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Role of AP4 in DNA damage repair, senescence and metastases in colorectal cancer

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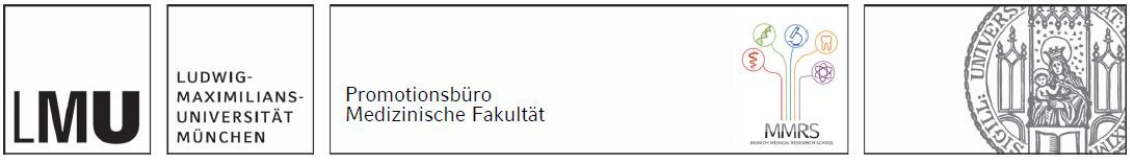

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Table of content

List of abbreviations

5-FU	5-Fluorouracil
AP-1	Activating protein-1
AP4	Transcription factor AP-4
APC	Adenomatous polyposis coli
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BER	Base excision repair
bHLH-LZ	Basic helix–loop–helix leucine zipper
CCLE	Cancer Cell Line Encyclopedia
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus molecular subtype
COAD	Colon adenocarcinoma
CRC	Colorectal cancer
CRIS	CRC intrinsic subtypes
DOX	Doxycycline
EMT	Epithelial-mesenchymal transition
HR	Homologous recombination
JNK1	c-Jun N-terminal protein kinase 1
LOH	Loss of heterozygosity
MAP2K7	Mitogen-activated protein kinase kinase 7
MAP3K13	Mitogen-activated protein kinase kinase kinase 13
MDC1	Mediator of DNA damage Checkpoint 1

Min	Multiple intestinal neoplasia
MIR22HG	MIR22 host gene
MMR	Mismatch repair
MSI	Microsatellite instability
MSS	Microsatellite stability
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PDX	Patient-derived xenografts
qChIP	Quantitative chromatin immunoprecipitation
SMS	Seed-matching sequence
TCGA	The Cancer Genome Atlas
TF	Transcription factor
TSS	Transcriptional start site
UTR	Untranslated region

List of publications

1. Chou J, Kaller M, Jaeckel S, Rokavec M, Hermeking H. AP4 suppresses DNA damage, chromosomal instability and senescence via inducing MDC1/Mediator of DNA damage Checkpoint 1 and repressing MIR22HG/miR-22-3p. *Molecular Cancer*. 2022 May 27;21(1):120.

2. Chou J, Kaller M, Rokavec M, Liu F, Hermeking H. AP4 promotes metastases of colorectal cancer by activating AP-1 via induction of the JNK1 pathway and a coherent miR-22-3p/FOSL1 feed-forward loop. *Cancer Communications*. 2024 https://doi.org/10.1002/cac2.12514.

Your contribution to the publications

Contribution to paper I

I generated all CRC cell lines with varying *AP4* gene status and inducible pRTR vectors as detailed in the paper. Subsequently, I prepared RNA samples for RNA-Seq analysis. I identified MDC1 as a potential AP4 target through a comprehensive analysis of RNA-Seq and ChIP-Seq data. To validate AP4 targets, I performed qChIP analysis. Expression levels of genes highlighted in the paper were assessed through qPCR and Western blot analyses. The impact of AP4 inactivation was investigated by me using various assays, including colony formation, β-galactosidase staining, immunofluorescence, comet assay, and homologous recombination assay. Additionally, I conducted MTT assays to evaluate the sensitivity of CRC cells to 5-FU treatment, following the different treatments outlined in the paper. I actively contributed to shaping ideas for the paper. I wrote the manuscript draft and prepared the figures showed in the paper.

Contribution to paper II

I generated all CRC cell lines with varying gene status and inducible pRTR vectors as detailed in the paper. Subsequently, I prepared RNA samples for RNA-Seq analysis. I identified FOSL1 and the components of JNK1 signaling as potential AP4 targets by analyzing RNA-Seq and ChIP-Seq data. I performed qPCR and Western blot analysis to determine the expression of genes presented in the paper. I performed qChIP analysis to validate AP4 targets. In the paper I utilized colony formation assay, CCK-8 assay, “Wound-healing” assay, modified Boyden-chamber assay and qPCR analysis to determine the effect of AP4 and its target on proliferation, migration, invasion and EMT in CRC cell lines. I performed dual-luciferase assays to determine AP-1 activity and to validate the regulation of *FOSL1* by miR-22-3p. For xenograft models, I prepared the cells used for tail-vein injection in the paper. I actively contributed to shaping ideas for the paper. I wrote the manuscript draft and prepared figures showed in the paper.

1. Introduction

1.1 Colorectal cancer (CRC)

CRC is one of the most frequently diagnosed cancers around the world. It accounts for around 8% of all diagnosed cancers and 9% of cancer-associated deaths in the United States as an example (**Figure 1.1**) [1]. Incidence and mortality of CRC are 25% higher in men than in women [2]. Today, more than 5.25 million people are living with CRC, and the number is only exceeded by breast cancer with 7.79 million cases [3]. Studies predicted that the annual incidence of CRC will increase from 1.9 million (2020) to 2.5 million (2035) [2, 4].

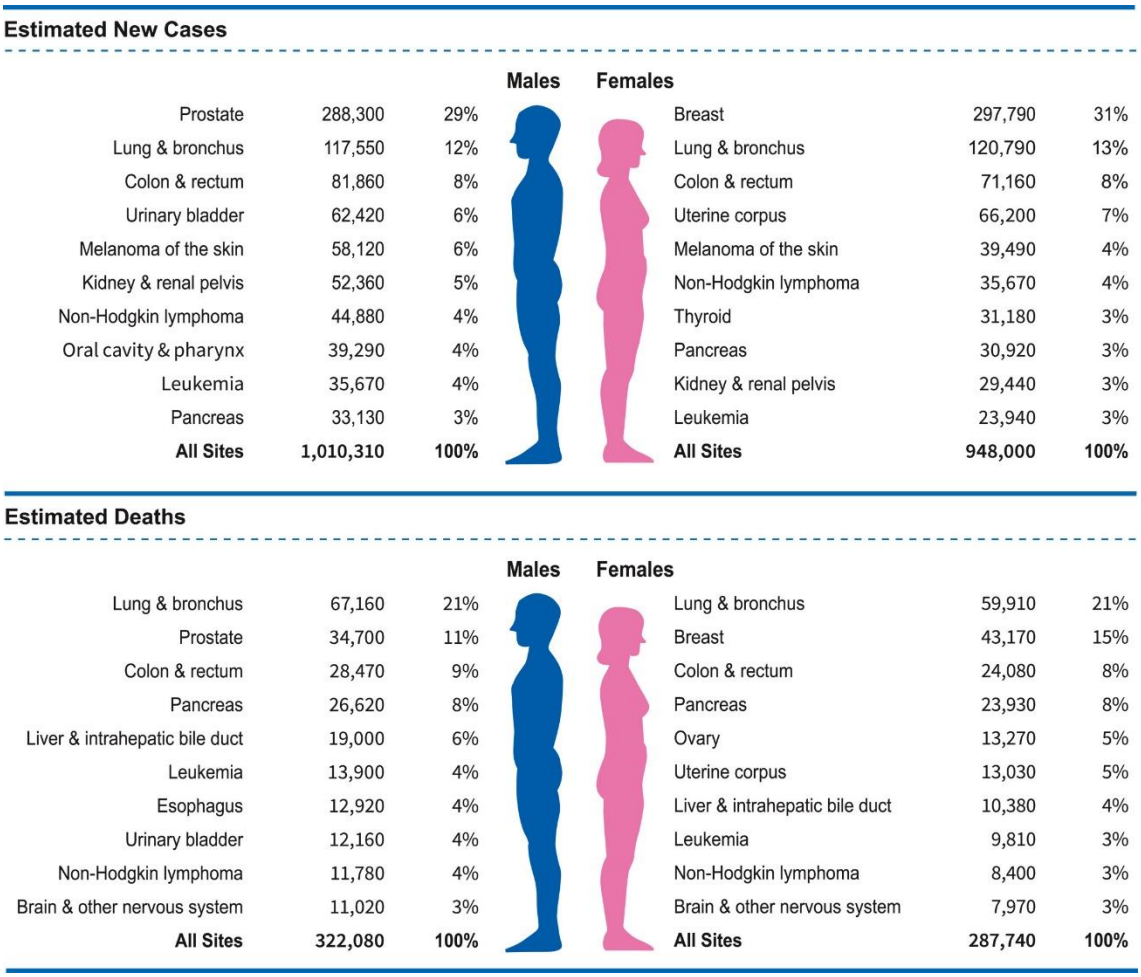

Figure 1.1 Top ten cancer types for the estimated new cancer cases and deaths by sex in the United States (2023). Colon and rectum cancer ranks third for estimated new cases and deaths among different tumor types in both males and females in the United States. Figure source:[1].

The incidence of CRC is positively correlated with the Human Development Index (HDI). The most developed countries have the highest rate of CRC incidence and mortality (**Figure 1.2**) but with a steady descending trend mainly due to nation-wide screening and widely used colonoscopy [3]. The incidence of CRC is increasing rapidly in low HDI countries owing to the increased exposure to CRC risk factors.

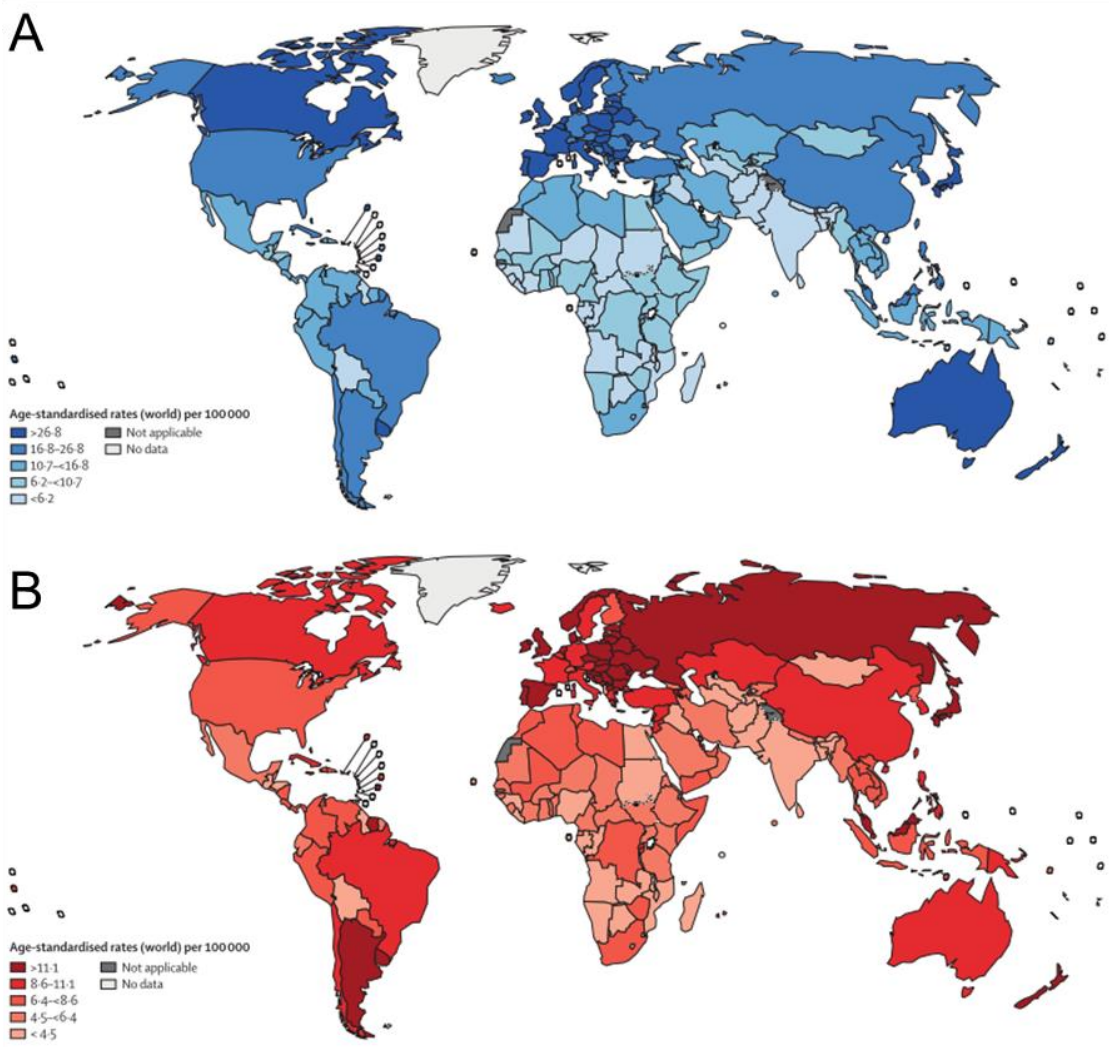

Figure 1.2 (A) Age-standardized cancer incidence in the world. **(B)** Age-standardized cancer mortality rates worldwide. The data was obtained from the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO). Figure source: [3].

A series of genetic and epigenetic changes facilitate the proliferation of intestinal epithelial cells, which results in CRC development [5]. CRC begins from ab-

errant crypts. It approximately takes over 10 years to develop CRC from the neoplastic precursor lesion (a polyp). 70-90% CRCs are caused by *APC* mutation, *RAS* activation, TGF-β pathway inactivation and loss of *p53* function [6]. Approximately 15% of CRCs display microsatellite instability (MSI) due to either epigenetic silencing of *MLH1* or germline mutations of the mismatch repair genes, such as *MLH1*, *MSH2*, *MSH6* and *PMS2* [7]. CRCs with MSI display a high CpG island methylation phenotype. Most MSI CRCs are clustered in the consensus molecular subtype 1 (CMS1) group, which is characterized by hypermethylation, hypermutation and strong infiltration of the tumor microenvironment with CD8+ cytotoxic T lymphocytes, CD4+ T helper 1 cells and natural killer cells [8]. The majority of CRCs exhibit genetic instability and are termed chromosomal instable (CIN) CRCs, which are manifested as the loss or gain of chromosome arms, chromosomal translocations, or gene amplifications [9]. The CIN CRCs can be categorized into three subgroups on the basis of gene expression signals: CMS2 (canonical subtype, 37% of early-stage CRC), CMS3 (metabolic subtype, 13% of early-stage CRC), and CMS4 (mesenchymal subtype, 23% of early-stage CRC) [8] (**Figure 1.3**).

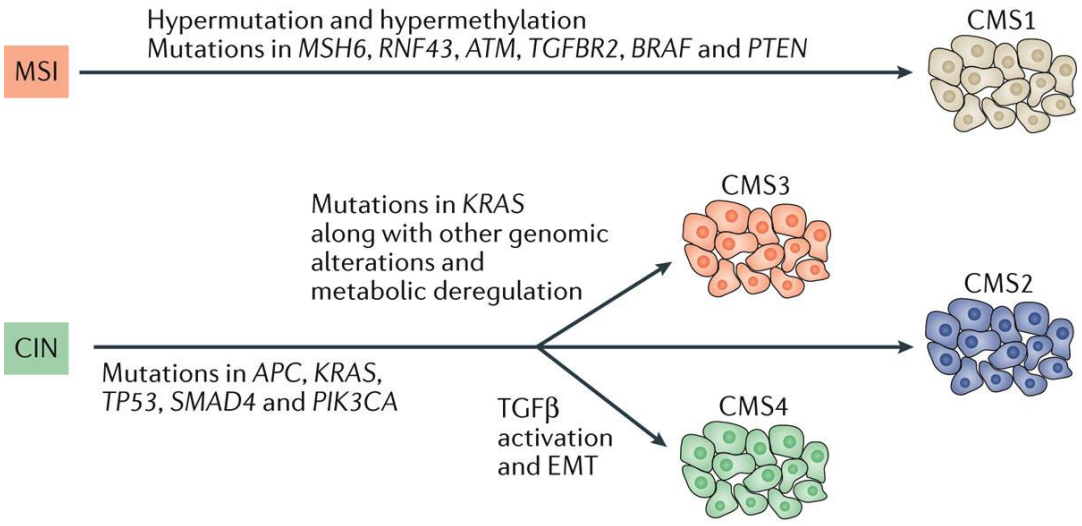

Figure 1.3 Colorectal carcinogenesis and transcriptomic subtypes. CRCs with microsatellite instability (MSI) mainly fall into consensus molecular subtype 1 (CMS1) associated with hypermutation and hypermethylation. CRCs with CIN are mostly categorized into CMS 2-4. A shift from the canonical CMS2 to CMS3 is thought to take place early, along with a unique combination of *KRAS* mutations and copy number events causing metabolic deregulation [8]. In CMS4 TGF-β activation by the stromal-enriched, inflamed microenvironment functions as a major driver of epithelial–mesenchymal transition (EMT) [8]. Figure source: [10].

1.2 c-MYC-AP4 transcription program and CRC

The *AP4* gene is a direct target of c-MYC [11] and AP4 mediates multiple functions of c-MYC (**Figure 1.4**).

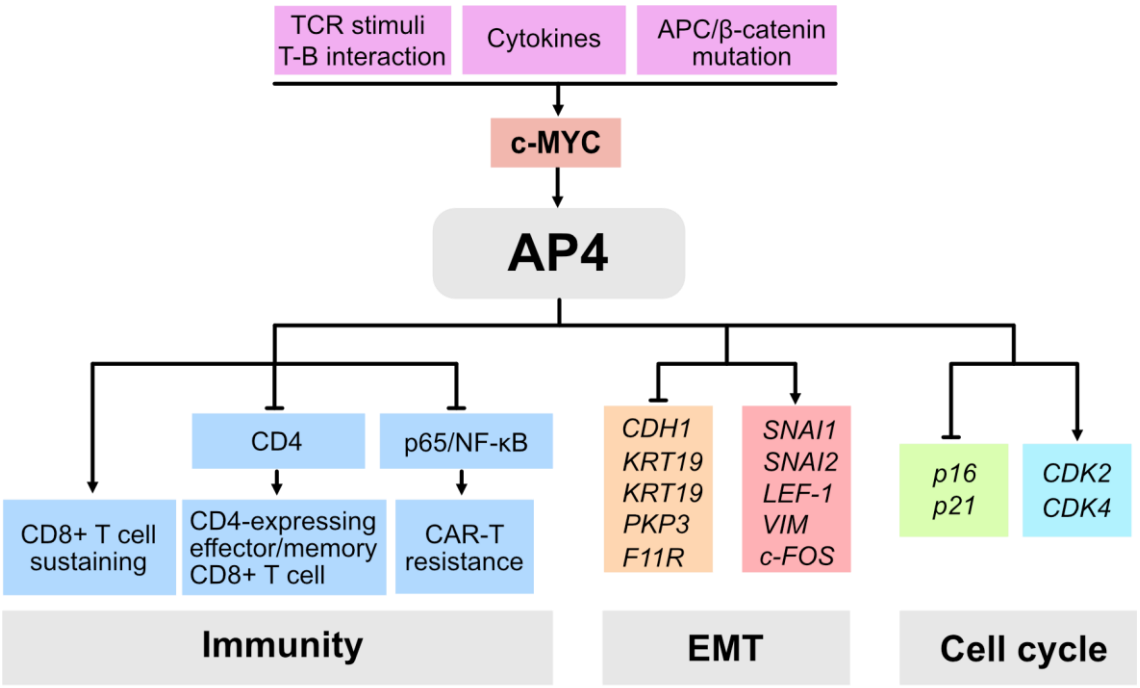

Figure 1.4 AP4 mediates effects of c-MYC and responses to different stimuli. AP4 mediates several processes downstream of c-MYC. AP4 sustains activation and population expansion of CD8+ T cells after down-regulation of c-MYC expression. AP4 mediates the effect of c-MYC on EMT and the cell cycle via directly targeting EMT-related effectors, p16, p21 and CDKs.

1.2.1 c-MYC

The *c-MYC* oncogene is deregulated in the majority of tumor types. The MYC family includes c-MYC, L-MYC and N-MYC proteins, which are basic helix-loop-helix leucine zipper (bHLH-LZ) DNA binding proteins [12]. Previous studies showed that elevated expression of c-MYC drives tumorigenesis. Aberrant c-MYC expression contributes to the initiation and maintenance of different types of tumors.

c-MYC structure

c-MYC protein consists of 439 amino acids [13]. The c-MYC protein contains a C-terminal DNA binding domain and an N-terminal transactivation domain

(TAD). c-MYC and its paralogues share several evolutionary-conserved segments, named MYC homology box (MB) 1 and 2. MB1 and 2 contributes to the function of c-MYC by facilitating the interactions of c-MYC with essential cofactors [14]. MB1 and 2 are located in the TAD and are heavily regulated by post-translational modifications. The C-terminus of c-MYC includes a bHLH-LZ domain responsible for DNA binding and heterodimerization between c-MYC and MAX [13]. The c-MYC/MAX heterodimer regulates the transcription and expression of genes via binding to E-box motifs at promoters of target genes [15]. The c-MYC-mediated transcriptional activation mainly relies on an intrinsically disordered domain in the N-terminal TAD comprising the MB0, MBI and MBII subdomains [16, 17]. The stability of c-MYC is determined by the phospho-degron consisting of the Ser62 and Thr58 residues in MBI. RAS/MEK/ERK/CDK2 initially phosphorylate the Ser62 residue and Thr58 is subsequently phosphorylated by GSK3β [18]. MBII provides a platform for other c-MYC-interactors, such as p400, GCN5, TIP48/49 and BAF53 [19].

Mechanisms of c-MYC-induced transcription

c-MYC induces transcription by enhancing histone acetylation at promoters [19, 20], as c-MYC binds to several histone acetyltransferases cofactors, such as TRRAP, GCN5, TIP60 and p300/CBP [21-23]. Chromatin is subsequently opened for the docking of acetyl-histone-binding proteins and transcription is activated. c-MYC can stimulate transcriptional elongation via inducing the release of paused RNA pol II from the promoter of genes [24]. Transactivation by c-MYC is dependent on phosphorylation of Ser62 and Thr58. Mutations of the residues Ser62 and Thr58 impairs oncogenicity of c-MYC [25].

Oncogenic function of c-MYC

c-MYC activation promotes cell proliferation, unscheduled DNA replication, protein synthesis, cellular senescence and imbalanced metabolism [26-28]. Therefore, c-MYC activation is considered as a hallmark of cancers.

Deregulated c-MYC induces unscheduled DNA replication and thereby triggers chromosomal instability. Many downstream genes are activated following the elevated c-MYC expression, which promote DNA synthesis and cell cycle

progression [29]. Previous studies have shown that c-MYC induces G_1/S transition and DNA replication by activating CDKs and by directly interacting with replication associated proteins [20, 30]. In addition, c-MYC activation results in changes at the chromosomal level, such as translocations, deletions, aneuploidy and fusions of centromere and telomere [31]. Increased double stranded DNA break (DSB) is a manifestation of genomic instability induced by c-MYC and is detectable via the increased formation of γH2AX foci [32, 33]. Accumulation of reactive oxygen species (ROS) also contributes to the DSB generation induced by c-MYC [34].

The genome of cancer cells is more vulnerable to DNA-damaging agent due to genomic instability. c-MYC activates the transcription of *NBS1*, *RAD50*, *RAD51* and *BRCA2,* which are components of the DNA damage response (DDR) signaling cascade activated by ATM, thereby c-MYC is presumably involved in the regulation of ATM-associated pathways in DDR [35-37].

1.2.2 AP4

AP4 structure

AP4 belongs to class A of bHLH-LZ proteins and recognizes the symmetrical DNA sequence CAGCTG [38]. AP4 contains two protein dimerization motifs, LZ1 and LZ2, which are leucine zipper elements and only allow the formation of AP4 homodimers [39].

AP4 function

AP4 was initially found to activate the transcription of the SV40 promoter [40]. However, several studies indicate that AP4 is frequently involved in the transcriptional repression of both viral and cellular genes [39, 41, 42]. *AP4* was identified as a direct transcriptional target of c-MYC by the Hermeking lab [11]. AP4 activates or represses genes by recognizing E-box motifs (CAGCTG) at their promoters and thereby regulates metabolism, apoptosis, proliferation, epithelial-mesenchymal transition (EMT) and metastasis [43].

Ap4 maintains the c-Myc-induced transcriptional program in murine T cells after exposure to IL-2, which sustains the acute activation of antigen-specific $CD8^+$T cells responding to pathogen [44]. Depletion of *AP4* in B cells reduces

germinal center sizes and represses somatic hypermutation, which results in an impaired control of chronic viral infection [45]. Loss of *AP4* represses the expression of genes inducing B cell differentiation. Therefore, *AP4* deletion blocks the differentiation during early B cell development and contributes to lymphoma development [46]. *AP4* deficiency results in the resistance to CAR T cell killing in pancreatic ductal adenocarcinoma [47]. Modular pooled knock in (ModPoKI) of *AP4* enhances the fitness of chronically stimulated CAR T cells and thereby contributes to the anti-cancer function of CAR T cells *in vitro* and *in vivo* [48].

Our previous results showed that *Ap4* is critical for adenoma initiation and growth by controlling the homeostasis of intestinal stem cells in the Apc^{min} mouse model of intestinal cancer [49]. Several types of cancers, such as hepatocellular carcinoma, breast cancer, prostate cancer and colorectal cancer, are promoted by AP4 via directly inducing downstream genes and activating different oncogenic signaling pathways, including PI3K/AKT and Wnt/β-catenin pathways [49-51]. Our previous studies indicated that AP4 is a central mediator and coordinator of cell cycle progression in response to mitogenic signals and c-MYC activation [52]. *AP4* depletion promotes premature senescence and resistance towards immortalization, as it leads to derepression of *p16* and *p21* [53]. *AP4*-deficiency increases both spontaneous and c-MYC-induced DNA damage and senescence in MCF-7 cells. Loss of *AP4* resulted in increased senescence, spontaneous and c-MYC-induced DNA damage due to the enhanced repression of DREAM and E2F target genes after c-MYC activation, which could be counteracted by depletion of *p21* or the DREAM component *LIN37* [51]. Therefore, AP4 functions as an oncogenic antagonist of cellular senescence. In addition, AP4 directly regulates EMT effectors, such as *SNAI1*, *CDH1, OCLN*, *VIM* and *FN1* [54]. Since elevated AP4 is associated with distant metastasis, lymph node metastasis and tumor grade in CRC [54], AP4 may therefore serve as a prognostic marker for predicting the risk of developing distant metastases of CRC.

1.3 DNA damage response and MDC1

c-MYC induces the G1/S transition and DNA replication stress by directly regulating the expression of cyclins, cyclin-dependent kinases and E2Fs [55]. c-MYC localizes to DNA replication origins and promotes the assemble of pre-replication complex via interacting with MCM proteins [56]. Overexpression of c-MYC is associated with the firing of oncogene-induced origins, which are potential sources of genomic instability [57]. DNA damage and chromosomal instability are increased following ectopic c-MYC [52, 58]. In addition, depletion of *Ap4* enhanced the c-Myc-induced DNA damage in MEFs [52]. Therefore, a goal of this thesis was to study the relevance of the c-MYC/AP4 axis for DNA damage and repair in CRC.

1.3.1 DNA damage response

Alterations of DNA damage response (DDR) pathway are involved in the progression of different cancer types [59]. Defects in the DDR pathway lead to genomic instability during the DNA replication. The frequency of DDR gene mutation is 10%-20% in somatic cells and 5% in germline cells [60]. DNA repair capacity confers the resistance to drugs inducing DNA damage in chemotherapy.

DNA damage

Ionizing radiation (IR) and environmental agents generate single-strand breaks (SSBs) and double-strands breaks (DSBs) in the DNA double helix backbone [61]. DNA damage response (DDR) signaling pathways are subsequently activated, which is mediated by members of phosphatidylinositol 3-kinase-related kinase (PI3KK) family, ATM (ataxia telangiectasia mutated) pathway, ATR (ataxia telangiectasia and Rad3-related protein) pathway, and DNA-PK (DNA-dependent kinase) pathway. The binding between MRE11-RAD50-NBS1 (MRN) complex and double-stranded DNA ends is an early event of DDR signaling following DSBs [62].

The DNA damage response to DSB

Following the activation of ATM at the DSB, the histone protein H2AX is phosphorylated on Ser139 and referred to γH2AX hereafter [63]. γH2AX provides a platform for the recruitment of DDR factors, and thereby amplifies DDR signaling.

γH2AX specifically interacts with the C-terminal BRCT domain of MDC1. The phosphorylated MDC1 is recognized by NBS1 FHA-BRCT region. Additional MRN and ATM are recruited to the region flanking DSBs subsequently [64]. Moreover, MDC1 recruits RNF8, an E3 ubiquitin ligase, to initiate the ubiquitination of H2AX, which provides the binding position to BRCA1 at the DSB site [65]. ATM helps to stabilize MDC1 on the chromatin. The MDC1-MRN-ATM recruitment at DSB amplifies the DDR signaling [66-68]. A model of the DNA damage response to DSB is provided in **Figure 1.5**.

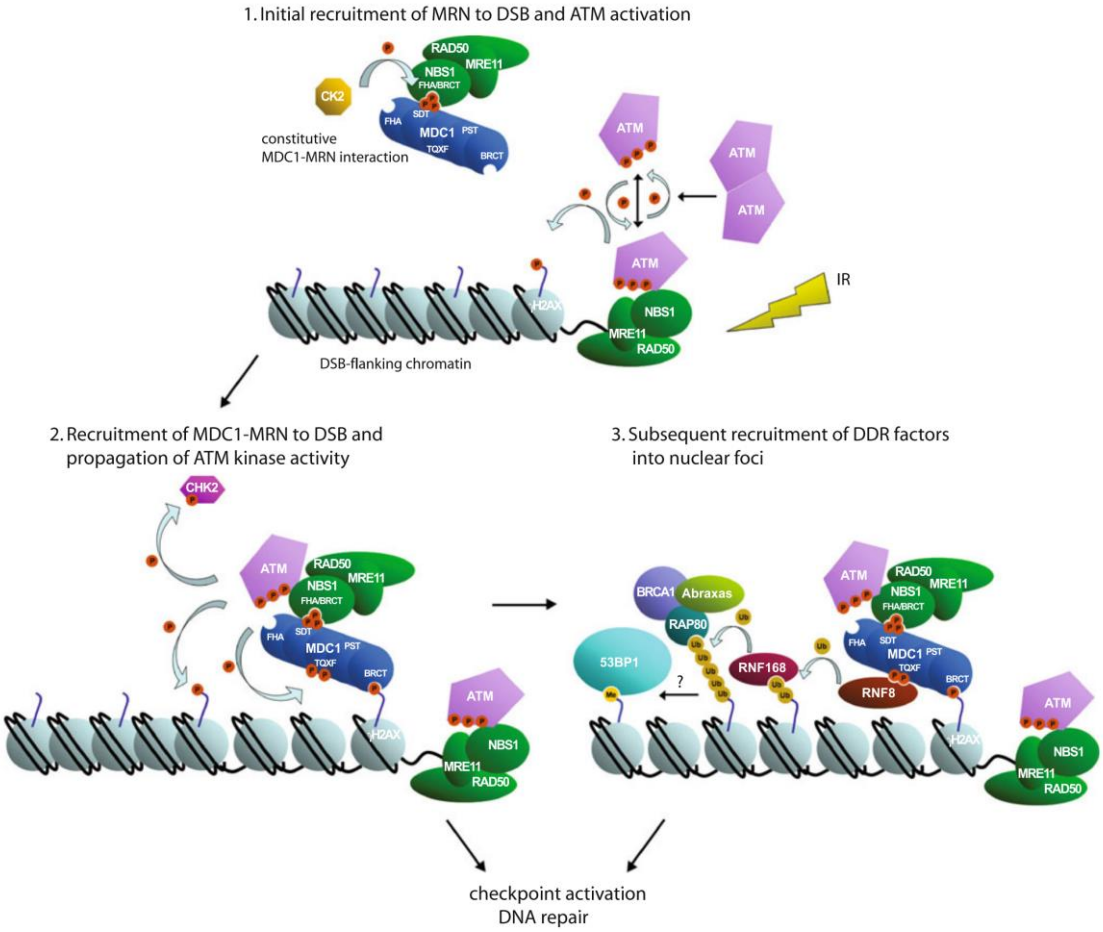

Figure 1.5 Model of the DNA damage response to DSB. MRN directly binds to free DNA ends when DSB occurs. Subsequently, ATM kinases are recruited and activated. H2AX flanking the DSB site is phosphorylated by ATM. MDC1 recognizes the γH2AX through direct interaction [69]. MDC1 recruits the ubiquitin ligase RNF8 and additional DDR factors, which triggers a signaling cascade and results in checkpoint activation and DNA repair [69]. Figure source: [69].

Homologous recombination (HR) is one of the major ways to repair DSBs. HR carries out the repair of DSBs mainly in the S and G2 phases of the cell cycle. The DNA sequence surrounding the DSB is resected and the homologous sister chromatid serves as a template to synthesize new DNA to replace the damaged DNA at DSB sites. BRCA1, BRCA2 and RAD51 are important components of HR [70].

DNA damage response and cellular senescence

Senescence represents a tumor suppressive mechanism. ARF/p53 [71] and INK4a/RB [72] are the two main pathways that mediate senescence. The *p53*- and *p16INK4a*-mediated cell cycle arrest prevents oncogene-induced proliferation of tumor cells [73].

Cellular senescence is one of the outcomes of DDR activation. The occurrence of senescence is accompanied by dramatic chromatin alterations. Cellular senescence is frequently associated with the formation of senescence associated heterochromatin foci (SAHF). The formation and function of SAHF are dependent on INK4a/RB pathway [74]. ATR is required for the induction of heterochromatic markers and SAHF in oncogene-induced senescence [75]. Chemotherapeutic drugs cause DNA damage and trigger cellular senescence in tumor cells. Therefore, senescence seems to be an integral part of the cellular response to chemotherapy.

DNA damage response and chemotherapy resistance

DDR maintains genome stability and integrity, conferring chemotherapy resistance in cancer treatment. Therefore, targeting components of DDR is a strategy to sensitize advanced CRCs to chemotherapy.

Disturbance of cell cycle checkpoints sensitizes tumor cells to DNA-damaging agents. Cell cycle checkpoints can be repressed by several approaches including inhibition of ATM, ATR or MAPK signaling pathways. The effect of inhibition of ATM on chemotherapy sensitivity is largely dependent on *p53* status. *p53*-proficienct cells fail to overcome doxorubicin resistance through ATM inhibition [76]. *p53* depletion also sensitizes *ATM*-deficient CRC cells to the treatment of PARP inhibitor (PARPi) [77].

Direct inhibition of CHK1, CHK2 or WEE1 overrides cell cycle checkpoints [78]. Since CHK1 is required to maintain genomic integrity, current studies are particularly focusing on repressing CHK1 to achieve sensitization towards chemotherapy. Double inhibition of CHK1 and CHK2 by a chemical inhibitor overcomes the radio-resistance of glioma stem cells [79]. An ongoing clinical trial (NCT02906059) is exploiting the WEE1 inhibitor AZD1775 combined with irinotecan as a second line of treatment for *RAS* or *BRAF* mutant CRC.

Targeting DNA repair is an alternative way to sensitize tumors towards chemotherapy. PARPi and DNA alkylating agents display a synergistic therapeutic effect in a *BRCA*-deficient cancer model [80, 81]. Repression of base excision repair (BER) factors, such as Pol β, MGMT, MPG and APE1, sensitizes tumors cells to alkylating chemotherapeutics [82]. Recently, histone deacetylases (HDACs) have been shown to promote DNA repair [83-85]. Therefore, HDACs inhibitors may serve as chemo-sensitizers.

1.3.2 MDC1

Mediator of DNA damage checkpoint 1 (MDC1) is a scaffold protein and accumulates at the DSB sites at the early stage of DDR. MDC1 is composed of several distinct domains and regions which are capable of recruiting or recognizing specific protein partners in the DDR [69] (**Figure 1.6**).

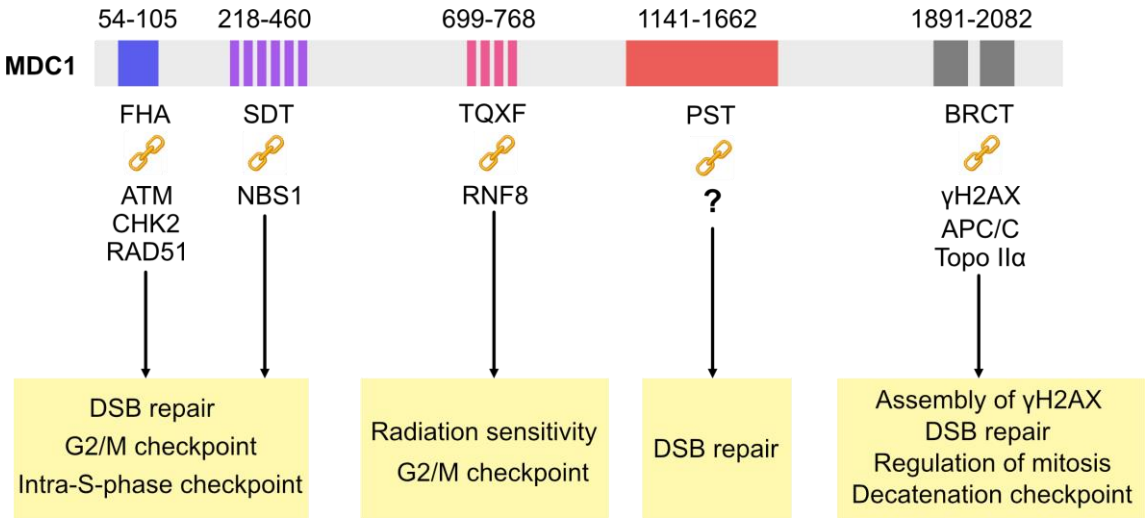

Figure 1.6 Schematic model of the MDC1 protein. MDC1 has several distinct domains which are responsible for the interaction with other phosphorylated proteins. MDC1 domains are also required for the phosphorylation of MDC1. FHA and BRCT domains serve as docking sites for other proteins with phospho-specific interaction modules. The figure is modified from [69].

The C-terminal tandem BRCT domain of MDC1 is required for the accumulation and amplification of phosphorylated γH2AX [86, 87]. As a factor responding at the early stage of DDR, MDC1 activates ATM by interacting with γH2AX within the proximity of the damaged chromatin and γH2AX subsequently spreads to distal regions [64, 88].

The TQXF motifs of MDC1 are bona fide targets of ATM after ionizing radiation (IR) [65, 89]. MDC1 is phosphorylated by ATM and subsequently interacts with RNF8 via a TQXF motif. The MDC1-RNF8 interaction is important for the ubiquitination of γH2AX, which is a prerequisite for recruiting BRCA1 and 53BP1 to MDC1 [65].

The proline-serine-threonine (PST)-rich repeat region of MDC1 is conserved in most vertebrate species. Unlike the BRCT domain, the PST domain is dispensable for the function of MDC1 in recruiting 53BP1, BRCA1 and the MRN complex. Previous studies have shown that PST is responsible for binding to DNA-PK flanking DSBs in chromatin [90]. Deletion of the PST domain results in non-homologous DNA end joining (NHEJ) defects [90]. In addition, the PST region is critical for HR and mitotic progression. Impaired HR and abnormal mitotic progression can be observed in *MDC1*-deficient cells [91, 92].

The SDT region of MDC1 consists of Ser and Thr residues upstream of the TQXF motifs. The accumulation and retention of the MRN complex strictly rely on the MDC1 SDT region [86, 93, 94]. Deletion of SDT region completely blocks the formation of the irradiation-induced MRN-foci [67, 68].

The N-terminal FHA domain is critical for MDC1 function in DDR. The MDC1 FHA domain is required for the interaction with the Thr68-phosphorylated CHK2 [95]. Previous studies suggest that MDC1 interacts with ATM kinase via the N-terminal FHA domain [88]. The specific interaction between the MDC1 FHA domain and RAD51 implies that the MDC1 FHA domain is involved in DSB repair through sister chromatid recombination [91, 96].

Since MDC1 is required for DDR, the loss of *MDC1* increases mutagenesis and malignant transformation. Several studies show that down-regulation of

MDC1 improves the sensitivity to DNA-damaging drugs in radio- and chemo-therapies [97-99]. Therefore, inhibition of *MDC1* may serve as a strategy to overcome the resistance of chemotherapeutic drugs by impairing DDR.

1.4 miR-22-3p

miR-22-3p is encoded by *MIR22HG,* which is located in chromosome 17p13 [100]. Numerous studies have characterized miR-22-3p as a tumor suppressor in different types of cancers. *MIR22HG* is directly repressed by c-MYC and SP1 in breast cancer [101]. miR-22-3p represses *CD147* via 3'-UTR targeting. Interestingly, c-MYC and SP1 promote the expression of CD147 by binding to the *CD147* promoter. Therefore, CD147 is up-regulated by a miR-22-3p/Sp1/c-MYC loop. In colorectal cancer, miR-22-3p inhibits autophagy and promotes apoptosis by targeting *BTG1,* which confers to the sensitivity to 5-FU treatment [102]. Furthermore, through targeting *CDK6*, *SIRT1* and *SP1*, miR-22-3p restores the senescence program [103]. In addition, *MDC1* is a direct target of miR-22-3p. The miR-22-3p-mediated inhibition of MDC1 leads to impaired homologous recombination repair and genomic instability, since MDC1 is a critical component of the DDR [104]. Collectively, miR-22-3p functions as a tumor suppressor in different types of cancers including CRC.

1.5 JNK signaling pathway

Mitogen-activated protein kinase (MAPK) pathway mediates the cellular responses to extracellular stimuli [105]. As one of the major MAPK pathways, the Jun N-terminal kinase (JNK) pathway is frequently deregulated in cancer.

1.5.1 JNK1 signaling cascade

JNK kinases consist of JNK1, JNK2 and JNK3. They are encoded by the *MAPK8*, *MAPK9* and *MAPK10* genes, respectively. JNK1 and JNK2 are expressed in most tissues whereas JNK3 is mainly expressed in brain, heart and testes [106]. JNK kinases are stress-activated protein kinases, which are phosphorylated following extracellular stimuli, such as toxins, stress, cytokines and drugs [107]. JNK1 and JNK2 are directly phosphorylated by two MAP2K kinases, MKK4 and MKK7, on threonine 183 (Thr183) and tyrosine (Tyr185) residues [108]. JNK1 controls several different cellular responses through downstream effectors, such as transcription factor activator protein-1 (AP-1) [109], c-MYC [110], p53 [111] and ATF-2 [112].

1.5.2 JNK1 pathway and EMT

Epithelial-to-mesenchymal transition (EMT) is an initial step of metastasis [113]. Down-regulation of adherent junction proteins and up-regulation of mesenchymal proteins are hallmarks of EMT [113]. JNKs have been shown to promote EMT and contribute stem cell maintenance. *Jnk1*- and *Jnk2*-deficiencies induce mesenchymal-to-epithelial transition (MET) in *p53*-/- MEFs [114]. JNK1 induces cellular migration in the brain via activating microtubule-associated proteins 1B/2 and stathmin-2 [115]. However, the effect of JNK1 on *in-vitro* migration and invasion is contradictory. Deletion of *JNK1/2* promotes migration and invasion of non-cancerous mammary epithelial cells [116].

1.6 FOSL1/FRA1

FOSL1/FRA1 has been characterized as a tumor promoting factor in different cancers. Previous studies have shown that FOSL1 is involved in the regulatory network of EMT-inducing transcription factors (EMT-TFs) [117]. In addition, FOSL1 is a member of AP-1 dimeric complex, which represents a critical factor in tumorigenesis [118].

1.6.1 Regulations of FOSL1

The transcription of *FOSL1* is controlled by oncoproteins via the regulatory elements localized in the first intron and upstream regions of *FOSL1* gene [117]. In response to cytokines and growth factors, STAT3 binds to the *FOSL1* promoter and increases the transcription of *FOSL1* [119]. As a component of AP-1, the transcription of *FOSL1* is auto-regulated by FOSL1 via the AP-1 binding sites at *FOSL1* promoter [120]. The oncoprotein c-MYC directly binds to the intronic region of *FOSL1* [121]. p53 binds within the first intron and enhancer region of the *FOSL1* gene [122]. The transcription of *FOSL1* is induced by p53 following genotoxic stress. In addition, the *FOSL1* 3'-UTR is targeted by miR-34a and miR-34c [123], which are encoded by p53-inducible genes [124]. Therefore, p53 antagonistically regulates *FOSL1*.

1.6.2 FOSL1 and EMT

FOSL1 drives metastasis by acting as a transcriptional regulator of EMT. Fosl1 promotes EMT by directly inducing *Zeb1* and *Zeb2* in mouse mammary epithelial cells [125]. FOSL1 maintains a mesenchymal state via transactivating *TGF-β* and *ZEB1/2* [125]. In CRC FOSL1 is required for SIRT1-induced EMT and metastasis [126]. Furthermore, FOSL1 induces EMT by targeting *VIM*, *SNAI2*, *AXL* and *SMAD3* in CRC [127]. miRNAs are also involved in the FOSL1-mediated EMT regulation. By transcriptionally inducing *miR-221/222*, FOSL1 down-regulates TRPS1, a zinc finger transcriptional repressor of ZEB2 [128]. In addition, FOSL1 reinforces the activity of AP-1 via inducing miR-134, and thereby activates JNK and ERK signaling [129].

1.7 Aims of the study

(1) Characterization of mRNAs/lncRNAs/miRNAs and downstream functions regulated by c-MYC in an AP4-dependent manner in human CRC cell lines.

(2) Functional characterization of the c-MYC-AP4-*MIR22HG*/miR-22-3p regulatory axis.

(3) Identification and characterization of relevant *MIR22HG*/miR-22-3p targets.

(4) Relevance of the AP4/miR-22-3p/MDC1 axis for the senescence, DNA damage and chemotherapy resistance in human CRC cell lines.

(5) Relevance of the miR-22-3p/FOSL1 axis for the regulation of EMT and metastasis by AP4.

(6) Functional characterization of the c-MYC-AP4-JNK1 regulatory axis in EMT and metastasis.

2. Summary (English)

AP4 is a basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factor and binds to CAGCTG motifs. As a direct target of c-MYC, AP4 is a central mediator of c-MYC-induced EMT and proliferation. Elevated AP4 expression is associated with tumor progression and poor patient prognosis of several types of cancers including CRC. Our previous research showed that inactivation of AP4 leads to premature cellular senescence, DNA damage, and impaired cell growth in mouse embryonic fibroblasts.

Here we investigated the role of AP4 in the regulation of DNA the damage response and cellular senescence, which affect the response to 5-fluorouracil (5-FU). Our findings revealed that AP4 prevents spontaneous and c-MYC-induced DNA damage in CRC cells. We identified that MDC1, a critical regulator of DDR, is a direct target of AP4. Therefore, AP4 represses DNA damage by promoting the expression of MDC1 and enhancing homologous recombination repair in CRC cells. Additionally, we identified that AP4 directly inhibits the expression of miR-22-3p, which represents a senescence-associated microRNA. Since *MDC1* is a known target of miR-22-3p, AP4, MDC1, and miR-22-3p form a coherent feed-forward loop, in which AP4 induces MDC1 and suppresses miR-22-3p. The regulation of DNA repair and cellular senescence by AP4, MDC1 and miR-22-3p is conserved in mice. c-MYC controls the levels of MDC1 and miR-22-3p in an AP4-dependent manner. By regulating MDC1, AP4 enhances the homologous recombination repair of DNA damage caused by 5-FU, which leads to 5-FU resistance in CRC cells.

Furthermore, we observed an association between poor response to chemotherapy and elevated *c-MYC*, *AP4* and *MDC1* expression, as well as low *MIR22HG* expression in primary CRCs in a cohort of patients. CRC patients displaying low levels of c-MYC, AP4, and MDC1, and high levels of miR-22-3p, are more likely to respond well to chemotherapy. Therefore, further studies are warranted to validate AP4 and MDC1 as predictive markers of chemotherapy response in CRC.

In addition, we characterized the transcriptional regulation of the JNK1 pathway and FOSL1 by AP4 in CRC cells. AP4 directly induces the expression of

MAP3K13, which subsequently activates the JNK1 pathway. Since *AP4*-deficiency largely abrogates the induction of MAP3K13 mediated by c-MYC, the JNK1 pathway is regulated by c-MYC in an AP4-dependent manner. AP4-induced lung metastases formation was suppressed by a JNK1 inhibitor in xenograft models. Therefore, JNK1 activation is presumably required for the AP4-promoted EMT and metastases in CRC.

Furthermore, the *FOSL1/FRA1* gene was characterized as an AP4 target. Silencing *FOSL1* suppresses the AP4-induced migration and invasion in CRC cells. In addition, silencing of *FOSL1* suppresses the AP4-induced lung metastases formation in xenograft models. We identified that *FOSL1* is a target of miR-22-3p, a miRNA directly repressed by AP4. *MIR22*-deficiency enhances migration and invasion of SW480 cells. AP4, miR-22-3p and their target *FOSL1* form a coherent feed-forward loop. In cells lacking miR-22-3p seed-matching sequence in the *FOSL1* 3'-UTR, ectopic miR-22-3p fails to repress FOSL1. CRC cells rendered *MIR22*-deficient display elevated basal FOSL1 levels and a defective inducibility of FOSL1 by AP4. Therefore, miR-22-3p is required to maintain FOSL1 expression in a range that allows induction by the c-MYC/AP4 pathway. Moreover, we showed that AP-1, a dimeric transcription factor containing JUN and FOS, is activated following ectopic AP4 expression. The intrinsic activity of AP-1 is increased in *AP4*-proficient CRC cells. *MIR22*-deficiency increases the activity of AP-1 in CRC cells.

In conclusion, AP4, miR-22-3p, and MDC1 work together in a coordinated manner to enhance DNA repair, prevent DNA damage and senescence, and contribute to 5-FU resistance in CRC. Since most CRC cases show increased levels of c-MYC and AP4, targeting the c-MYC/AP4/miR-22-3p/MDC1 pathway could be a promising strategy to sensitize CRC tumors to chemotherapy drugs. In addition, AP4, miR-22-3p and FOSL1 form a coherent, regulatory feed-forward loop to promote EMT and metastases in CRC. In conjunction with the AP4-mediated activation of JNK1 these regulations promote AP1 activation, ultimately promoting EMT and metastases formation. In the future, these findings may be exploited for the prevention and/or treatment of metastatic CRC.

3. Zusammenfassung (Deutsch)

AP4 ist ein basischer Helix-Loop-Helix-Leucin-Zipper (bHLH-LZ)-Transkriptionsfaktor, der an das CAGCTG-Motiv bindet. Als direktes Ziel von c-MYC ist AP4 ein zentraler Vermittler für c-MYC-Funktionen, wie EMT und Proliferation. Eine erhöhte AP4-Expression deutet auf Progression und eine ungünstige Prognose für verschiedene Krebsarten, einschließlich CRC, hin. Unsere vorherige Forschung zeigte, dass die Inaktivierung von AP4 zu vorzeitigem zellulärem Altern, DNA-Schäden und beeinträchtigtem Zellwachstum in murinen embryonalen Fibroblasten führt.

Hier untersuchten wir die Rolle von AP4 in der Regulation der DNA-Schadensantwort und des zellulären Alterns, der Reaktion auf Chemotherapeutika, welche DNA-Schäden verursachen, wie z. B. 5-Fluorouracil (5-FU). Unsere Ergebnisse zeigten, dass AP4 spontane und c-MYC-induzierte DNA-Schäden in CRC-Zellen verhindert. Wir identifizierten MDC1, einen kritischen Regulator der DDR, als direktes Ziel von AP4. Daher erreicht AP4 diese Wirkung, indem es die Expression von MDC1 fördert und die homologe Rekombinationsreparatur in CRC-Zellen verstärkt, einen Schlüsselregulator der DNA-Schadensantwort. Zusätzlich stellten wir fest, dass AP4 die Expression von miR-22-3p direkt hemmt, welches eine Seneszenz-assoziierte Mikro-RNA repräsentiert. Da MDC1 ein bekanntes Ziel von miR-22-3p ist, bilden AP4, MDC1 und miR-22-3p eine kohärente Vorwärtsschleife, bei der AP4 MDC1 induziert und miR-22-3p unterdrückt. Die Regulation der DNA-Reparatur und des zellulären Alterns durch AP4, MDC1 und miR-22-3p ist bei Mäusen konserviert. Da c-MYC AP4 direkt aktiviert, kontrolliert c-MYC die Expression von MDC1 und miR-22-3p indirekt durch AP4. Durch die Regulation von MDC1 verstärkt AP4 die homologe Rekombinationsreparatur von durch 5-FU verursachten DNA-Schäden, was zu 5-FU-Resistenz in CRC-Zellen führt.

Darüber hinaus beobachteten wir eine Assoziation zwischen einer schlechten Reaktion auf die Chemotherapie und einer erhöhten Expression von c-MYC, AP4 und MDC1 sowie einer niedrigen Expression von MIR22HG in primären CRCs in einer Patientenkohorte. Patienten mit CRCs, die niedrige Spiegel von c-MYC,

AP4 und MDC1 sowie hohe Spiegel von miR-22-3p aufweisen, neigen zu einer guten Reaktion auf die Chemotherapie. Daher sind weitere Studien erforderlich, um AP4 und MDC1 als prädiktive Marker für die Chemotherapie-Antwort bei CRC zu validieren. Diese Ergebnisse legen nahe, dass die Expressionsniveaus dieser Moleküle als Indikatoren für die Chemotherapie-Antwort bei CRC-Patienten dienen können.

Zusätzlich charakterisierten wir die transkriptionelle Regulation des JNK1-Wegs und von FOSL1 durch AP4 in kolorektalen Krebszellen. AP4 induzierte direkt die Expression des *MAP3K13* Gens, das für eine Kinase kodiert, die den JNK1-Weg aktiviert. Da die Inaktivierung von AP4 die durch c-MYC vermittelte Induktion des MAP3K13-Gens weitgehend aufhob, wird der JNK1-Weg auf eine AP4-abhängige Weise durch c-MYC reguliert. Die durch AP4 induzierte Bildung von Lungenmetastasen wurde durch die Hemmung von JNK1 in Xenotransplantat-Modellen unterdrückt. Daher ist die Aktivierung von JNK1 wahrscheinlich für die durch AP4 geförderte EMT und Metastasierung in CRC erforderlich.

Des Weiteren wurde das FOSL1/FRA1-Gen als Ziel von AP4 charakterisiert. Die Inaktivierung von FOSL1 unterdrückte die durch AP4 induzierte Migration und Invasion in CRC-Zellen. Darüber hinaus unterdrückte das Ausschalten von FOSL1 die durch AP4 induzierte Bildung von Lungenmetastasen in Xenotransplantatmodellen. Wir fanden auch heraus, dass FOSL1 ein Ziel von miR-22-3p ist, einer von AP4 reprimierten miRNA. AP4, miR-22-3p und ihr Ziel FOSL1 bilden daher eine kohärente Vorwärts-Verstärkungsschleife. In Zellen, welche die miR-22-3p-SEED-Paarungssequenz im 3'-UTR von FOSL1 nicht aufweisen, konnte eine ektopische miR-22-3p Expression FOSL1 nicht unterdrücken. CRC-Zellen, die MIR22-defizient waren, zeigten erhöhte, basale FOSL1-Spiegel und eine defekte Induzierbarkeit von FOSL1 durch AP4. Daher ist miR-22-3p erforderlich, um den Expression von FOSL1 in einem Bereich aufrechtzuerhalten, der eine Induktion durch den c-MYC/AP4-Weg ermöglicht. Darüber hinaus zeigten wir, dass AP-1 nach ektopischer AP4-Expression aktiviert wird. Auch MIR22-Defizienz erhöhte die Aktivität von AP-1 in CRC-Zellen. Die Aktivierung von AP4 induzierte Migration und Invasion von CRC-Zellen, aber die Hemmung des JNK1-Wegs oder das Ausschalten von FOSL1 hoben diese Effekte von AP4 weitgehend auf.

Darüber hinaus förderte die Aktivierung von AP4 die Bildung von Lungenmetastasen durch nicht-metastatische DLD-1-Zellen, was durch einen JNK1-Inhibitor blockiert wurde.

Zusammenfassend arbeiten AP4, miR-22-3p und MDC1 koordiniert zusammen, um die DNA-Reparatur zu verstärken, DNA-Schäden zu verhindern, Seneszenz zu unterdrücken und zur 5-FU-Resistenz bei CRC beizutragen. Da die meisten CRC-Fälle erhöhte Spiegel von c-MYC und AP4 zeigen, könnte die gezielte Beeinflussung des c-MYC/AP4/miR-22-3p/MDC1-Weges eine vielversprechende Strategie sein, um CRCs für Chemotherapeutika empfindlicher zu machen. Darüber hinaus bilden AP4, miR-22-3p und FOSL1 eine kohärente Vorwärts-Verstärkungsschleife, um EMT und Metastasen in CRC zu fördern. In Verbindung mit der durch AP4 vermittelten Aktivierung von JNK1 fördern diese Regelationen die Aktivierung von AP-1, was letztendlich die EMT und die Bildung von Metastasen unterstützt. AP4 aktiviert JNK1 und induziert FOSL1 direkt. Darüber hinaus erhöht AP4 indirekt die Expression von FOSL1 durch die Repression von miR-22-3p. AP4, miR-22-3p und FOSL1 bilden eine kohärente, Vorwärts-Verstärkerschleife. Diese Regulationen konvergieren in der Aktivierung von AP-1, die letztendlich die EMT, Migration und Invasion fördert und so die Bildung von Metastasen verstärkt. In der Zukunft könnten diese Erkenntnisse für die Prävention und/oder Behandlung von metastasierendem CRC genutzt werden.

4. Paper I

Chou J, Kaller M, Jaeckel S, Rokavec M, Hermeking H. AP4 suppresses DNA damage, chromosomal instability and senescence via inducing MDC1/Mediator of DNA damage Checkpoint 1 and repressing MIR22HG/miR-22-3p. *Molecular Cancer*. 2022 May 27;21(1):120.

5. Paper II

Chou J, Kaller M, Rokavec M, Liu F, Hermeking H. AP4 promotes metastases of colorectal cancer by activating AP-1 via induction of the JNK1 pathway and a coherent miR-22-3p/FOSL1 feed-forward loop. *Cancer Communications.* 2024 https://doi.org/10.1002/cac2.12514.

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