2;3). Signaling by constitutively active Ras proteins can either promote or prevent the induction of apoptosis, depending on the cellular context and on the specific isoform studied (4;5). For instance, expression of oncogenic N-ras but not oncogenic K-ras in the colonic epithelium provides protection against enterocyte apoptosis in a colitis model (6). In contrast, we and others have previously shown that oncogenic KRAS promotes apoptosis of human colorectal tumor cells exposed to either 5-FU or oxaliplatin (7;8). Although activating mutations in KRAS alone do not reliably predict the response of colorectal tumors to chemotherapy (9), these findings do suggest that the acquisition of KRAS mutations may be associated with an increased propensity to undergo apoptosis. Interestingly, several studies have connected Ras signaling to the activation of p53 (10-12). In non-transformed cells activation of p53 by oncogenic Ras induces a permanent cell cycle exit and induction of cellular senescence (13-15). However, p53 activation in (colorectal) tumor cells does not lead to senescence, but rather to apoptosis or to cell cycle arrest (16-18). The tumor suppressor protein p53 is a key player in determining the response of colorectal tumor cells to oncogenic stress and chemotherapy by oxaliplatin and 5-FU (16;19-21). The determinants specifying p53 signaling output are the subject of intense investigation. While p53-induced activation of the Cdk inhibitor p21 is sufficient to cause cell cycle arrest in most cell types, p53-induced apoptosis is more complex and involves the activation of different sets of target genes in different tissues and in response to different stimuli (22-25). In colorectal tumor cells the p53-upregulated modulator of apoptosis (Puma) plays an important role in the induction of apoptosis exposed to 5-FU or oxaliplatin (26). Puma belongs to the family of 'BH3-only' proteins that promote apoptosis by neutralizing Bcl-2 family pro-survival proteins, including Bcl-2, Bcl-xL, and Myeloid Cell Leukemia sequence 1 (Mcl-1) (27;28). p53 also promotes expression of Noxa, another BH3-only family member (29). Puma is generally considered to be a more potent apoptosis inducer than Noxa, because it can neutralize multiple Bcl-2 family members, while Noxa selectively neutralizes Mcl-1 (30-32). However, Noxa-dependent cell death may be particularly important in malignant cells (33) and Noxa could therefore represent a potential therapeutic target.

In the present report we have studied the contribution of p53 and p53 target genes to KRAS-induced sensitization of colorectal tumor cells to 5-FU and oxaliplatin. We find that mutant KRAS cooperates with p53 in the induction of Noxa, but not other pro-apoptotic p53 target genes. Furthermore, we show that Noxa does not control tumor growth *per se*, but is an important determinant of the tumor response to chemotherapy.

MATERIALS AND METHODS

Cell culture

The human colorectal cancer cell line HCT116$^{G13D/wt}$ and the isogenic cell line Hkh2$^{ko/wt}$ lacking the KRAS^{D13} allele were kindly provided by Dr Shirasawa (34). HCT116 p53KO cells were kindly provided by Dr Vogelstein. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 5% (v/v) fetal calf serum, 2 mM glutamine, 0,1 mg/ml streptomycin, and 100 U/ml penicillin. All cells were kept at 37ºC in a humidified atmosphere containing 5% CO_2. Oxaliplatin was obtained from Sanofi Aventis. 5-Fluorouracil was from TEVA.

Antibodies

The following antibodies were obtained from Cell Signaling Technology Inc, Danvers, MA, USA: anti-Puma (#4976), anti-Caspase-8 1C12 (#9746), anti-cleaved Caspase-3 ASP175 (#9661) and the secondary antibody peroxidase conjugated anti-rabbit IgG. The following antibodies were all obtained from Santa Cruz biotechnology, Heidelberg, Germany: anti-p53 DO-1 (sc-126), anti-p21 C19 (sc-397). anti-Noxa (IMG-349A) came from Imgenex Corporation San Diego, CA, USA; anti-α tubulin from Sigma Aldrich, St. Louis, MO, USA; anti-β-actin AC-15 (NB600-501) from Novus Biologicals, LLC, Littleton, CA, USA; Secondary antibody peroxidase conjugated anti-mouse IgG from Dako, Glostrup, Denmark; anti-phospho-Histone H3 (Ser10) (06-570) from Millipore, Billerica, MA, USA and the secondary antibody Cy5 AffiniPure F(ab')$_2$ Frag Donkey anti-Rabbit IgG from Jackson Immunoresearch Europe, Suffolk, UK. For immunohistochemical staining anti-Ki-67 clone SP-6 (#RM-9106S) from Lab Vision Products Thermo Fisher Scientific, Chesire, UK and anti-cleaved Caspase 3 (# 559565) from BD Pharmingen NJ, USA were used.

Lentiviral constructs and transduction

The lentiviral shRNA construct targeting p53 was kindly provided by Dr AG Jochemsen. Noxa-targeting lentiviral constructs were from the TRC-Mm1.0 library (Sigma Aldrich, St. Louis, MO, USA). The target set used for Noxa (NM_021127) included TRCN0000150555, TRCN0000155978, TRCN0000151311, TRCN0000153637 and TRCN0000155570. Of these constructs subsequent transduction of the cells with TRCN0000151311, followed by transduction with TRCN0000153637 gave the best knock down. As control vector we used the same vector containing a sequence targeting luciferase, TGACCAGGCATTCACAGAAAT. For virus production we used the third generation packaging system, kindly provided by Prof D Trono.

p53 phosphorylation status

The phosphorylation status of p53 was analyzed using the phospho-p53 Antibody Sampler Kit #9919 (Cell Signalling).

FACS analysis

Cells were fixed in 70% ice cold ethanol and incubated for 2 h at 4°C. To determine the percentage of mitosis, cells were washed in PBS-Tween (0,1%) and incubated with anti-phopho-Histone H3 antibody for 1 h at RT, after which cells were incubated with CY5-labelled secondary antibody for 1h at RT. To assess the cell cycle profile, fixed cells were treated with RNAse and DNA was stained with propidium iodide (PI). All samples were analysed using bivariate flow cytometry using Cell Quest and Modfit software (Becton Dickinson).

MLPA

The MLPA was performed according to the manufacturer's protocol (MRC-Holland BV).

RT-PCR

RT-PCR was performed by isolating RNA using Trizol, and conversion to cDNA using Superscript2 (Invitrogen). The primers used were for Noxa forward: 5'GGTACCCTGGGAAGAAGGCGCG3' and reverse: 5'GAATTCTCAGGTTCCTGCGCAGAAG3'. L32 served as a loading control with the forward primer: 5'GCCCAAGATCGTCAAAAAGA3', and reverse 5'ATTGTGGACCAGGAACTTGC3'.

Immunofluorescence

Immunofluoresence was performed as in (35). Images were acquired on a Zeiss LSM510 META microscope. MitoTracker (Invitrogen) was used according to the manufacturer's protocol.

Tumor model and chemotherapy

All experiments were performed in accordance with the guidelines of the Animal Welfare Committee of the University Medical Center Utrecht, The Netherlands. Male Balb/C Nu/Nu mice (10-12 weeks) were purchased from Charles River (Sulzfeld, Germany) and were housed in filter top cages. Tumor cells were injected subcutaneously (10^6 cells in 100 µl 1/3 diluted Matrigel (Becton Dickinson)). When tumors reached approximately 100 mm^3, mice were treated with 12,5 mg/kg oxaliplatin or vehicle intraperitoneally (day 0). A second gift of oxaliplatin (12,5 mg/kg) was administered on day 7. Tumor growth was assessed every 2 days, and mice were terminated 14 days following the start of treatment. The volume (V) was calculated by V=AxB2x0.5236 (A=largest diameter, B=diameter perpendicular to A).

RESULTS

KRAS^{D13} sensitizes tumor cells to chemotherapy-induced apoptosis without overriding cell cycle arrest

Oncogenic KRAS may sensitize tumor cells to apoptosis induction by forcing cell cycle progression in the presence of DNA damage. Therefore, we analyzed whether KRAS-facilitated apoptosis induction by 5-FU and oxaliplatin was accompanied by escape from cell cycle arrest. HCT116 (KRAS$^{wt/D13}$) and isogenic Hkh2 cells (KRAS$^{wt/ko}$) were treated with oxaliplatin and 5-FU for three consecutive days. Apoptosis induction and cell cycle distribution were then measured by FACS analysis of propidium iodide-stained cells. In addition, mitotic progression was measured by FACS analysis of phospho-histone H3-positive cells. We found that KRAS greatly facilitated apoptosis induction by both chemotherapeutics as described (7;8) (Figure 1A,B). HCT116 cells underwent a *bona fide* cell cycle arrest and did not enter mitosis prior to apoptosis induction (Figure 1C). This suggests that KRAS does not promote chemotherapy-induced apoptosis by forcing mitotic entry in the presence of DNA damage.

KRAS^{D13} promotes apoptosis without affecting p53 stabilization or p21 induction

Next, we tested whether KRAS-status would influence p53 stabilization. We found that p53 was stabilized to a similar extent in both HCT116 and Hkh2 cells in response to either oxaliplatin or 5-FU (Figure 2A). Nuclear accumulation of p53 was also similar in both cell types (Figure 2B). We were unable to detect p53 localization to the mitochondria in oxaliplatin- or 5-FU-treated HCT116 or Hkh2 cells (Supplementary Figure 1). However, whereas p53 stabilization correlated caspase-8 cleavage in HCT116 cells, this was not observed in Hkh2 cells (Figure 2A). Induction of the cyclin-dependent kinase inhibitor and p53 target p21 by chemotherapy was not affected by KRAS status (Figure 2A), which is in line with the finding that both apoptosis-prone and -resistant cells undergo cell cycle arrest.

KRAS promotes p53 phosphorylation of p53 on Ser37 and Ser392

The differential regulation of p53 target genes is controlled both by post-translational modification and by its binding partners. Here we tested whether KRAS status modified chemotherapy-induced phosphorylation of p53. We found that oxaliplatin- or 5-FU-induced p53 phosphorylation on Ser15 was not altered, but phosphorylation on Ser37 and Ser392 was far more pronounced in cells expressing oncogenic KRAS (Figure 2C). Again, p53 stabilization was not different between HCT116 and Hkh2 cells (Figure 2C).

p53 is required for KRAS^{D13}-stimulated tumor cell sensitization to apoptosis

Next, we tested whether p53 was required for the sensitizing effect of KRAS^{D13} on chemotherapy-induced tumor cell apoptosis. To this end, we created stable p53 knockdown cell lines by using lentiviral RNA interference. Knockdown of p53 in HCT116

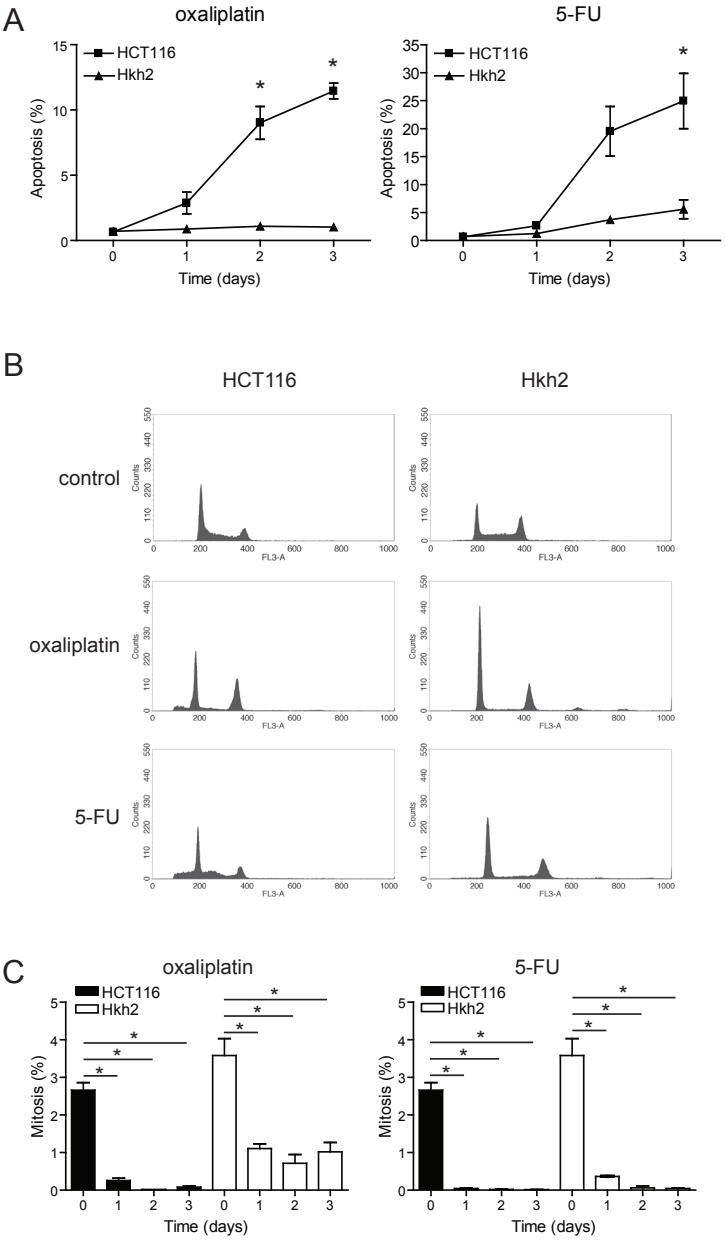

Figure 1. KRAS^{D13} sensitizes tumor cells to chemotherapy-induced apoptosis without overriding cell cycle arrest (**A**) HCT116 cells and Hkh2 cells were treated with 8μg/ml oxaliplatin or 8μg/ml 5-FU for three consecutive days. Cells were then fixed in formalin and stained with propidium iodide (PI). The sub-G1 fraction was determined by FACS analysis. Error bars represent SEM based on 3 independent experiments (*p<0.05). (**B**) Cells were treated as in A. After two days, cells were fixed and the cycle profiles of PI-stained cells were analyzed by FACS. (**C**) Cells were treated as in A and fixed on the indicated timepoints. Mitotic cells were then stained using anti-phospho- histone H3 and analyzed by FACS. Error bars represent SEM, based on 3 independent experiments (*p<0.001).

cells strongly reduced apoptosis in response to oxaliplatin or 5-FU in HCT116 cells (Figure 3A, upper panel). However, p53 knockdown did not affect 5-FU- or oxaliplatin-induced apoptosis in Hkh2 cells (Figure 3A, lower panel). In both cell types p21 induction was completely abrogated (Figure 3B). These results show that the apoptosis-promoting effect of $KRAS^{D13}$ depends on the presence of wildtype p53.

5-FU and oxaliplatin induction of Noxa requires oncogenic KRAS and wildtype p53

To gain further insight into how oncogenic KRAS facilitates apoptosis induction in response to chemotherapy, we performed a multiplexed RT-PCR analysis (MLPA) for the induction of apoptosis control genes by either 5-FU or oxaliplatin in HCT116 cells

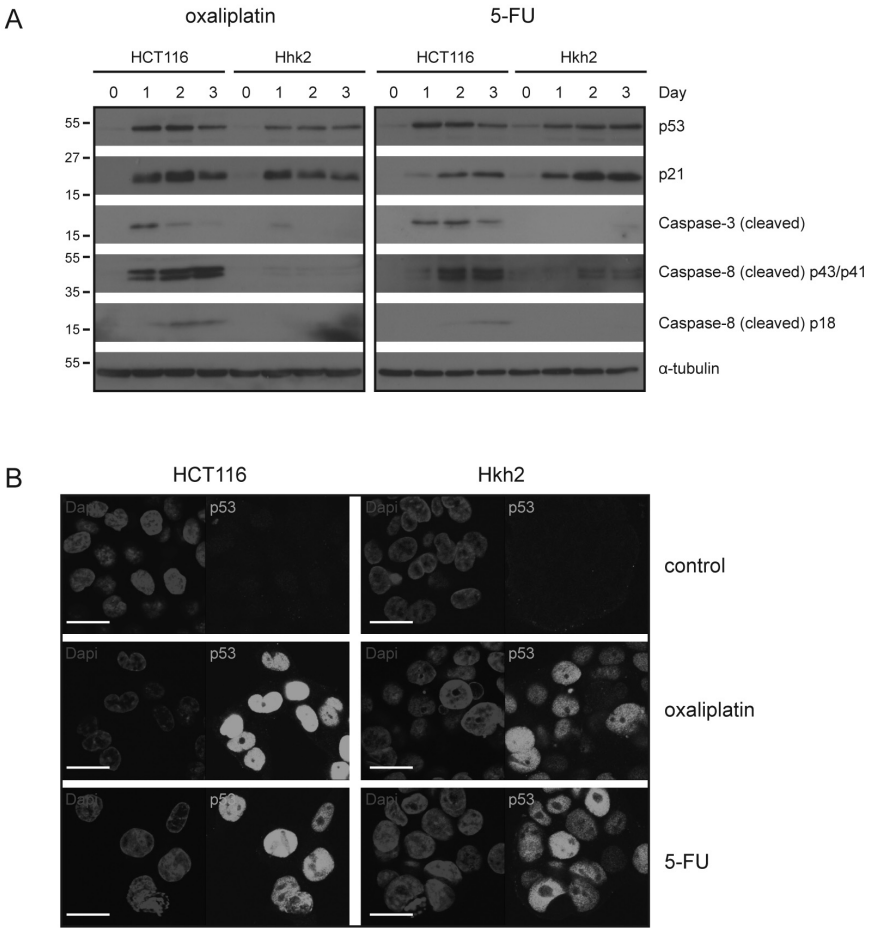

Figure 2. Oncogenic KRAS promotes apoptosis without affecting p53 stabilization or p21 induction (**A**) HCT116 cells and Hkh2 cells were treated with oxaliplatin (8μg/ml) or 5-FU (8μg/ml) for 0-3 days as indicated. Lysates were prepared and analyzed for the presence of p53, p21, cleaved Caspase 8, tubulin and actin by Western blotting. (**B**) HCT116 and Hkh2 cells were treated for 24 hours with oxaliplatin or 5-FU. Cells were then processed for immunofluorescence analysis of p53 localization. Bars represent 50μm. (**C**) Cells were treated as in B and cell lysates were analyzed for the presence of p53 (total p53) and p53 phosphorylated at residues Ser6, Ser9, Ser15, Ser20, Ser37, Ser46, and Ser392. The signals for Ser6, Ser9, Ser20 and Ser46 were below the detection limit. See page 135 for a full-color representation of this figure.

and Hkh2 cells (Supplementary Figure 2A). Of the 32 genes tested in this screen, only Noxa (PMAIP1) was selectively induced in HCT116 cells. The Noxa-related Puma gene was induced to a similar extent in HCT116 cells and Hkh2 cells by 5-FU or oxaliplatin.

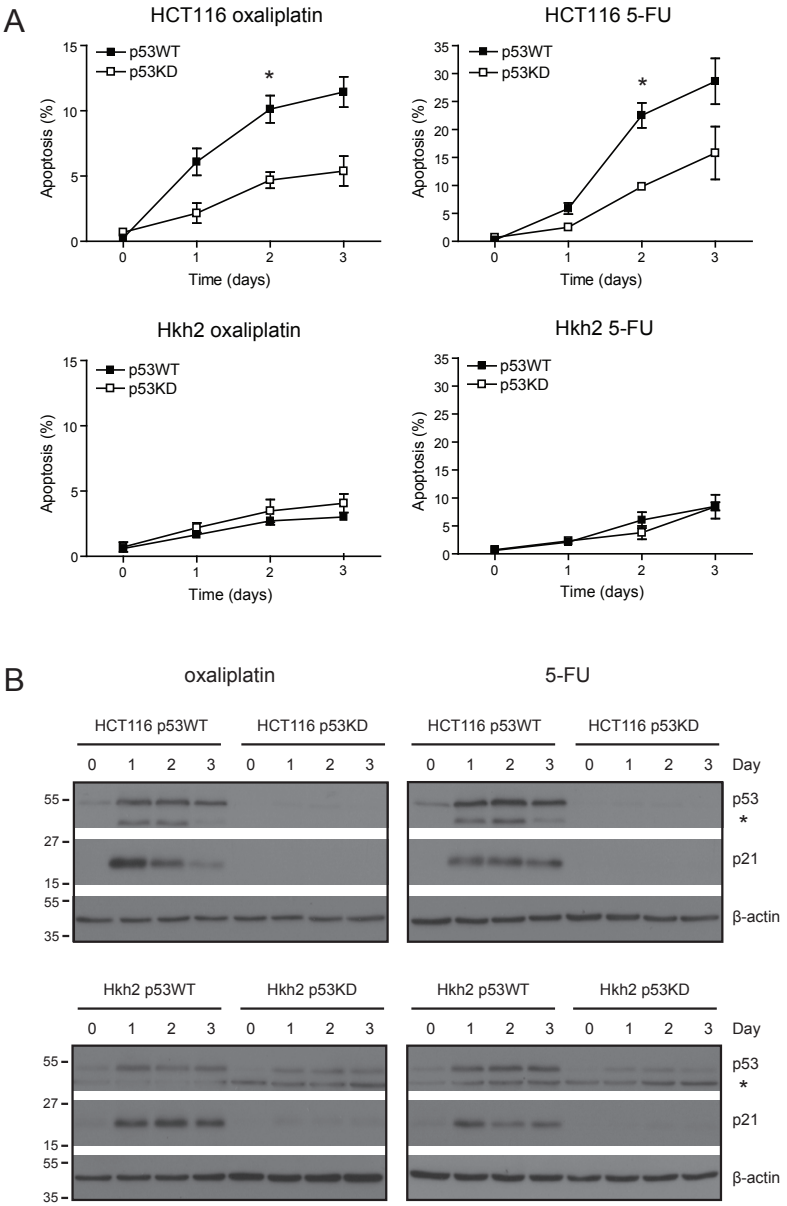

Figure 3. Wild type p53 is required for sensitization to apoptosis by oncogenic KRAS (**A**) HCT116 and Hkh2 cells were transduced with a lentiviral control shRNA vector, and with a vector targeting p53. All four cell lines were then treated for 0-3 days with oxaliplatin (8μg/ml) or 5-FU (8μg/ml). After fixation, cells were stained with propidium iodide and the sub-G1 fraction was determined by FACS analysis. Error bars represent SEM of 3 independent experiments (*p<0.05). (**B**) Cells were treated as in A, and cell lysates were prepared and analyzed for the presence of p53 and p21 by Western blotting.

However, chemotherapy induction of Puma mRNA levels was relatively low. The Puma/Noxa binding partner Myeloid Cell Leukemia sequence 1 (Mcl-1) was not induced by either drug (Supplementary Figure 2B). RT-PCR and Western blot analysis confirmed the selective induction of Noxa in HCT116 cells, but not Hkh2 cells (Figure 4A,B) and the relatively poor induction of Puma (Figure 4B). Thus, induction of the p53 target gene Noxa by 5-FU and oxaliplatin requires the presence of oncogenic KRAS.

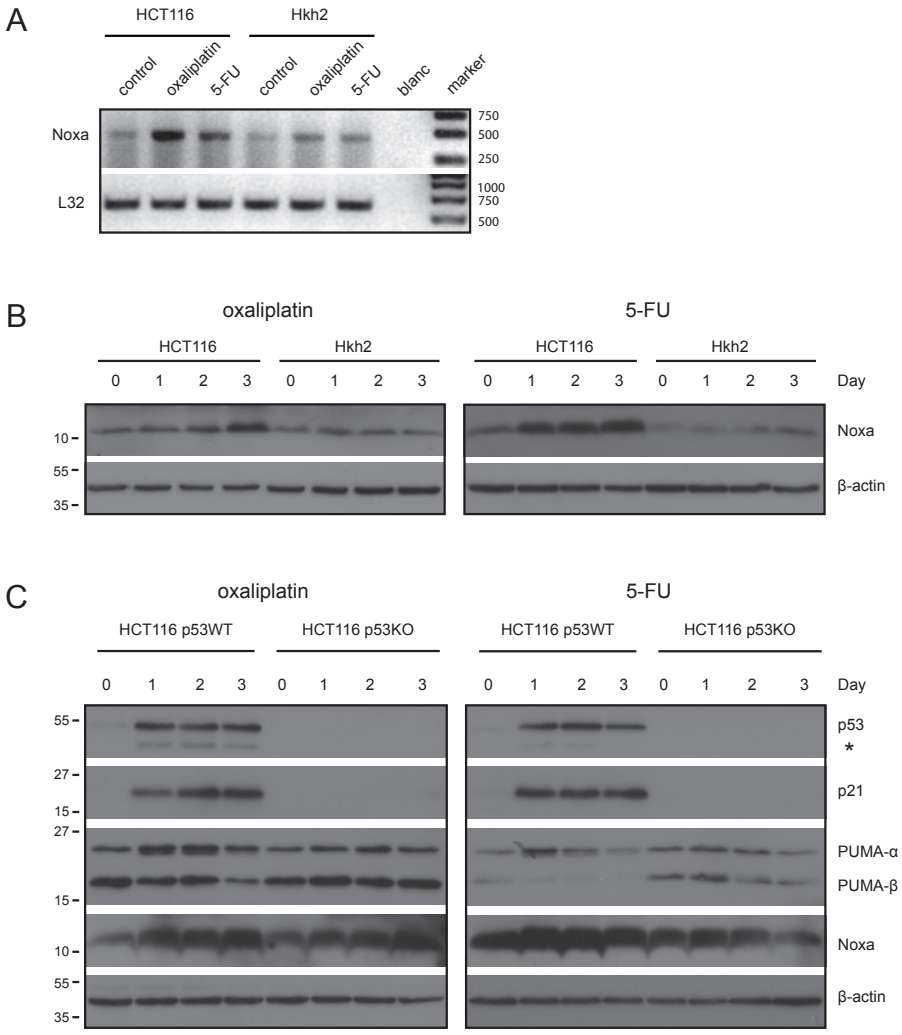

Figure 4. Oncogenic KRAS is required for p53-dependent induction of Noxa by chemotherapy (**A**) HCT116 and Hkh2 cells were treated with oxaliplatin (8μg/ml) or 5-FU (8μg/ml) for 24 hours. Total RNA was then isolated and was tested for the presence of Noxa and L32 mRNA by RT-PCR. (**B**) Cells were treated for 0-3 days with oxaliplatin or 5-FU, and Noxa protein levels were determined by Western blot analysis. (**C**) HCT116 expressing (HCT116 p53WT) or lacking p53 (HCT116 p53KO) were cultured in the presence of oxaliplatin or 5-FU for 0-3 days as indicated. Lysates were prepared and analyzed for the presence of p53, p21, Puma and Noxa protein levels. (**D**) The experiment described in C was repeated three times and all Noxa blots were scanned densitometrically to quantify the induction. Graphs represent % induction relative to day 0. * indicates statistically significant differences ($p<0.05$).

Next, we tested the dependency of Noxa induction on p53 in HCT116 cells. As expected, the induction of Noxa by oxaliplatin or 5-FU was strongly reduced in cells lacking p53 (Figure 4C). Quantification of Noxa induction in response to chemotherapy by densitometric scanning of the blots of three independent experiments showed that Noxa induction requires the presence of both wildtype p53 and oncogenic KRAS (Figure 4D).

A critical role for Noxa in apoptosis induction by oxaliplatin and 5-FU

We next tested whether KRAS-dependent induction of Noxa was instrumental in sensitizing HCT116 cells to oxaliplatin and 5-FU. To this end, we created stable cell lines in which Noxa was suppressed by RNA interference. Of a set of 5 Noxa-targeting shRNA constructs two (#2, #3) reduced Noxa protein levels to approximately 30% of control levels. Combination of these targeting constructs further reduced Noxa levels to approximately 10% of control levels (Figure 5A). Exposure of control cells and Noxa-suppressed cells to either oxaliplatin or 5-FU showed that Noxa suppression protected HCT116 cells from apoptosis induction by 50-70% over a period of three days. In contrast, the relatively inefficient targeting construct #1 (20-30% knockdown) had only a marginal protective effect (Figure 5A,B), when compared to control cells expressing luciferase-targeting shRNA.

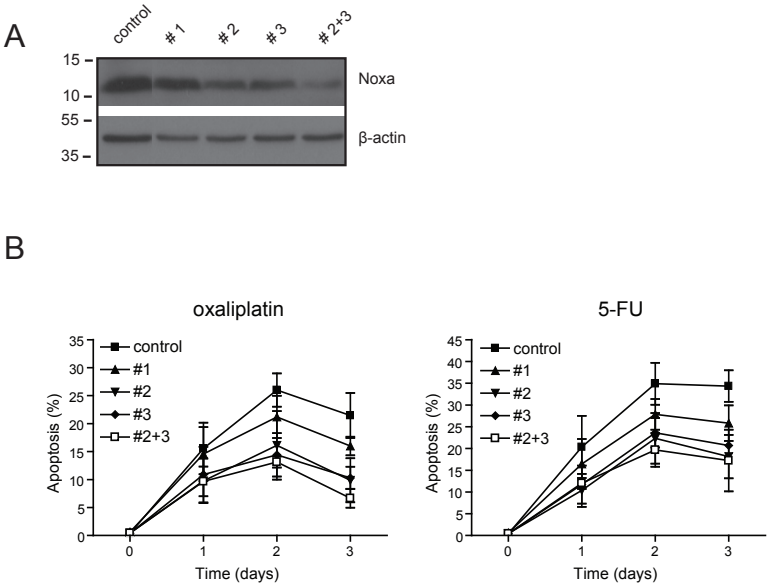

Figure 5. Contribution of Noxa to KRAS-facilitated apoptosis induction in response to chemotherapy (**A**) HCT116 cells were transduced with lentiviral vectors targeting Noxa (#1, #2, #3 and #2+#3), or with a vector targeting firefly luciferase (control). After selection in puromycin-containing medium for 1 week, cells were lysed and analyzed for Noxa protein levels by Western blot analysis. (**B**) HCT116 control and Noxa knockdown (NoxaKD) cells were treated with oxaliplatin (8μg/ml) or 5-FU (8μg/ml) for 0-3 days. The percentage apoptotic cells was then determined by FACS analysis as in Fig 1. Error bars represent SEM of three independent experiments.

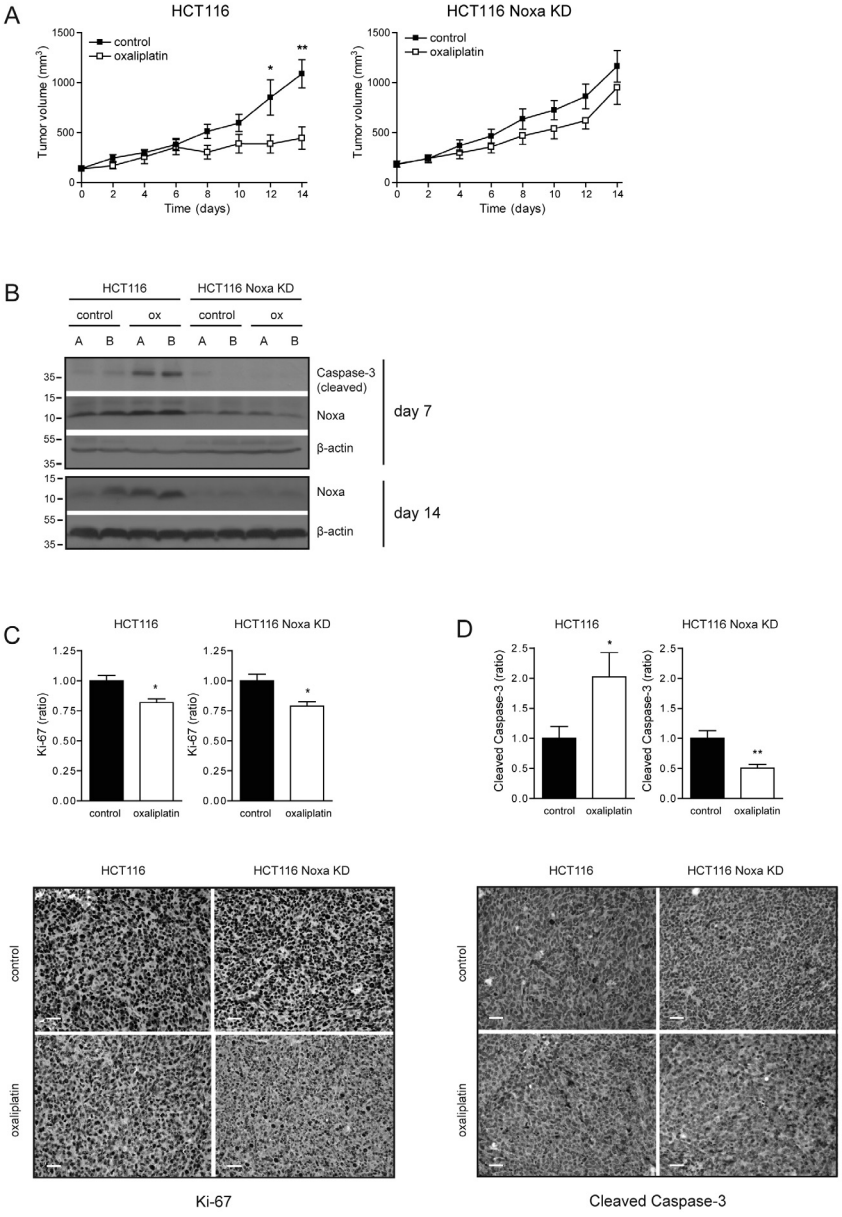

Figure 6. Contribution of Noxa to the therapeutic efficacy of oxaliplatin and 5-FU (**A**) Immune deficient nude mice (n=8/group) were injected subcutaneously with 10^6 HCT116 control or HCT116-NoxaKD cells. When tumors reached a size of 100 mm3, the mice received either PBS or oxaliplatin (12,5 mg/kg) intraperitoneally (Day 0). Seven days later, all mice received a second dose of either PBS or oxaliplatin. Tumor growth was followed by caliper measurements every 2 days (*p=0.048, **p=0.008). (**B**) Tumors were harvested on day 7 and day 14. Of each group, two tumors (A,B) were analyzed for the presence of cleaved Caspase-3 (as a marker for apoptosis) and for Noxa by Western blotting. All tumors were analyzed by immunohistochemistry for Ki-67 (**C**) and cleaved Caspase-3 (**D**) as markers for proliferation and apoptosis respectively. Ten random fields per tumor were scored and the positive tumor areas were determined by automated computer analysis. The bar graphs represent the ratios of Ki67 and Caspase-3-positive surface areas in oxaliplatin-treated xenografts versus control xenografts (*p<0.05). Representative images are shown on the right. Bars represent 50μm. See page 136 for a full-color representation of this figure.

Therapeutic efficacy of oxaliplatin and induction of tumor cell apoptosis requires Noxa

We next tested whether suppression of Noxa affected tumor growth and therapy resistance. To this end, control HCT116 cells (luc-shRNA) and Noxa-suppressed HCT116 cells were injected into the flanks of nude mice and tumor growth was followed over time. When tumors reached a size of 100 mm^3, PBS or oxaliplatin treatment was initiated. Tumor growth in control mice was unaffected by Noxa suppression (Figure 6A). Oxaliplatin treatment strongly reduced the growth of control HCT116 tumors, but had virtually no effect on Noxa-suppressed tumors (Figure 6A). Tumors were then excised and analyzed by Western blotting and immunohistochemistry, using Ki-67 and activated caspase-3 as markers for proliferation and apoptosis respectively. Noxa suppression was stable during the course of the experiment (Figure 6B). Induction of caspase-3 processing by oxaliplatin was completely abolished in Noxa-suppressed tumors (Figure 6B,D). Oxaliplatin had a marginal but statistically significant inhibitory effect on tumor cell proliferation, but this was not affected by Noxa suppression (Figure 6C). These results show that Noxa is a key determinant of the apoptotic, but not the cytostatic response of HCT116 tumors to oxaliplatin chemotherapy.

DISCUSSION

In the present study we provide evidence that oncogenic KRAS facilitates chemotherapy-induced apoptosis of HCT116 colorectal tumor cells by cooperating with p53 in the induction of the pro-apoptotic gene Noxa. p53-dependent induction of Puma did not require oncogenic KRAS. Puma and Noxa belong to the class of BH3-only proteins that function as neutralizers of the pro-survival Bcl-2-like proteins (36). The contribution of Puma and Noxa to apoptosis induction varies considerably between cell types (37-40). Apoptosis induction in colorectal cancer cells by chemotherapy is partly (~50%) dependent on the induction of Puma (26). Noxa is expressed both in the normal intestine as well as in most intestinal tumors (41), and Noxa-deficiency strongly reduces p53-dependent apoptosis in intestinal crypts following γ-irradiation (38). Our results show that Noxa also plays an essential role in chemotherapy-induced KRAS/p53-dependent apoptosis induction in intestinal (colorectal) cancer cells. Taken together, optimal p53-dependent apoptosis in colorectal cancer cells presumably requires induction of both Noxa and Puma to achieve maximal neutralization of Bcl-2 pro-survival proteins.

Importantly, p53 is not the only regulator of Noxa gene expression. Transcription factors such as c-Myc, E2F1 and HIF1α regulate Noxa expression in a p53-independent manner (42-45). Interestingly, oncogenic KRAS is critical for maintaining high c-Myc levels in the HCT116/Hkh2 system (46), and can promote expression of E2F1 (47) and stabilization of HIF1α (48). The stimulatory effect of oncogenic KRAS on one or more of these transcription factors may explain why basal Noxa levels are low in $KRAS^{D13}$-deleted tumor cells. p53 is

not required for maintaining basal levels of Noxa, but cooperates with oncogenic KRAS to induce Noxa expression in response to chemotherapy.

Expression of the H-Ras oncogene in primary cells causes stabilization of p53 via induction of the tumor suppressor $p19^{ARF}$ ($p14^{ARF}$ in human cells) (49). However, despite the presence of a mutant *KRAS* allele in HCT116 cells, its $p14^{ARF}$ levels are relatively low (50) and we did not observe overt differences in basal or chemotherapy-induced p53 stabilization in cells with or without $KRAS^{D13}$. The control of p53 signaling output by oncogenic KRAS may therefore involve alterations in p53 post-translational modifications and/or binding partners. Our study showed that deletion of $KRAS^{D13}$ strongly reduced chemotherapy-induced phosphorylation of p53 at Ser37 and Ser392. Phosphorylation of both residues has been associated with transcriptional output of p53, albeit not with apoptosis-specific gene regulation. Since KRAS status did not affect p53 stabilization or p21 induction, $KRAS^{D13}$-controlled phosphorylation of Ser37 and/or Ser392 may contribute to specifying p53 target gene induction. Interestingly, Ser37 phosphorylation augments p53 acetylation by p300 (51) and this promotes Noxa induction and apoptosis (52). Phosphorylation of p53 at Ser46, Ser15 and Ser20 have also been implicated in apoptosis-specific p53 signalling (53). However, phosphorylation of these residues was either not detected (Ser20, Ser46) or not regulated by KRAS status (Ser15) in the HCT116 cell system.

Several additional determinants of the tumor cell response to p53 activation have been identified. These include p53 post-translational modifications, interaction partners, and proteins that occupy p53 target gene promoters independently of p53 (54). Whether oncogenic KRAS alters any of these additional pathways or whether differential phosphorylation at Ser37 and/or Ser392 is sufficient to skew p53 signaling output towards Noxa induction and apoptosis will be the subject of further studies.

Oxaliplatin and 5-FU are the mainstay chemotherapeutics in the treatment of patients with disseminated colorectal cancer. However, there are currently no reliable predictors for response to chemotherapy (55-57). Meta-analyses of the literature on the impact of p53 abnormalities and KRAS mutations on therapy outcome revealed that neither p53 nor KRAS status had predictive value (9;58). Our results suggest that tumors expressing oncogenic KRAS in combination with wildtype p53 may respond best to chemotherapy. To the best of our knowledge, this has so far not been addressed in large patient cohorts. Noxa expression in a cohort of colorectal cancer patients treated with 5FU was unrelated to treatment response (59). However, such negative correlations are hard to interpret, given that Noxa induction in response to chemotherapy is transient. Colorectal tumors express Noxa at normal levels (60), and inactivating mutations have so far not been reported. Loss of Noxa function therefore does not seem to be required during colorectal tumor development. Indeed, the Noxa-dependent apoptosis pathway is intact in colorectal tumor cells (61;62). Furthermore, Noxa has been identified as a tumor-specific inducer of breast carcinoma cell death that spares non-transformed mammary cells (63). These observations suggest that the Noxa pathway may be an attractive target for therapeutic exploitation.

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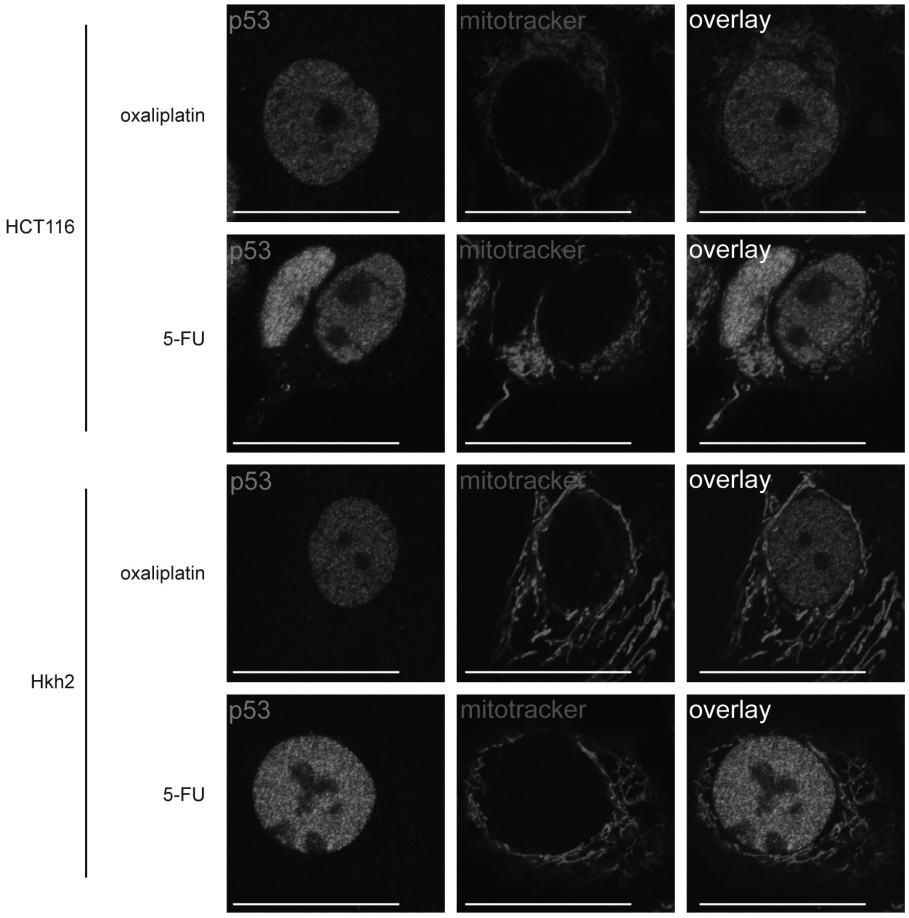
SUPPLEMENTARY FIGURES

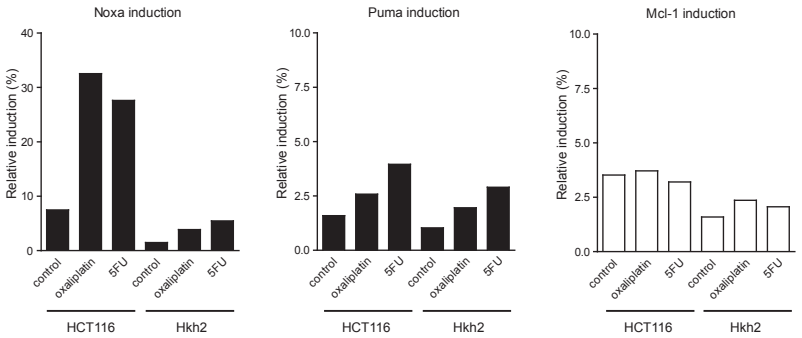


Supplementary Figure 1. p53 appears not co-localize with mitochondria following oxaliplatin or 5-FU
HCT116 and Hkh2 cells were treated for 24 hours, stained for p53 and mitochondria and analyzed by immunofluorescence microscopy. See page 137 for a full-color representation of this figure

A

	Induction					
Genes	HCT116 control	HCT116 oxaliplatin	HCT116 5-FU	HKH2 control	HKH2 oxaliplatin	HKH2 5-FU
Tnfrsf21	6,43	4,11	2,69	0,57	1,56	1,25
Cdkn1a	16,58	76,94	61,15	5,30	23,84	34,18
Htra2	2,10	1,47	0,97	0,63	1,09	0,97
Bcl2L2	0,42	0,00	0,35	0,13	0,28	0,28
Cflar v11	0,36	1,19	0,93	0,25	0,56	0,71
Bcl2L1	6,70	8,92	6,91	2,80	5,92	5,51
PMAIP1	7,48	32,52	27,59	1,47	3,86	5,47
Cflar v22	0,27	0,00	0,00	0,11	0,21	0,00
MCL1	3,52	3,71	3,20	1,59	2,36	2,06
B2M	1,00	1,00	1,00	1,00	1,00	1,00
BAD	1,66	1,29	0,91	0,75	1,02	0,95
BAX	5,60	8,94	4,66	1,34	4,54	3,75
BOK	2,17	1,68	0,71	1,12	2,16	1,17
Bcl2L11	4,54	5,55	5,40	1,68	3,25	3,95
BID	3,04	3,38	3,16	1,64	3,27	3,69
MIL1	3,16	2,54	1,83	1,46	2,32	1,65
HRK	0,00	0,00	0,00	0,35	0,31	0,00
Birc1	0,24	0,00	0,00	0,11	0,00	0,00
Birc3	0,90	1,35	2,25	0,39	1,99	3,28
Birc5	8,96	1,58	0,00	1,91	2,86	1,04
Birc6	1,58	1,45	1,41	0,47	1,09	0,88
SerpinB9	2,99	1,25	0,97	0,39	0,61	0,38
Bbc3	1,59	2,58	3,96	1,03	1,95	2,90
Parn	1,48	1,10	0,91	0,50	1,09	0,74
Pdcd8	2,96	2,37	1,97	0,73	1,60	1,28
Moap1	0,59	0,96	1,07	0,30	0,61	0,66
Gusb	1,01	0,00	0,00	0,30	0,32	0,00
Bnip3L	1,37	1,06	1,14	0,72	1,12	1,07
APAF1	2,06	2,03	2,22	0,37	1,19	0,98
Diablo	3,61	2,69	2,57	1,21	2,57	2,13
Bmf	0,00	0,00	0,00	0,16	0,00	0,00
Bik	0,58	1,13	1,47	0,20	0,42	0,54

B



Supplementary Figure 2.

5

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Chapter 5

Combined KRAS and TP53 mutation status is not predictive in CAPOX treated metastatic colorectal cancer

ABSTRACT

Background

The response of colorectal tumors to chemotherapy is highly variable. Our preclinical work shows that the *KRAS* oncogene sensitizes colorectal tumor cells to oxaliplatin and capecitabine in a wild type *TP53* dependent manner. Therefore, we tested whether the combined mutation status of *KRAS* and *TP53* could predict response to chemotherapy in metastatic colorectal cancer.

Patients and methods

We selected a subgroup of patients form the CAIRO II study that received capecitabine plus oxaliplatin (CAPOX) treatment in combination with bevacizumab. The tumors were analyzed for *KRAS* and *TP53* mutations by PCR/sequencing. The relationship between tumor response and genotype was analyzed.

Results

The following KRAS/TP53 genotypes were identified: $KRAS^{mut}/TP53^{mut}$ n=21, $KRAS^{mut}/TP53^{wt}$ n=20, $KRAS^{wt}/TP53^{mut}$ n=25, $KRAS^{wt}/TP53^{wt}$ n=15. No genotype was associated with a significantly better or worse progression-free and overall survival.

Conclusion

The combined mutation status of *KRAS* and *TP53* does not predict response to CAPOX in patients with metastasized colorectal cancer.

INTRODUCTION

In the Western society, colorectal cancer is the second leading cause of cancer related deaths. In Europe alone in 2008, 212.000 patients died as a consequence of colorectal cancer (1). The incidence of colorectal cancer for males is 57.1 and 37.4 for females in Europe, with increasing mortality rates of 26.8 and 16.4 per 100.000 inhabitants, respectively (1;2).

Irresectable disseminated colorectal cancer is currently treated with a chemotherapy regimen that is based on 5-fluorouracil (or its oral prodrug analogue, capecitabine) in combination with oxaliplatin or irinotecan. Unfortunately, response rates do not exceed 40-50% (3;4), and by using these chemotherapeutics, the percentage of long term survivors has increased with a mere 10% (5).

Treatment response is influenced both by patient and tumor characteristics and depends on the chosen therapeutic regimen. The specific contribution of the tumor's genetic blueprint has been the subject of many studies (6-9). Nevertheless, due to a lack of useful biomarkers, it is currently impossible to predict tumor responses to the standard chemotherapy regimens (10).

The results from preclinical studies by us and others suggest that oncogenic KRAS sensitizes colorectal cancer cells to oxaliplatin and 5-FU (11-13). However, since *KRAS* and *TP53* mutations individually do not predict response to chemotherapy in colorectal cancer patients, clinical implementation of both genes has not occurred (14). The tumor suppressor gene *TP53* is generally acknowledged to be a key mediator of tumor cell apoptosis in response to chemotherapy. However, the value of *TP53* status as a predictive marker is still unclear and this has prevented its implementation in clinical practice (15-20).

Recently, we showed that mutant *KRAS* increases the sensitivity of colorectal tumor cells to 5-FU or oxaliplatin, but only in the presence of wildtype *TP53* (13). This suggested to us that a combination of mutant KRAS and wildtype TP53 might predict response to chemotherapy in colorectal patients. Therefore, we tested the *KRAS* and *TP53* mutation status in a cohort of colorectal cancer patients receiving a fluoropyrimidine and oxaliplatin (CAPOX) as first-line treatment and correlated the combined results of these two genes with outcome.

PATIENTS AND METHODS

Patient Selection

From the CAIRO II study (21), we randomly selected 46 patients with wild type *KRAS* and 47 with mutant *KRAS*. All patients received CAPOX with bevacizumab. DNA was isolated and analyzed for *TP53* mutations. The response criteria for disease-free and overall survival were described previously (21).

Mutation Analysis

For the detection of *TP53* mutations, DNA was amplified with primers flanking exons 4, 5, 6, 7, 8 and 9. For *KRAS*, primers flanking codons 12 and 13 were used (all primer sequences available on request). Then, PCR products were sequenced in both sense and antisense directions using the BigDye Terminator v1.1 sequencing kit on ABI 3130 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequences were analyzed using Mutation Surveyor software (SoftGenetics, LLC, State College, PA, USA). The functional impact of the different p53 mutations was assessed by consulting the database of the International Agency for Research on Cancer (www-p53.iarc.fr) (22).

Statistics

Tumors were divided in four groups, based on their genotype ($KRAS^{mut}/TP53^{mut}$ n=21, $KRAS^{mut}/TP53^{wt}$ n=20, $KRAS^{wt}/TP53^{mut}$ n=25, $KRAS^{wt}/TP53^{wt}$ n=15). Baseline characteristics between groups were compared using the chi-square test. Progression-free survival was considered as primary endpoint and overall survival as secondary endpoint. The progression-free and overall survival were estimated by the Kaplan-Meier method and compared by means of the log-rank test. All analyses were performed using SAS software, version 8.2.

RESULTS

Sequence analysis of the KRAS and TP53 genes allowed us to distinguish four tumor genotypes: i) $KRAS^{mut}/p53^{mut}$, ii) $KRAS^{mut}/p53^{wt}$, iii) $KRAS^{wt}/p53^{mut}$ and iv) $KRAS^{wt}/p53^{wt}$ (Table I). In 12 tumors the *TP53* status could not be determined due to repeated failure in the amplification of one or more exons. These patients were excluded from further analysis. The four groups were comparable in baseline characteristics, including age, gender, WHO performance status, serum lactate dehydrogenase (LDH), previous adjuvant chemotherapy, primary tumor site and number of affected organs at baseline (Table II). The tumors in 46 patients contained one or more mutation(s) in *TP53*. 39 different codons were affected with the highest frequency of 5 mutations in a single codon (Figure 1A). Exons 4 and 5 were most frequently affected (Figure 1B). Thirteen patients had more than one mutation in *TP53*, with a maximum of three mutations. The distribution of mutations in the *KRAS* oncogene showed codon 12 to be primarily affected, with 40 out of 47 mutations (Figure 1C).

Table 1. Stratification of patients according to tumor KRAS and TP53 mutation status

	TP53WT	TP53MUT	TP53 status unknown	Total
KRASWT	15	25	6	46
KRASMUT	20	21	6	47
Total	35	46	12	93

The median progression-free survival in the KRAS^{mut}/TP53^{wt} group was not significantly better than in the KRAS^{wt}/TP53^{wt} group (both 13 months, Figure 2A). Similarly, no significant median progression-free survival difference was found when comparing the KRAS^{mut}/TP53^{wt} with the KRAS^{mut}/TP53^{mut} group, the KRAS^{wt}/TP53^{wt} with the KRAS^{wt}/TP^{53}mut group and the KRAS^{mut}/TP53^{mut} with the KRAS^{wt}/TP53^{mut} group (all 13 months, Figure 2B-D).

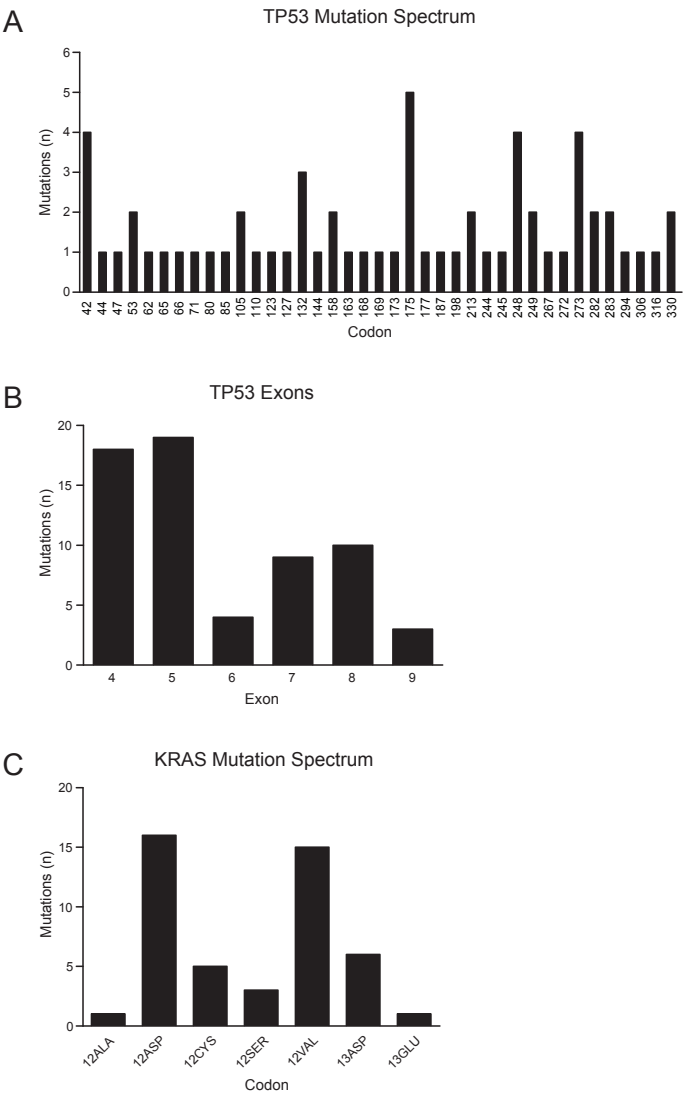

Figure 1. TP53 and KRAS mutation spectrum (**A**) Tumor samples were analyzed by RT-PCR, followed by direct sequencing for *TP53* exons 4-9. Absolute numbers of mutations in the different codons are shown. (**B**) Mutations were grouped according to their presence in the different exons. (**C**) Tumor samples were analyzed by RT-PCR, followed by direct sequencing for *KRAS* codons 12 and 13. Absolute numbers of mutations in the different codons, as well as the amino-acid substitution are shown.

The overall survival in patients with tumors harboring mutant KRAS and wild type TP53 was not significantly better or worse than that in any of the other groups. All groups showed a median overall survival of just above 20 months (Figure 3A-D), with a median follow-up of 32.8 months.

Table 2. Baseline characteristics of the defined patient groups. All groups were compared for the indicated parameters by using the Chi-square test.

	Overall (n=81)	Groups				
		KRASWT/TP53WT	KRASMUT/TP53WT	KRASWT/TP53MUT	KRASMUT/TP53MUT	p-value
Age (yrs)						
Median	65	62	65	61	67	
Standard deviation	9	10	8	10	7	0.50
Gender (no. (%))						
Male	50 (62)	7 (47)	14 (70)	17 (68)	12 (57)	
Female	31 (38)	8 (53)	6 (30)	8 (32)	9 (43)	0.45
WHO performance status (no. (%))						
0	53 (66)	9 (60)	13 (65)	20 (80)	11 (55)	
1	27 (34)	6 (40)	7 (35)	5 (20)	9 (45)	
Missing	1					0.32
Serum LDH (no. (%))						
Normal	48 (60)	10 (67)	14 (70)	14 (58)	10 (48)	
Above normal	32 (40)	5 (33)	6 (30)	10 (42)	11 (52)	
Missing	1					0.48
Prior adjuvant therapy (no. (%))						
No	65 (80)	10 (67)	16 (80)	21 (84)	18 (86)	
Yes	16 (20)	5 (33)	4 (20)	4 (16)	3 (14)	0.50
Site of primary tumour (no. (%))						
Colon	31 (46)	6 (46)	9 (60)	10 (48)	6 (33)	
Rectum	13 (19)	3 (23)	2 (13)	3 (14)	5 (28)	
Rectosigmoid	23 (34)	4 (31)	4 (27)	8 (38)	7 (39)	
Missing	14					0.80
No. of affected organs (no. (%))						
1	37 (46)	5 (36)	10 (50)	13 (52)	9 (43)	
>1	43 (54)	9 (64)	10 (50)	12 (48)	12 (57)	
Missing	1					0.76

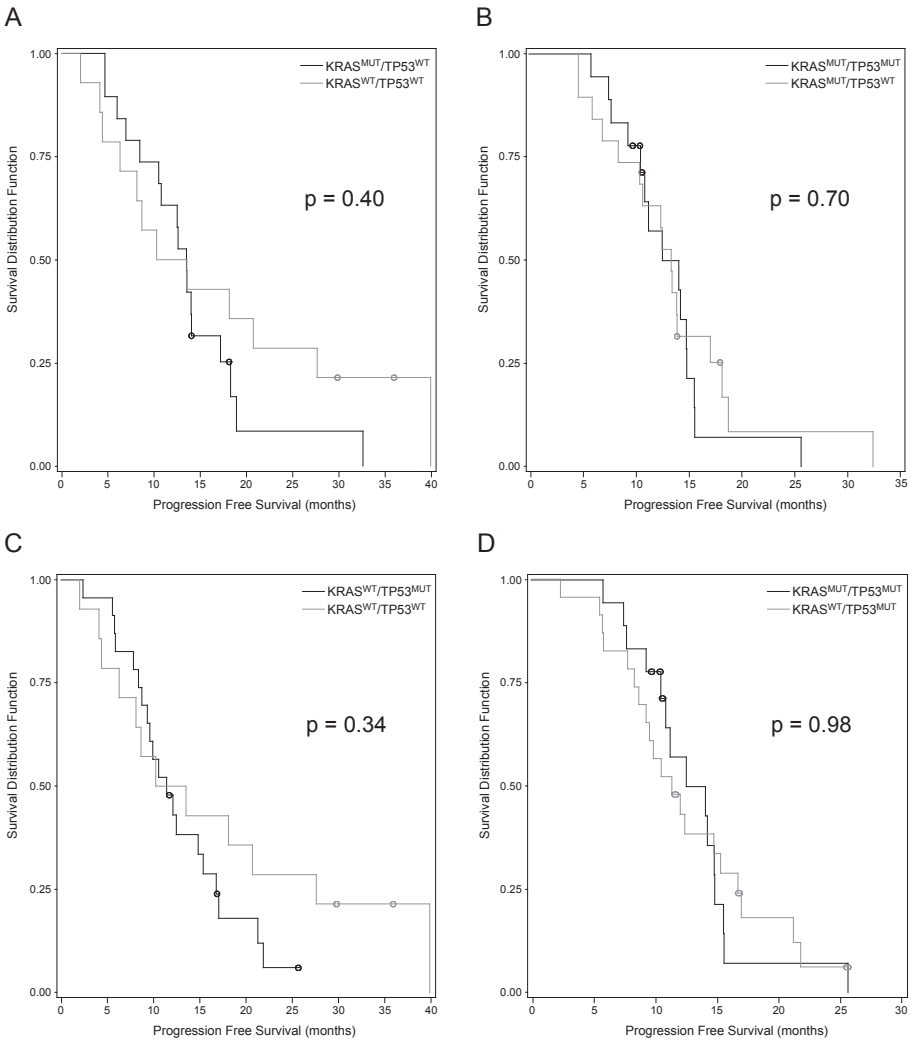

Figure 2. Effect of *KRAS* and *TP53* mutation status on progression-free survival Kaplan-Meier graphs showing progression-free survival to assess the effect of (**A**) mutated KRAS in a p53 wild type backround, (**B**) mutant p53 in a mutant KRAS background, (**C**) mutant p53 in a KRAS wild type background, and (**D**) mutant KRAS in a mutant p53 background. No statistically significant differences were found.

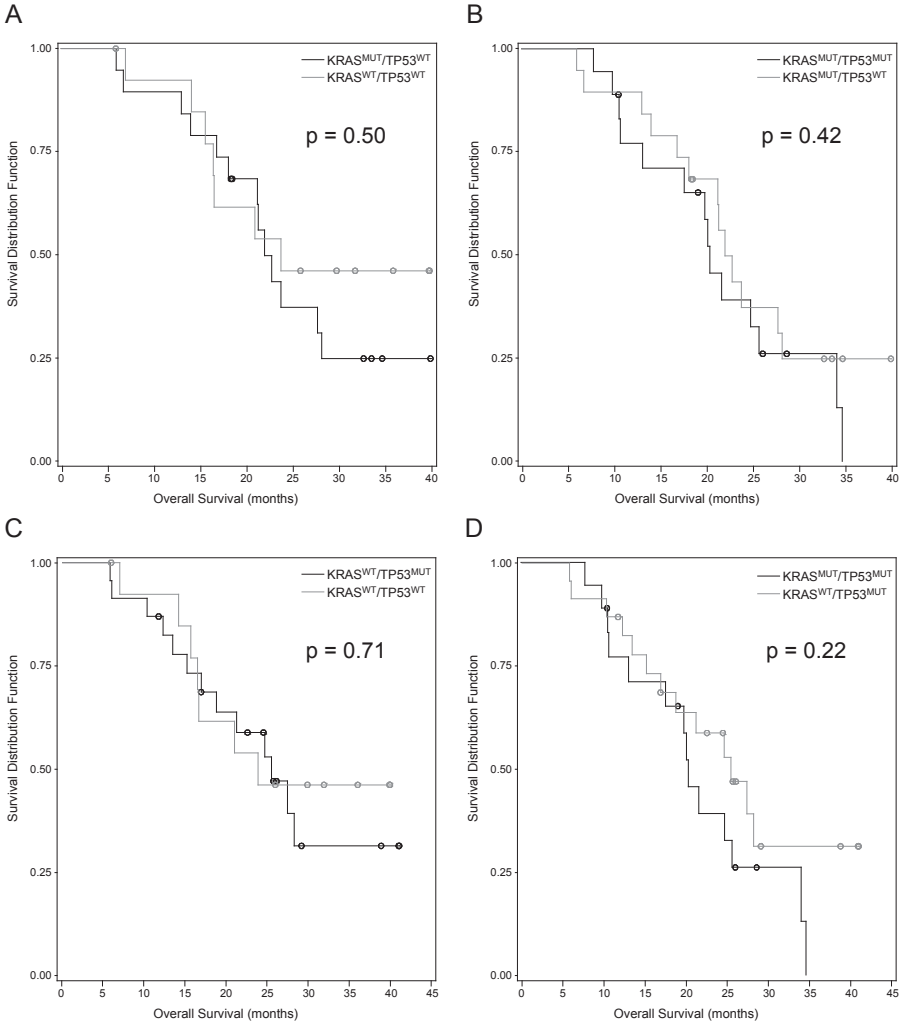

Figure 3. Effect of *KRAS* and *TP53* mutation status on overall survival Kaplan-Meier graphs showing overall survival to assess the effect of (**A**) mutated KRAS in a p53 wild type backround, (**B**) mutant p53 in a mutant KRAS background, (**C**) mutant p53 in a KRAS wild type background, and (**D**) mutant KRAS in a mutant p53 background. No statistically significant differences were found.

DISCUSSION

Based on our previous work we hypothesized that tumors with mutant *KRAS* and wild type *TP53* would respond best to a fluoropyrimidine and oxaliplatin containing regimen (13). However, we show here that upon such treatment progression-free and overall survival of patients with colorectal cancer is unaffected by *KRAS* and *TP53* status, either alone or in combination. There are several potential explanations for the apparent discrepancy with the preclinical results.

Firstly, the *in vitro* and xenograft studies were performed with colorectal cancer cell lines either expressing wildtype *TP53* or lacking *TP53*. However, human colorectal tumors do not usually lose p53 expression, but rather acquire mutations in the gene.

A wide spectrum of mutations has been detected throughout the *TP53* gene. The full range of effects of many of these mutations is poorly understood. Mutations in *TP53* can result in loss of function, but can also result in the acquisition of dominant traits such as invasive and metastatic potential (23). Furthermore, some p53 mutants act in a dominant negative fashion, neutralizing the function of wild type p53. Of all 63 mutations found in this patient cohort, only 27 have known functional consequences (22). The number of patients in our series is too small to allow meaningful conclusions on these functional categories.

Secondly, although the KRAS mutations identified in this study can all be classified as activating mutations, differences in the biological behavior of the different mutants have been identified (24;25). Overall they show no effect on tumor response to chemotherapy, however subgroups analyses have not yet been made.

Thirdly, in addition to the functional heterogeneity among the specific *TP53* and *KRAS* mutations, other levels of heterogeneity are likely to influence the tumor response to chemotherapy. Colorectal tumors have mutations in, on average, 12 cancer-driving genes of which *KRAS* and *TP53* only constitute two (26). In addition, the tumor subgroups defined in this study are likely to be characterized by heterogeneity at the level of genomic deletions and amplifications and by epigenetic diversity. This implies that classification of colorectal tumors on the basis of their *KRAS* and *TP53* mutations still generates highly heterogeneous tumor subgroups.

Multidimensional tumor analysis which combines large scale sequence analysis with analysis of genomic rearrangements and epigenetic alterations may be required to identify tumors that respond well to conventional chemotherapy, and those that do not.

ACKNOWLEDGEMENTS

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Chapter 6

Synergistic killing of Colorectal Cancer Cells by Oxaliplatin and ABT-737

ABSTRACT

Oxaliplatin is frequently used in the treatment of metastatic colorectal cancer (CRC). However, many tumors do not respond to oxaliplatin, possibly due to expression of Bcl-2-like survival proteins. ABT-737 is a Bcl-2-family inhibitor that has shown efficacy as a sensitizer to chemotherapy in various tumor models. Here, we investigated whether ABT-737 could improve oxaliplatin efficacy in CRC cells.

A panel of CRC cell lines was treated with oxaliplatin and ABT-737, either alone or in combination. Treatment with oxaliplatin or ABT-737 alone had marginal effects on cell viability. Combination of the two drugs lead to a strong synergistic apoptotic response, but only in the subset of cell lines expressing wildtype *TP53*. HCT116 cells lacking either *wildtype TP53* or oncogenic *KRAS* responded very poorly to single or combination treatments. Combination treatment strongly enhanced Noxa expression in a *wildtype TP53*- and oncogenic *KRAS*-dependent manner. Suppression of Noxa by RNA interference prevented apoptosis after combination treatment. Finally, treatment of mice with subcutaneous tumor xenografts showed that combination treatment drastically reduced tumor volume, while single drug treatment had no effect.

We conclude that the combination of oxaliplatin and ABT-737 is very effective in killing colorectal tumor cells and that this requires induction of Noxa by wildtype *TP53* and oncogenic *KRAS*. Further studies should explore the potential benefit of adding BH3-mimetics to oxaliplatin-based chemotherapy of colorectal tumors.

INTRODUCTION

Oxaliplatin and 5-fluorouracil (5-FU) are essential in the chemotherapeutic treatment of colorectal cancer. Although these drugs increase the lifespan of patients with metastatic disease, overall survival rarely exceeds two years (1). Increasing the therapy response to prolong overall survival therefore remains a major challenge.
Previously, we showed that the efficient killing of colorectal cancer cells by oxaliplatin or by 5-FU was dependent on the presence of oncogenic *KRAS* and wild type *TP53*. This was caused by induction of the pro-apoptotic *TP53* response gene Noxa (2).

Noxa belongs to the family of BH3-only proteins, which induce apoptosis by binding to and neutralizing survival proteins like Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and Bfl1/A1. In doing so, these proteins allow the pro-apoptotic proteins Bax and Bak to form oligomers within the mitochondrial membrane which causes mitochondrial outer membrane depolarization, release of cytochrome C, formation of the apoptosome, and activation of effector caspases that mediate cell destruction (3). In normal untransformed cells Noxa is not generally considered to be potent inducer of cell death when compared to other members of the BH3-only proteins, like Puma and Bim. However, in tumor cells Noxa does play a major role in the induction of apoptosis (2;4). The capacity of Noxa to induce apoptosis is strongly enhanced when anti-apoptotic proteins that are not bound by Noxa, such as Bcl-2, Bcl-X_L and Bcl-w, are neutralized (5). A potent inhibitor that binds and neutralizes Bcl-2, Bcl-X_L and Bcl-w (but not Mcl-1 or Bfl1/A1) is the small molecule inhibitor ABT-737 (6). Interestingly, ABT-737 is particularly effective in cells in which the Mcl-1 survival protein is neutralized by high Noxa levels (7-9). Based on our previous study in which oxaliplatin caused apoptosis in a Noxa-dependent fashion (2), we tested whether ABT-737 could synergize with oxaliplatin in stimulating apoptosis in CRC cells. Furthermore, we tested the contribution of mutant KRAS and wild type TP53 to Noxa induction and sensitization to apoptosis.

MATERIALS AND METHODS

Cell culture

The human CRC cell-line HCT116$^{G13D/wt}$ and the isogenic cell line Hkh2$^{ko/wt}$ lacking the mutant KRAS^{D13} allele were kindly provided by Dr Shirasawa (10). The murine C26 and human DLD1, Ls174T and HT29 CRC cell-lines were purchased from ATCC (Manassas, VA, USA). HCT116 p53$^{wt/wt}$ and p53$^{-/-}$ cells were kindly provided by Dr B Vogelstein (11). HCT116 Noxa knockdown and control cells were described before (2). All cells were cultured in Dulbecco's modified Eagle's medium (Dulbecco, ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 5% (v/v) fetal calf serum and 2mM Glutamine both purchased from Cambrax (New Jersey, USA).

FACS analysis of the cell cycle profile and apoptosis

The cell cycle profile was determined to asses the percentage of apoptotic cells (sub-G1 population) and G2 arrest after treatment with oxaliplatin (Sanofi Aventis, Gouda, The Netherlands) and ABT-737 (kindly provided by ABBOT Laboratories, Illinois, USA) by FACS analysis using propidium ioide (Sigma Aldrich). Cells ($2x10^5$) were seeded in 6-well plates (Costar Corning, NY, USA) and incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. The following day the cells were treated with either 25μM oxaliplatin, 2.5μM ABT-737 or the combination for 24 hours (Ls174T were treated for 48 hours). Floating and attached cells were harvested and fixed as single cells in ice-cold 70% Ethanol for 15 minutes at 4°C followed by an incubation of 30 minutes at 37°C with 20μg/ml propidium iodide and 100μg/ml RNase A (Roche Diagnostics, Basel, Switzerland). All samples were analysed by bivariate flow cytometry using Cell Quest software (Becton Dickinson, Breda, The Netherlands).

Determination of synergistic drug responses

Combination index (CI) values were calculated by Calcusyn software (Biosoft, Cambridge, UK) to determine synergy. To this end the cells were treated as described above with the drugs at varying doses of a constant ratio 50:5, 25:2.5, 12.5:1.25 and 6.25:0.625 μM oxaliplatin:ABT-737 respectively, in triplicate. FACS analysis of sub-G1 content was then used to determine the % apoptotic cells in all these cultures. These values were used in Calcusyn to asses the CI values. CI values < 0.30 indicate strong synergy. CI values > 0.80 indicate no synergy.

Western blotting and antibodies

Lysates of treated and untreated tumor cells were prepared in lysisbuffer (20mM HEPES pH7.4, 1% NP40, 150mM NaCl, 5mM $MgCl_2$, 10% glycerol). Equal amounts of protein were run out on SDS-PAA gels and were analyzed by Western blotting using antibodies directed against p53 (clone DO-1), p21 (clone C19) obtained from Santa Cruz (CA, USA), cleaved caspase-3 (clone Asp175), Puma (polyclonal) obtained from Cell Signaling Technology (Danvers, MA, USA), Noxa (clone 114C307.1, Imgenex, San Diego, CA, USA) and Mcl-1 (polyclonal, BD Pharmingen, San Diego, CA, USA). β-Actin (clone AC-15) was used to control for protein load and was obtained from Novus Biologicals (Littleton, CO, USA).

Xenograft tumor mouse model

All experiments were conducted in accordance with the guidelines of the Animal Welfare Committee of the University Medical Centre Utrecht, The Netherlands. C26 cells ($1x10^6$) were subcutaneously injected into the flanks of 8-10 weeks old male BALB/c mice (Charles River; Sulzfeld, Germany). Tumor volume (V) was calculated by $V=AxB^2x0.5236$, whereas A indicates the largest diameter and B the diameter perpendicular to A. Tumors were grown for 7 days until a volume of approximately $50mm^3$ was reached. At day 7 the mice were injected intraperitonally (i.p.) with one dose of 5mg/kg oxaliplatin followed

by daily i.p. injections of 50mg/kg ABT-737 in 30% propylene glycol (Sigma Aldrich), 5% Tween-80 (Merck, New Jersey, USA) and 65% 50mg/ml dextrose (Sigma Aldrich) in water at pH4. Mice that did not receive oxaliplatin or ABT-737 received the same injections of the vehicles (PBS for oxaliplatin and PG/TX100/dextrose for ABT-737).

Statistical analysis

All *in vitro* experiments were performed at least three times independently. *In vivo* experiments consisted of 8 mice per group. Figures depict group means ± SEM. Statistical differences were analyzed by one-way ANOVA followed by a Bonferroni multiple comparison test using GraphPad Prism software (GraphPad, San Diego, CA). A p value < 0.05 was considered to be statistically significant (*).

RESULTS

ABT-737 synergizes with oxaliplatin to kill colorectal cancer cells

A panel of 5 colorectal cancer cell lines (C26, HCT116, LS174T, DLD1, HT29) was treated with oxaliplatin and ABT-737 either alone or in combination. Apoptosis was measured by FACS analysis of sub-G1 content. In three cell lines (C26, HCT116, LS174T) the combination treatment showed a strong synergistic apoptosis response, indicated by low combination indices (CI) (Figure 1A), while in two others (DLD1, HT29) this effect was not observed. Interestingly, the synergistic response to combination treatment correlated with wild type *TP53* status (Figure 1A).

TP53 is required for synergistic tumor cell killing by oxaliplatin and ABT-737

To test whether *TP53* is required for synergistic tumor cell killing by oxaliplatin and ABT-737, we used HCT116 $p53^{wt/wt}$ and HCT116 $p53^{-/-}$ cells. These isogenic cell lines, only differing in p53 status, were treated with either drug alone or with the combination. FACS analysis of sub-G1 DNA content and Western blotting for active Caspase-3 showed that HCT116 $p53^{-/-}$ cells were completely resistant to apoptosis induced by the combination treatment (Figure 1B).

Noxa is required for Caspase-3 processing by oxaliplatin and ABT-737

Since oxaliplatin induces Noxa to promote apoptosis (2) we next tested how the single and combination treatments affected expression levels of Noxa, the related protein Puma, and the Noxa/Puma-target Mcl-1. Single treatment of *TP53* wildtype cells with oxaliplatin or ABT-737 alone induced expression of Noxa, and this was strongly enhanced during combination treatment. Puma levels were unaffected by treatment with either drug alone, but were reduced by the combination treatment. Mcl-1 was marginally induced by ABT-737, but this was not observed in cells treated with oxaliplatin alone or in cells treated with both drugs.

Apoptosis-resistant p53$^{-/-}$ cells were unable to induce Noxa expression following single or combined drug treatment, and they did not display Caspase-3 activation. p53-deficiency did not affect Puma levels but reduced Mcl-1 levels by approximately 50%. (Figure 2A). Our previous results had indicated that induction of Noxa by oxaliplatin not only depends on *TP53* but also on oncogenic *KRAS*. Therefore, we tested whether the synergistic induction of Noxa following combined treatment with oxaliplatin and ABT-737 required the presence of this oncogene. Indeed, mutant *KRAS*-deficient HCT116 cells were unable to induce Noxa and failed to process Caspase-3 in response to either drug alone or to the combination (Figure 2B).

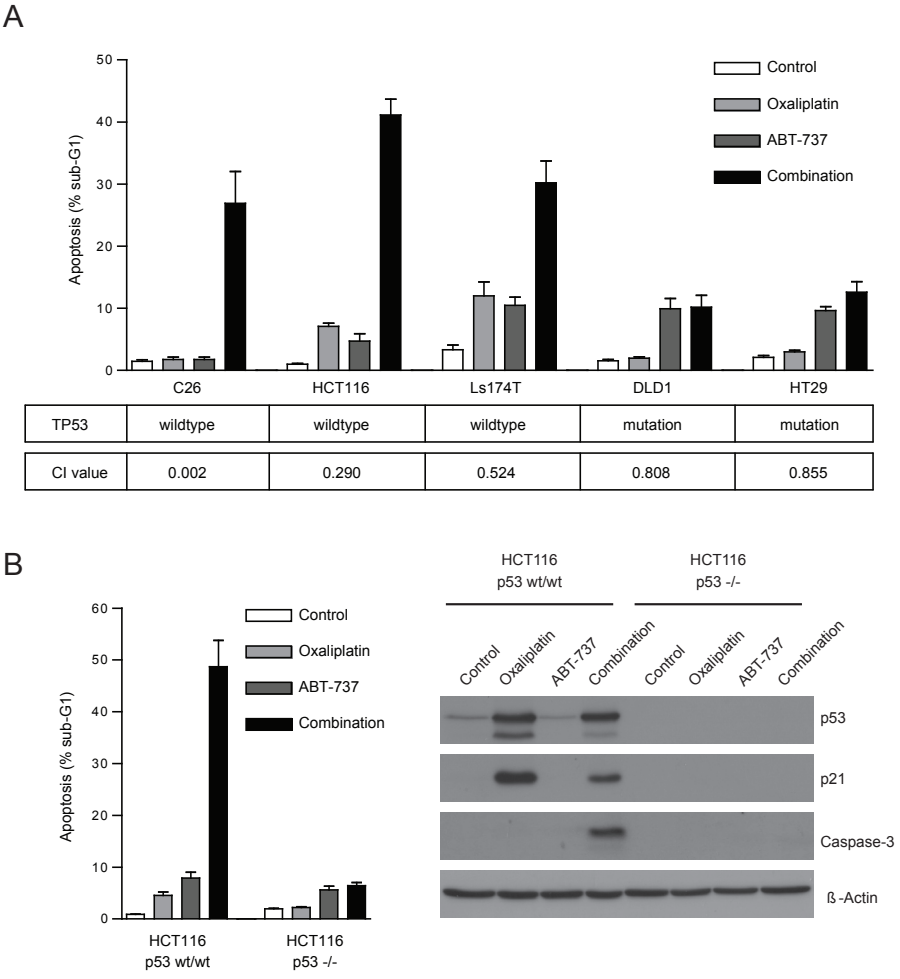

Figure 1. Synergistic p53-dependent killing of colorectal cancer cells by oxaliplatin and ABT-737 (**A**) Cells were treated with oxaliplatin and/or ABT-737 for 24 hours (C26, HCT116, DLD1, HT29) or 48 hours (Ls174T). Cells were then fixed in formalin and stained with propidium iodide (PI). The sub-G1 fraction was determined by FACS analysis. Error bars represent SEM based on 3 independent experiments. Combination-index (CI) values were calculated using Calcusyn. CI values < 0.30 indicate strong synergy. CI values > 0.80 indicate no synergy. (**B**) Cells were treated as in A. Lysates were prepared and analyzed for the presence of p53, p21, cleaved Caspase-3 and β-Actin by Western blotting.

To assess the contribution of Noxa to apoptosis induced by the combination treatment we made use of HCT116 cells in which Noxa was stably suppressed by RNA interference (2). Neither oxaliplatin alone nor the combination with ABT-737 increased Noxa levels under these conditions and Caspase-3 processing was not observed (Figure 2C).

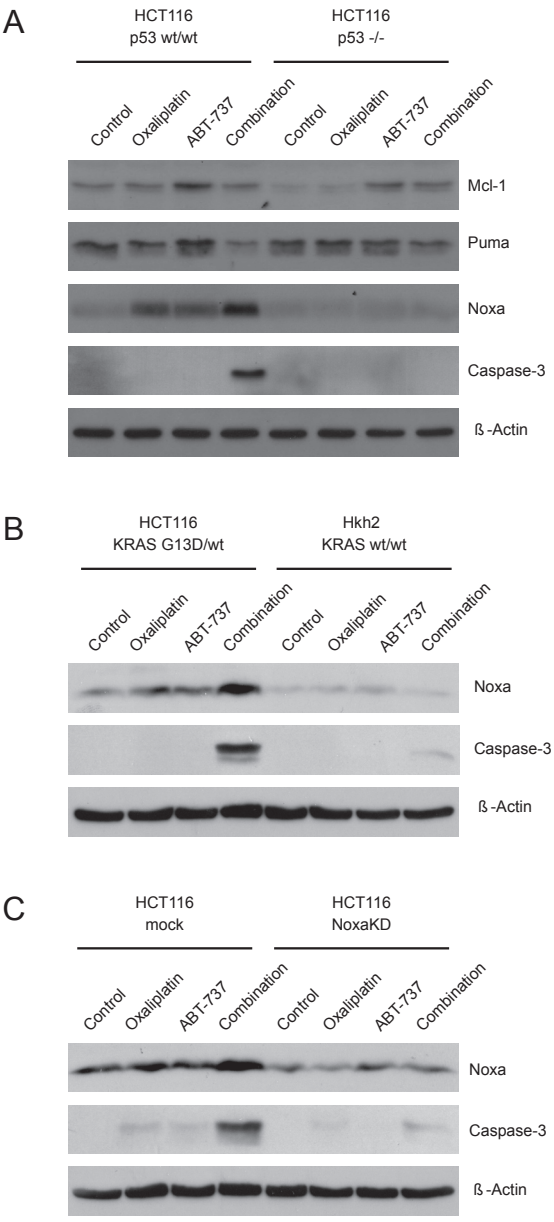

Figure 2. Oxaliplatin and ABT-737 induce Noxa in a p53- and mutant KRAS-dependent manner (**A**) HCT116 $p53^{wt/wt}$ and $p53^{-/-}$ cells were treated with oxaliplatin and/or ABT-737. Lysates were prepared and analyzed for the presence of Mcl-1, Puma, Noxa, cleaved Caspase-3 and β-Actin by Western blotting. (**B**) As in (A) but using HCT116 $KRAS^{G13D/wt}$ and $p53^{-/wt}$ cells. (**C**) As in A but using HCT116 Noxa-knockdown and control cells.

Oxaliplatin and ABT-737 synergistically reduce tumor growth

Finally, we assessed whether oxaliplatin and ABT-737 could also affect tumor growth in a synergistic fashion. To this end BALB/c mice were injected subcutaneously with the syngenic colorectal cancer cell line C26. Treatment with oxaliplatin, ABT-737 or the combination started seven days following tumor inoculation and the mice were sacrificed on day 15. Tumor growth was not significantly affected by oxaliplatin or ABT-737 alone at the doses used. However, treatment of the mice with both drugs strongly and significantly reduced tumor growth resulting in a 75% reduction of tumor volume on day 15 (Figure 3).

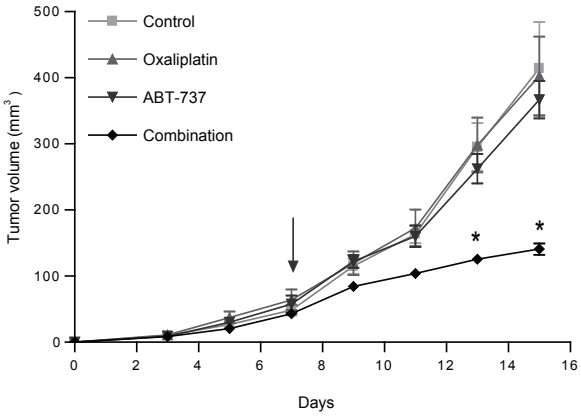

Figure 3. Synergistic reduction of tumor growth by oxaliplatin and ABT-737 Mice carrying subcutaneous C26 tumors were treated with oxaliplatin on day 7 (arrow) and ABT-737, either alone or in combination. Control mice received both vehicles. Treatment started 7 days following subcutaneous tumor cell injection. Tumor growth was analyzed by caliper measurements. Observers were blinded to treatment. * p <0,05.

DISCUSSION

Our results show that ABT-737 and oxaliplatin kill colorectal tumor cells in a synergistic fashion, and that this depends on KRAS/p53-dependent induction of the pro-apoptotic protein Noxa. ABT-737 selectively inhibits the anti-apoptotic proteins Bcl-2, Bcl-X_L, and Bcl-W, but leaves the Noxa targets Mcl-1 and A1 unaffected (6;12;13). Treatment with ABT-737 caused an upregulation of both Noxa and Mcl-1. While addition of oxaliplatin further enhanced Noxa induction, it reduced Mcl levels. Given that apoptosis induced by the combination treatment depends on Noxa, we propose a model in which oxaliplatin and ABT-737 cooperate to induce Noxa to neutralize the Mcl-1 branch of survival proteins, while ABT-737 targets the Bcl-2 branch in addition (Figure 4). Interestingly, the pro-apoptotic protein Bad inhibits the same pro-survival proteins as ABT-737 and cooperates with Noxa to induce potent tumor cell killing (5).

Recently it was shown that Puma (but not Noxa) is sensitive to caspase-3-dependent proteolytic destruction (Hadji et al, 2010). In line with these results, we found that caspase-3 processing in cells treated with the drug combination was associated with strongly reduced levels of Puma.

A number of studies have demonstrated the importance of the Mcl-1/Noxa axis in determining the tumor cell response to ABT-737. This relationship was shown in renal cell carcinoma cells (9), small cell and non small cell lung cancer cells (7;8;14-16), lymphoma and acute lymphoblastic leukemia cells (17-19), acute and chronic myeloid leukemia cells (12;20), prostate cancer cells (21;22), melanoma cells (23-25), glioblastoma cells (26) and colorectal cancer cells (27). Our study demonstrates that the widely used chemotherapeutic drug oxaliplatin induces Noxa expression, and that this makes cells highly sensitive to killing by ABT-737. In addition, we have identified two genetic determinants of synergistic tumor cell killing by oxaliplatin and ABT-737: wildtype TP53 and oncogenic KRAS. The molecular basis for this dependency is that both proteins are required for drug-induced Noxa expression. Further work using patient derived colonospheres and xenografts is needed to asses the potential therapeutic benefit of oxaliplatin/ABT-737 combination therapy in (a subpopulation of) human colorectal tumors.

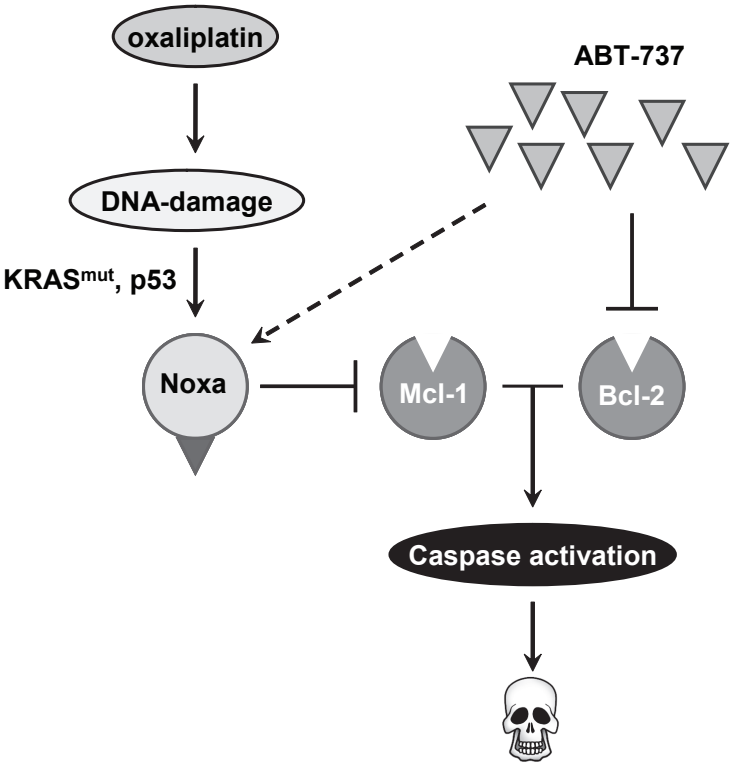

Figure 4. Model for the synergistic response of colorectal tumor cells to oxaliplatin and ABT-737 Oxaliplatin and ABT-737 cooperatively induce Noxa in a p53- and KRAS-dependent manner. This neutralizes the Mcl-1 branch of Bcl-2-like survival proteins. In addition, ABT-737 neutralizes the Bcl-2 branch as well.

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Chapter 7

General discussion and future perspectives

Targeting the MAPK pathways in KRAS mutant cells

The rationale for targeting the RAS/RAF/MEK/ERK MAPKinase pathway in tumors with mutant KRAS is based on the fact that signaling through this pathway has been implicated in RAS induced cell transformation and oncogenesis (1). Unfortunately, the objective response rate following MEK inhibiting therapies have been disappointing (2).

Interestingly, in cells with a mutation at the level of RAF ($BRAF^{V600E}$), direct inhibition of $BRAF^{V600E}$ or inhibition at the level of MEK is very effective in suppressing the growth of tumor xenografts in mice (3-6). However, we (see chapter 2) and others have shown that tumor cells with an endogenous KRAS mutation, are not or less sensitive to MEK inhibition (7-9). This demonstrates that mutant KRAS has options to bypass signaling through the RAF/MEK/ERK pathway, while mutant BRAF has not. Indeed, as shown in chapter 2 and by others, RAS, has the opportunity to signal through the p38 MAPK pathway (10). By activating p38, mutant KRAS can activate shared transcriptional targets of both the MER/ERK and the p38 MAPK pathway. Examples are the MNK1 and MNK2 kinases. MNK1/2 phosphorylate the eukaryotic translation initiation factor 4E (eIF4E), which can cause tumor development in transgenic mice overexpressing eIF4E (11). MNK1/2-mediated phosphorylation of other substrates, including Sprouty2, cPLA2, and hnRNPA1, may also enhance tumorigenesis (12-15).

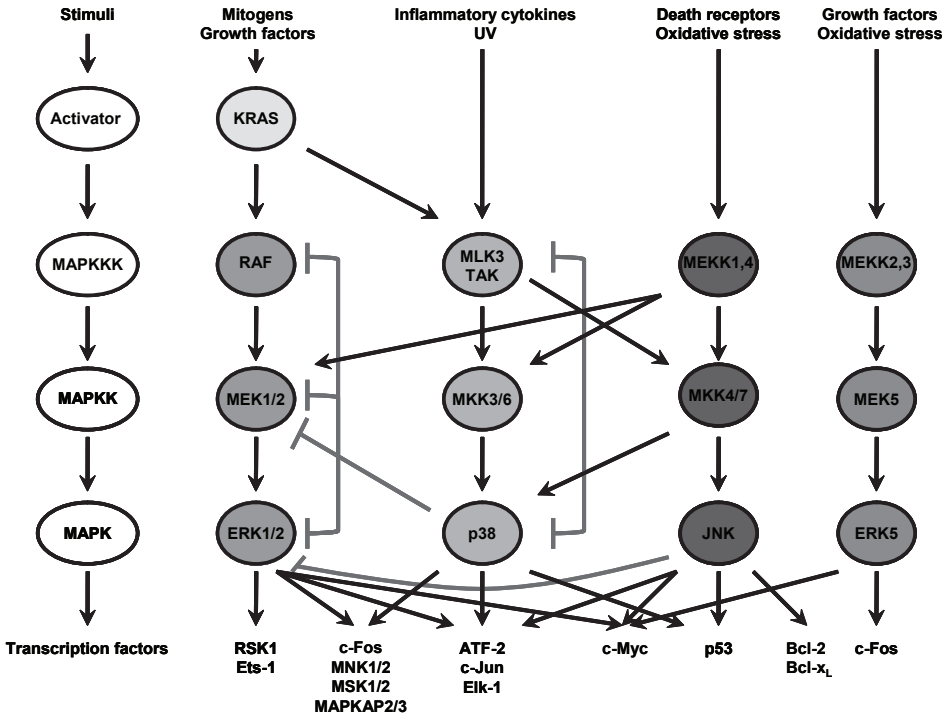

Figure 1. The currently known MAPK signaling pathways Black arrows indicate activational action, where the inhibitory phosphatases are shown in grey. Adapted from Junttila *et al*, FASEB Journal, 2008 and Jeffrey *et al*, Nature Reviews on Drug Discovery, 2007.

All MAPKinase pathways are organized in modular cascades in which activation of upstream kinases by cell surface receptors lead to sequential activation of a MAPK module (see Figure 1). To date 4 MAPK signaling pathways have been identified, of which three have been characterized in detail (the MEK/ERK, the p38 and the JNK pathway). It has been suggested that in normal physiological contexts, MAPK cascades would function independently, in a "linear" fashion, with no crosstalk between them. Interplay between pathways would be induced only during pathological situations when signal strength exceeds the capacity of the pathway (16). This theory is supported by findings that upstream activating kinases of p38 and JNK, the MAPK-kinases-kinases MEKK1-3, activate MEK1/2 (the MAPKK of the ERK module) by direct phosphorylation (17-19). In addition, many more "non-linear" connections of positive and negative cross-regulation between distinct MAPK modules have been identified (20). Furthermore, MAPK signaling modules are stringently controlled by protein phosphatases, adding another level of complexity to the control of MAPK signaling (20).

In view of this great complexity of MAPK pathway cross-regulation, it remains questionable whether it is possible to target more than the tumor cell can adapt to. If tumor cells are not dependent on a single pathway for survival or growth, it is likely that inhibition of that pathway will not result in a lasting therapeutic effect. As we have seen in chapter 2, this is the case for colorectal tumor cells expressing oncogenic KRAS, where KRAS allows p38 to be activated in MEK-suppressed cells, allowing these to recover from the G1-arrest imposed by MEK inhibition. Second, the reversibility of cell cycle arrest is of importance. To prevent tumor cell recovery from MAPK pathway inhibition, it would be logical to target factors that have an impact on multiple of these pawthays. Such a possible therapeutic target is the protein phosphatase 2A. Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase that can both negatively and positively regulate the activity of the known MAPK pathways. For example, the upstream activator of p38, MKK3, as well as MEK1/2 and ERK1/2 are dephosphorylated by PP2A *in vitro*, causing inhibition of the p38 and ERK pathways. RAF-1 (one of the three RAF-kinases, also known as C-RAF) on the other hand, the activator of MEK1/2, is induced by PP2A mediated dephosphorylation of its inhibitory phosphorylation site (21;22). Inhibition of PP2A causes cell transformation and mutations in PP2A occur at low frequency in several human cancers (23-25). PP2A-mediated dephosphorylation of ERK1/2 induces apoptosis in leukemia cells. Dephosphorylated (inactive) ERK can no longer phosphorylate the pro-apoptotic Bcl-2 family member Bad, resulting in accumulation of dephosphoryled (active) Bad (26-28). In KRAS mutant colorectal cancer cells (HT29, HCT116, SW480) PP2A activation also leads to apoptosis through Bad dephosphorylation (29). Due to the pro-apoptotic effect of PP2A activation, and the inhibition of multiple MAPK-pathways, this strategy has the potential to prevent tumor cell adaptation.

Wip1 as a therapeutic target

Wild type p53 induced protein 1 (Wip1), a member of the protein phosphatase 2C (PP2C) family, was found to be amplified and overexpressed in many primary breast cancers (30;31), neuroblastomas (32) and ovarian clear cell adenocarcinomas (33). In addition, Wip1 is highly expressed in intestinal stem cells and in a mouse model of APC^{min} driven polyposis, a premalignant stadium of colorectal cancer. Wip1 removal significantly increased the lifespan of the mice by reducing the number of polyps (34). Also Wip1-deficient mouse embryonic fibroblasts showed resistance to oncogene driven transformation. Finally, RAS- and Erbb2-driven mammary gland tumors in Wip1-deficient mice showed a delay in onset (35). Together with Wip1's function as a checkpoint recovery competence gene (see chapter 3), these data support the notion that Wip1 may act as an oncogene. Importantly, this oncogenic potential is shown selectively in the presence of wild type p53 (31-33;35;36). An explanation for this is that Wip1 counteracts p53 directly and indirectly (37). For instance, Wip1 dephosphorylates and stabilizes Mdm2 which causes p53 destabilization (38). This could make Wip1 an interesting target in tumor cells expressing wild type p53.

The following aspects could make Wip1 an interesting target. First, Wip1's amino-acid sequence is only distantly related to the rest of the PP2C phosphatase subfamily, suggesting that specific Wip1 inhibition may be possible (39). Second, genetic ablation of Wip1 in mice is well tolerated, suggesting a minimal occurrence of side effects following specific inhibition (34). Third, Wip1 knockout mice are resistant to tumorigenesis. Finally, the fact that Wip1 is overexpressed in tumors may provide a good therapeutic window (toxic dose compared to effective dose) for intervention (39). Although phosphatases are traditionally considered to be difficult drug targets (40), an effective and specific Wip1 inhibitor has been developed (39). This inhibitor (CT007093) caused cell death in Wip1 overexpressing breast cancer cells (MCF7), through induction of p38. The dual role of p38 as an inducer of tumor cell proliferation (chapter 2) and as an inducer of G2 checkpoint arrest, cell death and senescence, remains puzzling and most likely depends on the cellular background (41). This might prove to be an obstacle as KRAS mutant cells use p38 to stimulate proliferation. Whether Wip1 inhibition will be effective in (colorectal) cancer cells with mutant KRAS needs to be established.

Overcoming tumor cell resistance to apoptosis

During tumor development, cancer cells acquire many abnormalities, like genomic instability or oncogene activation, which should normally induce cell death through the intrinsic apoptosis pathway. The fact that cancer cells survive, suggests that a block in apoptosis may be required for carcinogenesis. Three distinct ways by which tumor cells can block the activation of apoptosis through the intrinsic pathway have been described (42). One way is by reducing the levels of the pro-apoptotic BH3-only proteins or by preventing their activation. This may involve deletion of the genes themselves or inactivation of p53, which is required for their transcriptional activation (class A block).

The second option is to reduce or eliminate Bax and Bak (class B block), which are the effectors of mitochondrial outer membrane permeabilization (MOMP), which is the rate-limiting step in the intrinsic apoptosis pathway. The third possibility is by increasing the expression of pro-survival proteins like Bcl-2 (class C block), which neutralize Bak and Bax and by doing so prevent MOMP and apoptosis.
Tumor cells which survive by using the latter option are considered to be "primed for death", as they are uniquely dependent on pro-survival proteins of the Bcl-2 family. This is more common among malignant cells than among nonmalignant cells. In the colorectal cancer cell system we investigated, we observed apoptosis caused by an increased induction of Noxa following oxaliplatin and 5-FU treatment. This was dependent on mutant KRAS and wild type p53. Mcl-1, the pro-survival binding partner of Noxa, failed to compensate for increased Noxa levels. If chemotherapeutics like 5-FU and oxaliplatin neutralize this branch of the survival proteins, further neutralization of the Bcl-2-branch could have great therapeutic impact.

The observation that Noxa-mediated Mcl-1 neutralization does not occur in cells lacking p53 suggests that cells without functional *TP53* could use a class A block to prevent apoptosis. Consequently, these cells need to be targeted in a different manner to enhance the apoptotic response. A strategy to tackle this problem is to reactivate the p53 pathway. Functional p53 may be restored in different ways. First, virus-mediated introduction of wild type p53 through gene therapy was effective in the treatment of patients with head and neck cancer in one trial In this trial, the treatment of head and neck squamous cell carcinoma with adenoviruses encoding wild type p53 combined with radiotherapy was more effective than radiotherapy alone (43). Sixty-four percent of the group receiving the combination treatment showed complete remission compared to 20% in the group with radiotherapy alone. Second, stabilization of p53, by inhibition of Mdm2 mediated degradation of p53, in cells which overexpress MDM-2 and/or reactivation of mutant p53, through introduction of the wild type p53 C-terminal peptides, can induce apoptosis in preclinical studies (reviewed in (44)). More clinical trials are currently underway to further assess the therapeutic efficacy of restoring the p53 signaling pathway.
Mcl-1 overexpression is common in human cancers, and high expression could correlate with chemotherapy resistance and disease progression (45). Oxaliplatin strongly induced the Mcl-1 neutralizer Noxa. Furthermore, the combination of oxaliplatin and the Bcl-2-inhibitor ABT-737 synergistically enhanced apoptosis of colorectal cancer cells in a Noxa-dependent manner (see chapter 6). This effect was dependent on the presence of wild type p53. In addition to oxaliplatin, ABT-737 synergizes with bortozemib, vinblastine, epotoside, imatinib, CPT-11 and TRAIL (46-50). This suggests that the small molecule ABT-737 could be applied to enhance the effectiveness of a broad range of therapeutics. Importantly, it appears that in different tumor cell types both the pro-apoptotic proteins have to be induced and the pro-survival proteins have to be inhibited, in order to induce apoptosis (46-50). The promising perspective ABT-737 offers, is that it neutralizes Bcl-2, Bcl-x_L and Bcl-w with subnanomolar affinity and exhibits marked cytotoxicity in

combination with several chemotherapeutics and radiation, while sparing non-tumor cells (51). Nonetheless, ABT-737 itself is not likely going to be clinically implemented because of poor physiochemical and pharmaceutical properties. It is not orally available and has low aqueous solubility, which makes intravenous delivery challenging. In our experiments we have administered ABT-737 to the mice via intraperitoneal injections. However, for human administration, an orally bioavailable alternative is preferred because of dose flexibility and the possibility of chronic use. Alternatively, ABT-263, an orally bioavailable BH3 mimetic, has shown comparable activity as an apoptosis inducer in small cell lung carcinoma and acute lymphoblastic leukemia in mice. ABT-263 also enhances the activity of chemotherapeutic agents *in vivo* (52). This creates great expectations and to date, two phase I trails have finished recruiting volunteers and two phase II studies have started recruiting patients.

KRAS and TP53 as predictive biomarkers

The *KRAS* oncogene can be a functional biomarker. The mutations are limited to a small part of the gene, and they can be relatively easily detected. *KRAS* is currently used as a negative predictor for the addition of anti-EGF receptor treatment to the current standard therapy in colorectal cancer (53-56). *TP53's* function on the other hand, shows to be far more difficult to assess. Mutations occur throughout the gene, without any absolute hotspots. On top of that, little is known about the effects of most of the mutations. More than 80% of p53 alterations are missense mutations that lead to the synthesis of a stable full length protein (57). Prediction of the consequences of all these different mutations on p53 function is still very difficult (58). In the preclinical studies we used a model in which p53 can be knocked down or knocked out. Based on the observed increase in apoptosis we hypothesized that the combination of mutant *KRAS* and wild type *TP53* could correlate with an increase in response to capecitabine (the orally administrable pro-drug of 5-fluorouracil) and oxaliplatin. To this end we analyzed a subgroup of patients with metastasized colorectal cancer from the CAIRO2 study (see chapter 5). However, mutant *KRAS* in combination with wild type *TP53* did not predict an increase in response to therapy.

To be able to assess the predictive value of the *TP53* tumor suppressor gene, screening methods must be developed for high throughput functional analysis of specific p53 mutations. Until that time *TP53* either alone or in combination with KRAS, will probably have limited or no function as a predictive biomarker. Nevertheless, therapies which can reinstate the functionality of the p53 signaling pathway may offer a new perspective for the predictive value of the *KRAS* oncogene. Furthermore, such drugs may allow synergistic tumor cell killing by chemotherapy and Bcl-2-inhibitors in mutant p53 cells. Reinstatement of functional p53 could ultimately lead to the application of Bcl-2 inhibitors in colorectal cancer patients with mutant *KRAS* and mutant *TP53*.

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8

Chapter 8

Nederlandse samenvatting

Review Committee

Acknowledgements

List of publications

Curriculum vitae auctoris

NEDERLANDSE SAMENVATTING

Het colorectaal carcinoom (dikke darmkanker) en de metastasering daarvan, vormt in de westerse samenleving de tweede meest voorkomende doodsoorzaak als gevolg van kanker. In 2008 stierven er alleen al in Europa 212.000 mensen aan de gevolgen van colorectale kanker. Niet resectabele gemetastaseerde ziekte wordt behandeld met chemotherapie, welke bestaat uit 5-fluorouracil (5-FU), in combinatie met oxaliplatin of irinotecan. Helaas laten niet meer dan 40-50% van de patiënten een respons op de behandeling zien, en is het het aantal lange termijn overlevenden hierdoor slechts toegenomen met 10%.

Terwijl de term “colorectaal carcinoom” een enkelvoudige entiteit suggereert, zijn de achterliggende genetische oorzaken van deze ziekte zeer heterogeen. Deze heterogeniteit in “genetische blauwdruk” veroorzaakt een wisselende respons op chemotherapie.
Tot op heden zijn we onvoldoende in staat om op basis van de genetische blauwdruk te voorspellen hoe een individuele patient, of patientenpopulatie, gaat reageren op de toegepaste chemotherapie. Hierdoor behandelen wij alle patiënten met gemetastaseerde ziekte, indien hun conditie het toestaat, met een standaard chemotherapeutisch regime, alsof zij aan een homogene ziekte lijden. Dit heeft als gevolg dat patiënten die geen baat zullen hebben bij de therapie wel worden blootgesteld aan de bijwerkingen hiervan. Tevens leidt dit tot onnodig hoge zorgkosten.

Om ons inzicht in het al dan niet optreden van een therapeutische respons te vergroten, is in dit proefschrift de voorspellende waarde van verschillende genetische factoren bij de behandeling van colorectale tumorcellen onderzocht. Ook is gekeken naar de mechanistische achtergond van de reactie van tumorcellen bij blootstelling aan chemo- en andere anti-tumorale therapie. Uiteindelijk zou dit kunnen leiden tot identificatie van patiëntengroepen op basis van het genetisch profiel van hun tumor, met een daarop toegespitst chemotherapeutisch behandelschema.

Van alle colorectaal carcinomen heeft zo’n 40% een mutatie in het KRAS oncogen. Deze mutatie leidt tot ongebreidelde celdeling, metastasering en invasie in het omliggende weefsel. Het belang van de KRAS mutatie in de ontwikkeling van een normale cel naar een uiteindelijke tumorcel is uitvoerig bestudeerd en aangetoond. Dit heeft ertoe geleid dat er verschillende pogingen zijn en worden ondernomen om de gevolgen van deze mutatie specifiek te remmen. Een van de gekozen strategiën is het remmen van de belangrijke MEK/ERK signaleringsroute, welke een belanrijke rol speelt in de stimulatie van groei, invasie en overleving van de tumorcel. Specifieke remmers van deze signaleringsroute blijken echter in klinische studies onvoldoende resultaat te laten zien. In **hoofdstuk 2** van dit proefschrift hebben we onderzocht, wat de mechanistische achtergrond zou kunnen zijn voor het gebrek aan klinisch resultaat. Hieruit komt naarvoren dat colorectale tumorcellen met een KRAS mutatie de signalering door de MEK/ERK route kunnen

laten overnemen door een andere, namelijk de signaleringsroute via p38. Wanneer wij beide routes tegelijk remmen zien wij een langdurig remmend effect op de celgroei, wat ervoor pleit om verder onderzoek te doen naar de effectiviteit van deze combinatie van remmers bij de behandeling van het colorectaal carcinoom.

Chemotherapeutica induceren DNA-schade door verschillende mechanismen. Na DNA-schade te hebben opgelopen activeert een cel het *TP53* tumor suppressor gen, wat ertoe leidt dat de cel in zijn celcyclus wordt gestopt. Hierna wordt de schade gerepareerd of ondergaat de cel geprogrammeerde celdood, als de schade te groot blijkt. In **hoofdstuk 3** van dit proefschrift blijkt dat Wip1, geïnduceerd door *TP53*, van essentiëel belang is voor een cel om na reparatie van de schade de celcyclus weer te kunnen hervatten. Mogelijk speelt Wip1 een rol in het ontwikkelen van een cel tot een tumorcel, omdat als gevolg van een overactieve functie cellen met onherstelbare DNA-schade (waaronder mutaties) weer aan de celcyclus zouden kunnen deelnemen.

In eerder verricht onderzoek hebben wij geobserveerd dat coloractale tumorcellen met een mutatie in het *KRAS* oncogen gevoeliger zijn voor behandeling met de standaard chemotherapeutica oxaliplatin en 5-FU. In **hoofdstuk 4** hebben onderzocht wat de mechanistische verklaring is voor deze verhoogde gevoeligheid. In de aanwezigheid van gemuteerd *KRAS* en functioneel *TP53* wordt de verhoogde gevoeligheid veroorzaakt door de specifieke activering van Noxa (Latijn voor “schade”). Dit eiwit is onderdeel van een balans van eiwitten die geprogrammeerde celdood induceren of voorkomen. Door de toename van Noxa slaat de balans door in het voordeel van de eiwitten die het celdood programma activeren, met als gevolg de grotere gevoeligheid voor de chemotherapeutica.

In **hoofdstuk 5** hebben we onderzocht of onze bevindingen uit het laboratorium- en proefdierstudies vertaald konden worden naar de klinische situatie. Hiervoor hebben we de mutatiestatus voor *KRAS* en *TP53* in een groep patiënten bepaald die behandeld waren met capecitabine (een oraal toe te dienen vorm van 5-FU) en oxaliplatin. Uit de analyse bleek echter dat patiënten met een *KRAS* gemuteerde tumor die wel normaal functionerend *TP53* had, niet correleerde met een betere progressievrije overleving ten opzichte van de patiënten zonder *KRAS* mutatie en/of gemuteerd *TP53*. Een mogelijke verklaringen voor de discrepantie tussen laboratorium en kliniek zou kunnen liggen in de diversiteit van gevolgen van een mutatie in *TP53*. Een mutatie in *TP53* kan diverse invloeden kan hebben op de functie. Dit in tegenstelling tot de laboratoriumopstelling, waarbij er slecht een vergelijking wordt gemaakt tussen functioneel en niet functioneel *TP53*.

Zoals boven beschreven, zagen we in hoofdstuk 4 dat gemuteerd *KRAS* bij blootstelling aan 5-FU of oxaliplatin, door middel van Noxa de balans van eiwitten betrokken bij de voorkoming of inductie van geprogrammeerde celdood verstoorde. In **hoofdstuk 6**

testten we de hypothese of additionele remming van de beschermende eiwitten het effect kon versterken. Dit bleek inderdaad het geval. Wanneer ABT-737 werd toegevoegd, werden de beschermende eiwitten geremd. Hierdoor werd het effect van de toename van Noxa als gevolg van de chemotherapie synergistisch versterkt. Voor deze toename in effect was het wel noodzakelijk dat tumorcellen zowel gemuteerd *KRAS* als normaal functioneel *TP53* in hun genetisch profiel hadden. Deze resultaten zouden na verdere studie kunnen leiden tot een verbeterde behandeling van patienten met gemetastaseerd colorectaal carcinoom en het beschreven genetisch profiel.

REVIEW COMMITTEE

Prof.dr. E.E. Voest

Prof.dr. P.J. van Diest

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Prof.dr. L.M.A. Akkermans

Dr. V.B. Nieuwenhuijs

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LIST OF PUBLICATIONS

Synergistic killing of Colorectal Cancer Cells by Oxaliplatin and ABT-737
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**These authors contributed equally*

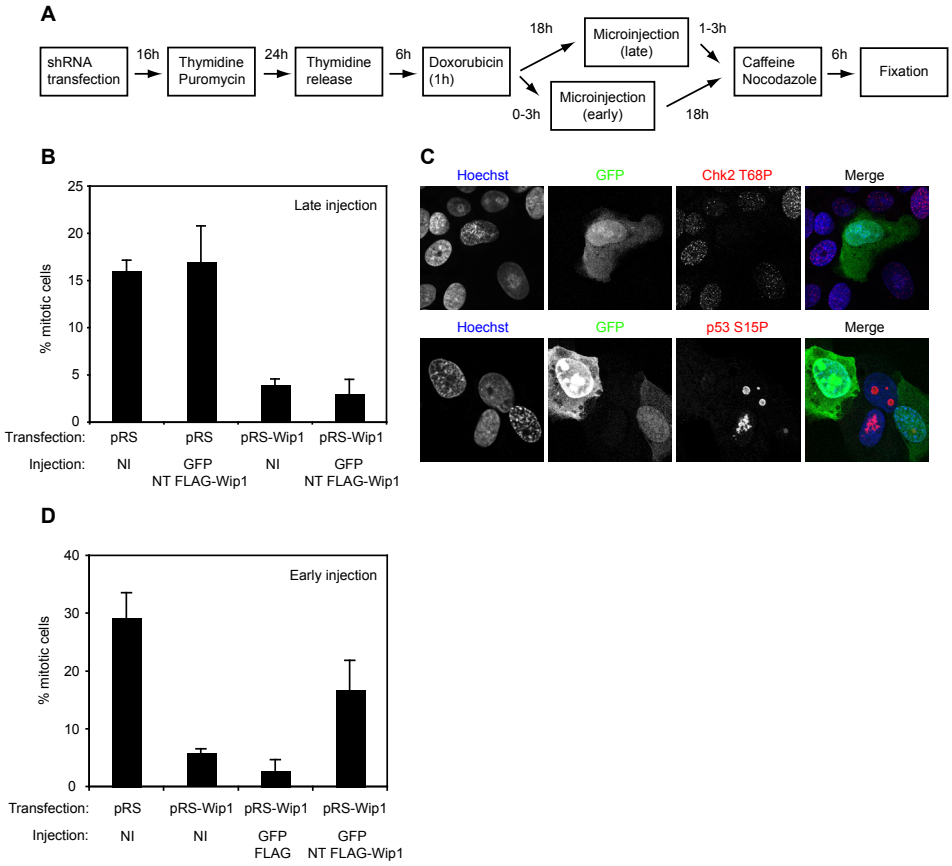
CURRICULUM VITAE AUCTORIS

Menno Theo de Bruijn was born in Almelo on the 2nd of October, 1980. He grew up in Nijverdal and attended the primary school, Prinses Marijke School in Nijverdal and the secondary school, O.S.G. Erasmus in Almelo. He graduated from the HAVO in 1997 and VWO in 1999. After graduation het started medical school at the University of Utrecht and began his clinical rotations in 2002. At the end of 2004 he performed his research elective at the Flinders University and Flinders Medical Centre in Adelaide, Australia (supervisors: Prof. dr. G.J. Barritt, Dr. V.B. Nieuwenhuijs and Dr. R.T. Padbury). He obtained his medical degree in November, 2005. In that same year he was awarded the prize for best presentation at the congress of The Royal Australasian College of Surgeons in Perth, Australia. He was also awarded the Alexandre Suerman scholarship for talented starting researchers of the UMC Utrecht, which funded his first two years of PhD research with the Surgical Oncology Research Group (supervisors: Prof. dr. I.H.M. Borel Rinkes and Dr. O. Kranenburg). His last two research years were funded by the Association for International Cancer Research. After completion of his PhD research he started his clinical career in the St. Antonius Hospital, Nieuwegein (supervisor: P.M.N.Y.H. Go) in November 2009. In August 2010 Menno started his surgical training in the UMC Utrecht (supervisor: Prof. dr. I.H.M. Borel Rinkes), and he is scheduled to perform the last four years in the Ziekenhuis Groep Twente in Almelo (supervisor: Dr. J.G. van Baal).

9

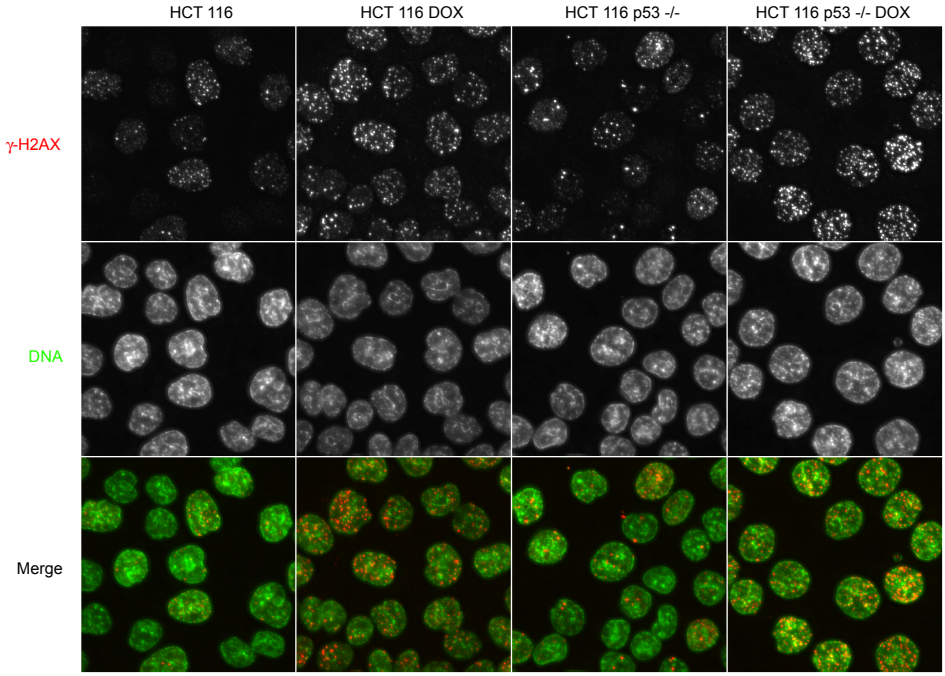
Chapter 9

Color appendix



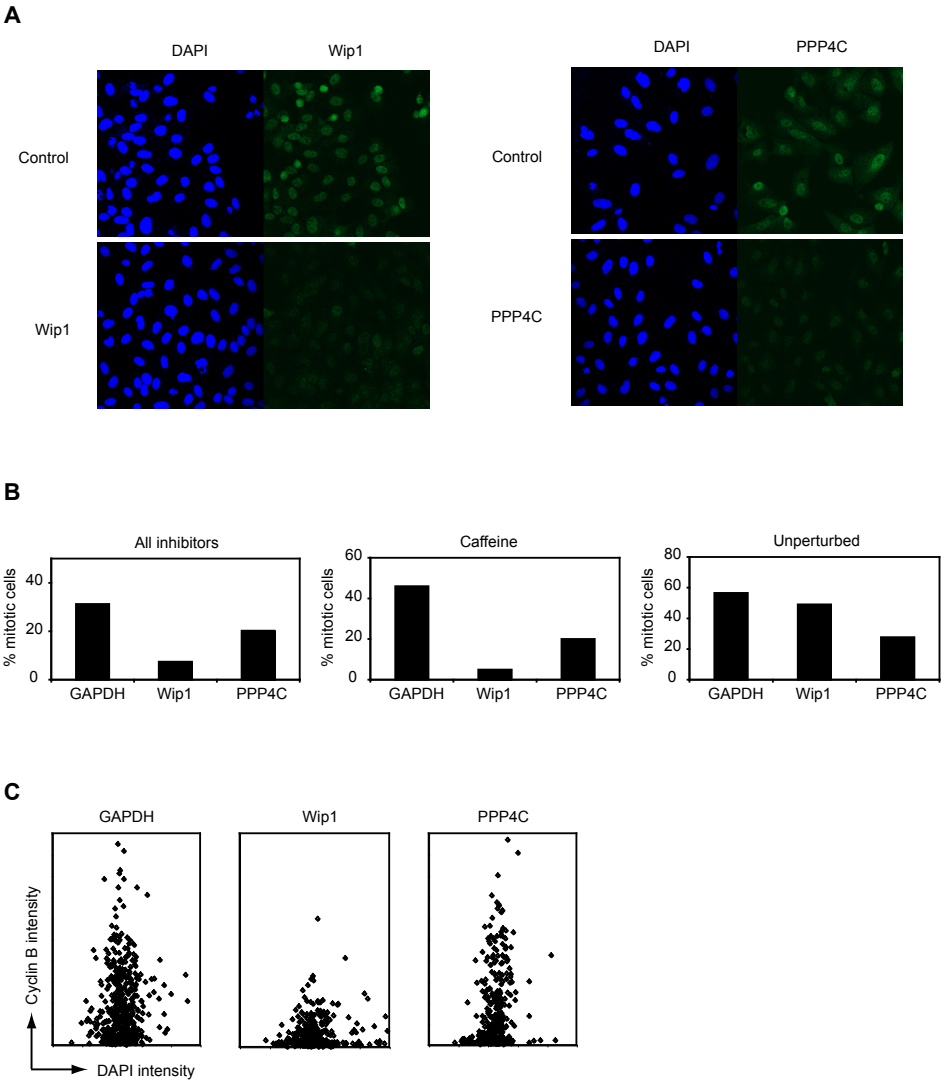
Chapter 3

Supplementary Figure 1. Late reconstitution with Wip1 cannot rescue checkpoint recovery. (**A**) Schematic outline of experiments in B-D. (**B**) Late injection of non-targetable FLAG-Wip1 cannot rescue Wip1 RNAi recovery deficient phenotype. pRS or pRS-Wip1 transfected U2OS cells were selected with puromycin, exposed to doxorubicin, 18 hours later co-injected with GFP (to mark successfully injected cells) and non-targetable FLAG-Wip1 and treated with caffeine for 6h, as outlined in A. NI, not injected cells. 57-144 cells were counted for each injected construct in each of three independent experiments. Error bars represent standard error. (**C**) Late injected Wip1 is active since Chk2 T68 (top panels) and p53 S15 (bottom panels) are dephosphorylated in the injected, but not in the surrounding cells. Images show pRS-Wip1 transfected cells co-injected with GFP and non-targetable FLAG-Wip1, as outlined in A. (**D**) Early injection of non-targetable FLAG-Wip1 partially rescues Wip1 RNAi recovery deficient phenotype. Experiments were performed as in B, but injections were performed immediately after doxorubicin wash-out, followed by caffeine addition 18 hours later. 46-135 cells were counted for each injected construct in each of three independent experiments. Error bars represent standard error.



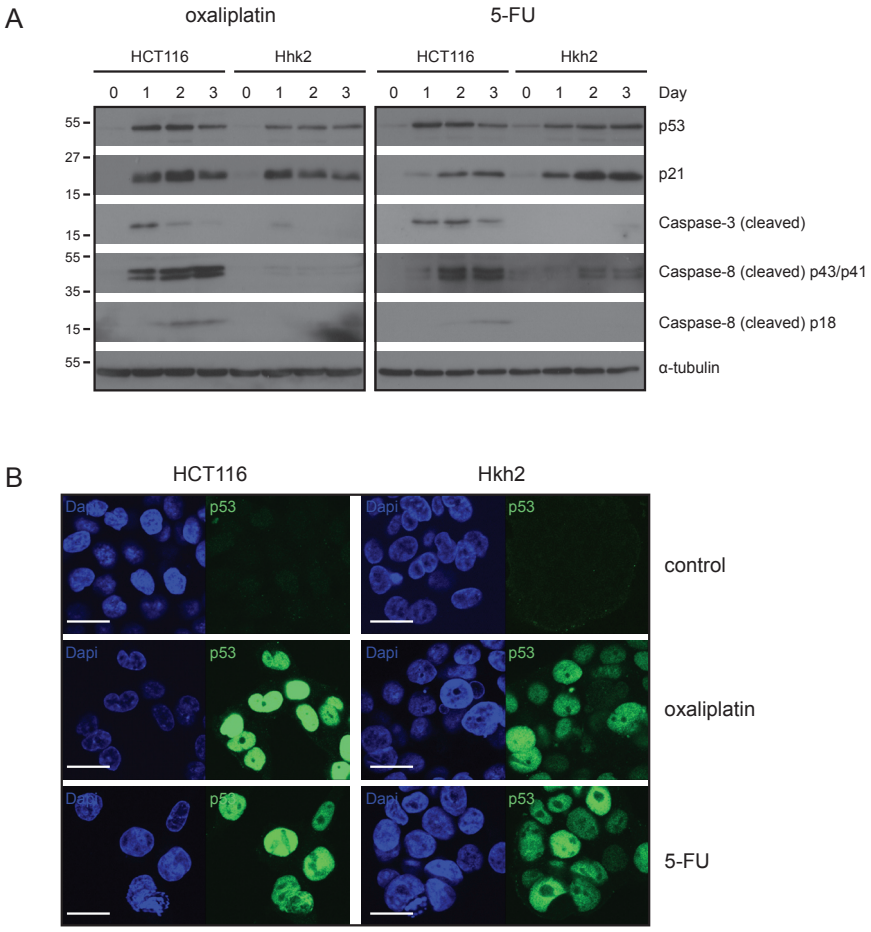
Chapter 3

Supplementary Figure 3. A subset of untreated HCT116 cells contain γH2AX foci. HCT116 and HCT116 p53-/- cells were left untreated or treated with 0.5 μM doxorubicin for 18 hours, fixed and stained for γH2AX.



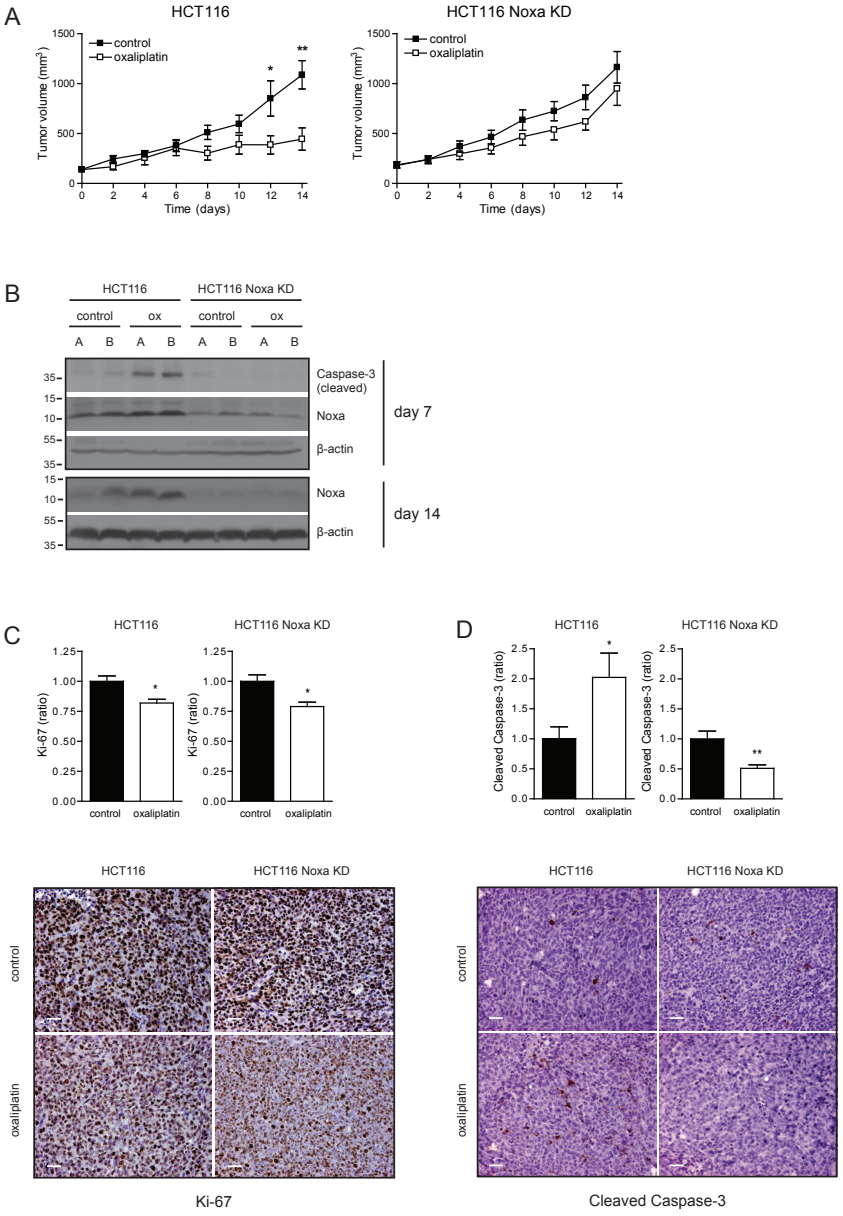
Chapter 3

Supplementary Figure 5. Not all phosphatases counteracting checkpoint kinases confer checkpoint recovery competence. (**A**) U2OS cells were transfected with siRNAs targeting GAPDH, Wip1 or PPP4C. Images show immunofluorescence stainings using Wip1 or PPP4C antibodies. (**B**) U2OS cells were transfected with siRNAs targeting GAPDH, Wip1 or PPP4C, and treated as in figure 1F (all inhibitors and caffeine). Alternatively, cells were synchronized by a thymidine block and fixed after 20h release into taxol-containing medium (unperturbed). The amount of mitotic cells was assessed by counting MPM2-positive cells using automated image analysis (materials and methods). (**C**) U2OS cells were treated as in B, but in the absence of checkpoint kinase inhibitors. Cells were stained with DAPI and Cyclin B antibodies and the fluorescence intensities of 500 cells per sample were analyzed using automated image analysis (materials and methods). Graphs show the cytoplasmic Cyclin B intensities plotted versus the nuclear DAPI intensities.



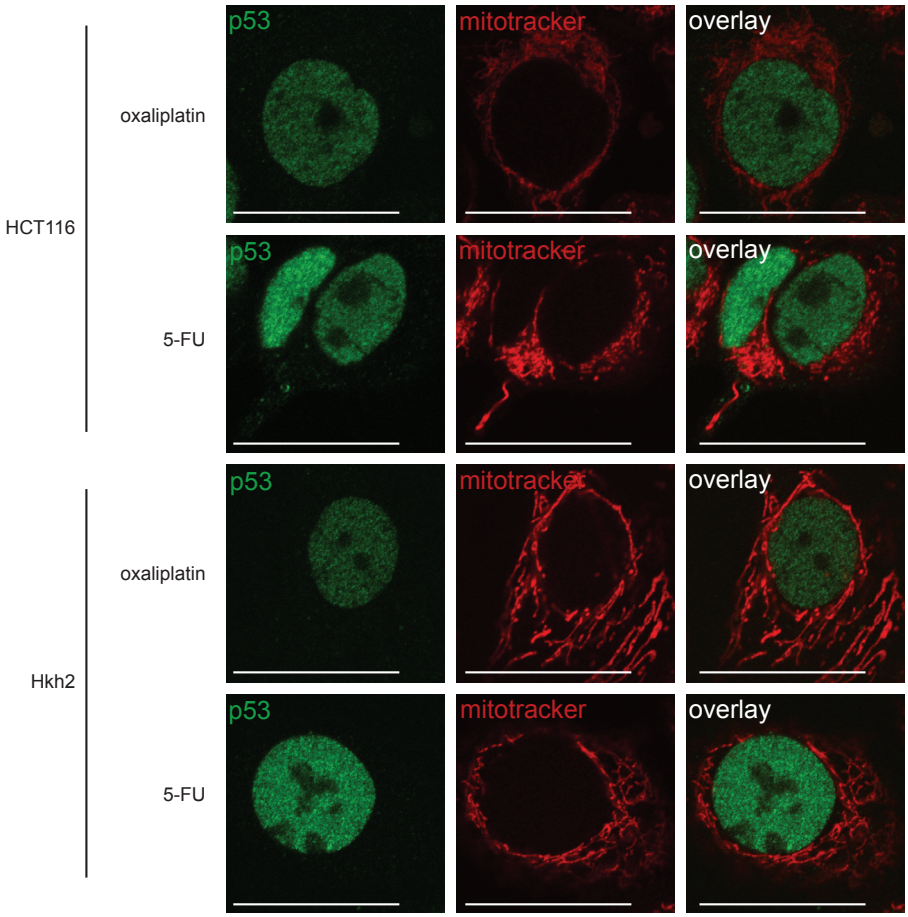
Chapter 4

Figure 2. Oncogenic KRAS promotes apoptosis without affecting p53 stabilization or p21 induction (**A**) HCT116 cells and Hkh2 cells were treated with oxaliplatin (8µg/ml) or 5-FU (8µg/ml) for 0-3 days as indicated. Lysates were prepared and analyzed for the presence of p53, p21, cleaved Caspase 8, tubulin and actin by Western blotting. (**B**) HCT116 and Hkh2 cells were treated for 24 hours with oxaliplatin or 5-FU. Cells were then processed for immunofluorescence analysis of p53 localization. Bars represent 50µm. (**C**) Cells were treated as in B and cell lysates were analyzed for the presence of p53 (total p53) and p53 phosphorylated at residues Ser6, Ser9, Ser15, Ser20, Ser37, Ser46, and Ser392. The signals for Ser6, Ser9, Ser20 and Ser46 were below the detection limit.



Chapter 4

Figure 6. Contribution of Noxa to the therapeutic efficacy of oxaliplatin and 5-FU (**A**) Immune deficient nude mice (n=8/group) were injected subcutaneously with 10^6 HCT116 control or HCT116-NoxaKD cells. When tumors reached a size of 100 mm3, the mice received either PBS or oxaliplatin (12,5 mg/kg) intraperitoneally (Day 0). Seven days later, all mice received a second dose of either PBS or oxaliplatin. Tumor growth was followed by caliper measurements every 2 days (*p=0.048, **p=0.008). (**B**) Tumors were harvested on day 7 and day 14. Of each group, two tumors (A,B) were analyzed for the presence of cleaved Caspase-3 (as a marker for apoptosis) and for Noxa by Western blotting. All tumors were analyzed by immunohistochemistry for Ki-67 (**C**) and cleaved Caspase-3 (**D**) as markers for proliferation and apoptosis respectively. Ten random fields per tumor were scored and the positive tumor areas were determined by automated computer analysis. The bar graphs represent the ratios of Ki67 and Caspase-3-positive surface areas in oxaliplatin-treated xenografts *versus* control xenografts (*p<0.05). Representative images are shown on the right. Bars represent 50µm.



Chapter 4

Supplementary Figure 1. p53 appears not co-localize with mitochondria following oxaliplatin or 5-FU
HCT116 and Hkh2 cells were treated for 24 hours, stained for p53 and mitochondria and analyzed by immunofluorescence microscopy.