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Oral examination…….

The role of the p38-FOXM1 pathway in the regulation of cancer cell invasion

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Summary

Head and neck cancer is the sixth most common cancer worldwide and associated with a poor clinical prognosis, due to development of recurrent tumors and metastasis. Tumor recurrence and low patient survival are strongly linked with the ability of tumor cells to invade and infiltrate the surrounding tissue. Stress-activated protein kinases (SAPK), particularly p38, are known to regulate a wide range of cellular phenotypes, including cell invasion via the activity of secreted proteases. The proliferation-associated Forkhead box protein M1 (FOXM1) transcription factor, a p38 downstream target, plays a role in the development and growth of many cancer types. However, only very little is known about the role of p38 and FOXM1 in invasive processes of head and neck cancer and the exact mechanism underlying this process. In this work we examined the downstream events of p38 signaling primarily focusing on the role of FOXM1 transcription factor in regulation of the urokinase-type plasminogen activator (uPA) gene and invasion of head and neck squamous cell carcinoma (HNSCC) cells. Using different HNSCC cell lines, we confirm that p38 regulates FOXM1 expression and provide evidence that p38 signaling driven *in vitro* invasion of HNSCC cells requires FOXM1 expression. Furthermore, siRNA-mediated FOXM1 knockdown is sufficient to inhibit the invasive behavior of HNSCC cells *in vitro*. By using reporter gene assays, bioinformatical analysis of the publically available ChIP-Seq data, chromatin immunoprecipitation assays, and transplantation-based mouse model of oral cancer, we identified the molecular mechanism of FOXM1-mediated invasion of HNSCC cells. FOXM1 controls the uPA-dependent invasion via activation of c-Fos and thus drives AP-1 activity on the uPA promoter, which enhances its expression and proteolytic activity. Further, an activated Ras signaling is necessary for a potent FOXM1-mediated uPA activity and tumor formation. The data are supported by a bioinformatical study, demonstrating concomitant up-regulation of FOXM1 and uPA in oral dysplasia and SCCs of head and neck, oesophagus, lung and cervix. In the mouse model of oral cancer we show that uPA expression is upregulated in recurrent tumors compared to primary tumors, giving further evidence for a crucial role of the p38-FOXM1-uPA axis in the development of recurrent tumors. Taken together, we conclude that the stress signalling cascade requires a FOXM1-dependent intermediate step preceding the activation of AP-1 transcription factor to enhance invasive behaviour of tumor cells. This novel mechanism promotes invasion of HNSCC and may provide a potential target for the adjuvant therapy of these highly invasive cancers.

Zusammenfassung

Kopf-Hals-Krebs gehört zu der sechst häufigsten Krebsart weltweit und ist assoziiert mit einer schlechten klinischen Prognose, für welche die Entstehung von Rezidivtumoren die Hauptursache sind. Die Entstehung von Tumorrezidiven und die geringe Überlebensrate von Patienten hängen stark mit der Fähigkeit von Tumoren zusammen umliegendes Gewebe zu infiltrieren. Stress-aktivierte Protein Kinasen (SAPK), vor allem p38, sind dafür bekannt, dass sie eine Reihe von zellulären Phänotypen regulieren, darunter auch Zellinvasion mit Hilfe von Proteasen. Der Proliferations-assoziierte Forkhead box protein M1 (FOXM1) Transkriptions-faktor, ein "Target" von p38, spielt eine große Rolle in der Entwicklung und dem Wachstum von vielen Krebsarten. Jedoch ist nur wenig bekannt über die Rolle von p38 und FOXM1 bei invasiven Prozessen von Kopf-Hals-Tumoren und dem genauen Mechanismus hinter diesem Prozess. In dieser Arbeit haben wir Vorgänge unterhalb des p38 Signalweges untersucht und uns dabei auf die Rolle von FOXM1 bei der Regulation des Urokinase-Typ Plasminogen Activator (uPA) Gens und der Invasion von Plattenepithelkarzinomen des Kopf-Hals-Bereichs (HNSCC) konzentriert. Unter der Verwendung von mehreren HNSCC Zelllinien konnten wir bestätigen, dass p38 die Expression von FOXM1 reguliert, dass FOXM1 für p38-gesteuerte *in vitro* Invasion von Kopf-Hals-Tumoren benötigt wird und das eine Inhibierung von FOXM1 durch siRNA genügt um das invasive Verhalten von HNSCC Zellen *in vitro* herunterzuregulieren. Durch den Gebrauch von Reportergenuntersuchungen, bioinformatischen ChIP-Seq Daten, Chromatin Immuno-prezipitation und Mausmodellen konnten wir den genauen molekularen Mechanismus der FOXM1-abhängigen Invasivitätsregulierung identifizieren. FOXM1 kontrolliert uPA-abhängige Invasion über eine erhöhte AP-1 Aktivität am uPA Promoter und erhöht so dessen Expression und proteolytische Aktivität. Ferner ist ein aktiviertes Ras Protein für eine starke FOXM1-abhängige uPA Aktivität und Tumorformation nötig. Zur Stützung unserer Daten, konnten wir in bioinformatischen Studien beobachten, dass in Dysplasien und Plattenepithelkarzinomen des Kopf-Hals-Bereichs, Öesophagus, Lunge und Gebärmutterhals FOXM1 und uPA überexprimiert sind. In einem vorher etablierten Mausmodel konnten wir zudem zeigen, dass uPA in rezidiven Tumoren, verglichen mit Primären, überexprimiert ist, welches weiter die wichtige Rolle der p38-FOXM1-uPA Achse bei der Entwicklung von Rezidivtumoren bestärkt. Zusammenfassend folgern wir, dass Stress-induzierte Signalkaskaden einen FOXM1-abhängigen Zwischenschritt benötigt, welcher der Aktivierung von AP-1 Transkriptionsfaktoren vorangeht und welcher das invasive Verhalten von Tumoren fördert. Dieser neue Mechanismus begünstigt invasives Verhalten von Plattenepithelkarzinomen und könnte sich womöglich als potentielles Ziel für die adjuvante Therapie von solchen, hochinvasiven, Krebstypen anbieten.

Abbreviations

AP-1	activator protein-1
APS	ammonium persulfate
ATF	activating transcription factor
BCA	bicinchinonic acid
BSA	bovine serum albumine
CAT	chloramphenicol acetyltransferase
CD	cluster of differentiation
Cdc	cell division cycle kinase
Cdh	cadherin
Cdk	cyclin dependent kinase
cDNA	complementary DNA
CENP	centromere protein
ChIP	chromatin immunoprecipitation
Cks	cyclin dependent kinases regulatory subunit
COX	cyclooxigenase
Cyfip	cytoplasmic FMR1 interacting protein 1
DBD	DNA binding domain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl suofoxide
DNA	desoxyribonucleic acid
dNTPs	desoxy adenine tri-phosphate
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
Elk	ETS containing protein
EMT	epithelial mesenchymal transition
ERK	extracellular signal-regulated kinase
Fos	FBJ osteosarcoma oncogene
FOXM1	Forkhead box M1
Fra	other aliases: fol-like antigen 1 (Fosl1)
GAPDH	glyceralaldehyde-3phosphate dehydrogenase
GPI	glycophosphatidylinositol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus

hsp	heat shock protein
IGF	insuline-like growth factor
IHC	immunohistochemistry
Il	interleukin
IR	infrared
JNK	c-Jun N-terminal kinases
kb	kilobase
kDa	kilodalton
KIS	kinase-interacting stathmin
LB	lysogeny broth
LOH	loss of heterozygosity
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEK	MAP kinase-ERK kinase
MEM	minimum essential medium
MKK	putative mitogen activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger RNA
MTT	4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide
myc	myelocytomatosis viral oncogene
PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinotiside-3-kinase
PLK	polo-like kinase
PMA	phorbol 12-myristate 13 acetate
PTEN	phosphatase and tensin homolog
Raf	rapidly accelerated fibrosarcoma
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	rounds per minute
RT-PCR	real-time PCR
SAPK	stress-activated protein kinase
SCC	squamous cell carcinoma
SD	standard deviation
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
Smad	mothers against decapentaplegic homolog

SRC	rous sarcoma
STAT	signal transducer and activator of transcription
TBP	TATA-binding protein
TBS	tris-buffered saline
TEMED	Tetramethylethylenediamine
TGF	transforming growth factor
tPA	tissue plasminogen activator
TPA (PMA)	12-O-tetradecanoylphorbol-13-acetate
TRE	thyroide hormone response elements
Tris	tris(hydroxymethyl)aminomethane
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
UV	ultraviolett
VEGF	vascular endothelial growth factor
wt	wild-type

1 Introduction

1.1 Head and neck cancer

1.1.1 Epidemiology and risk factors

Head and neck cancer is the sixth most common cancer, worldwide affecting 650,000 new patients annually. In the United States approximately 50,000 new cases occur each year, with around nearly 10,000 deaths, accounting for over 3% of all incident malignancies (Jemal A et al., 2007). The incidence rates vary internationally with the highest rates found in Melanesia, South-Central Asia and Eastern Europe and lowest rates observed in Africa and Central America for both males and females (Jemal A et al., 2011). The term head and neck cancer characterizes all malignancies that can arise from various anatomic structures of the lip, tongue, nasopharynx, larynx, oropharynx, or hypopharynx (Figure **1**), including tissue of craniofacial bones, soft tissue, salivary glands, the epithelium lining and mucosal membrane (Pai SL et al., 2009). Over 90% of all head and neck cancers are of squamous cell histology, arising from the squamous mucosa of the upper aerodigestive tract. Therefore this disease is generally often described as head and neck squamous cell carcinoma (HNSCC) (Shiboski CH et al., 2005).

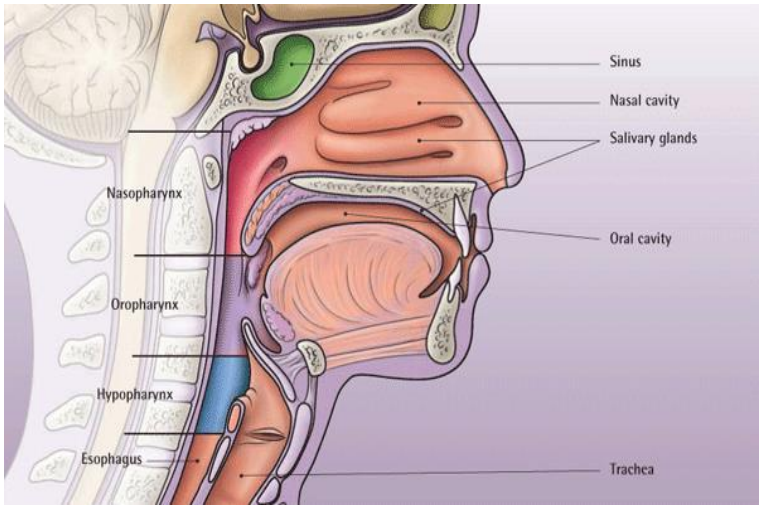


Figure 1: The head and neck region: HNSCC can arise from different structures within the head and neck region, including the oral cavity, nasopharynx, oropharynx, hypopharynx, and nasal cavity. *Picture was taken from Raju Rao MD Radiation Oncology Center web page*.

The most important risk factors for the development of head and neck cancer are tobacco use and alcohol consumption, which are implicated in 75% of all HNSCC and have a multiplicative combined effect (Tuyns AJ et al., 1988; Vineis P et al., 2004). For a subgroup of HNSCC, particularly for oropharyngeal squamous cell carcinoma, infection with high-risk types of human papilloma viruses (HPV) has been recognized as an increasingly important risk factor (Chaturvedi et al., 2011). While in the last couple of years the incidence of HNSCC from specific sites has declined, mainly due to a decrease in tobacco consumption, the incidence of HPV-infection related cancers, like oropharyngeal cancer, has increased and is probably independent of other carcinogens (Rothenberg et al., 2012). Some sexual practices, because of their higher risk for transmitting the HPV virus, are also risk factors for the development of oropharyngeal cancer (Argiris A et al., 2008).

1.1.2 Molecular mechanisms of HNSCC development

It is generally accepted that the initiation and progression of HNSCC is a multistep process of accumulated genetic and epigenetic alterations, including the activation of proto-oncogenes and inactivation of tumor-suppressor genes, by deletion, point mutations, promoter methylation, and gene amplification (Califano J et al., 1996). Those particular genetic alterations are associated with the histopathological stages of HNSCC development from squamous hyperplasia, through graded dysplasia and carcinoma in situ, to invasive carcinoma (Pai SL et al., 2009) (Figure **2**).

Especially loss of heterozytocity (LOH) of certain chromosomal regions can lead to genetic instability and upregulation of oncogenes, or downregulation of tumor-suppressor genes (Williams HK, 2000). In squamous dysplasia and HNSCC loss of chromosomal region 9p21, which harbors several tumor suppressor genes, is the most common genetic alteration, occurring in 80% of all cases and appears to be an early event in the development of HNSCC (Nawrotz H et al., 1994). This region encodes the two transcripts p16 and $p14^{ARF}$, which are important regulators of the cell cycle. Despite LOH, p16 can also be inactivated through promoter methylation, or point mutations (Reed AL et al., 1996).
The loss of the chromosome region 17p, which harbors the TP53 tumor suppressor gene, appears to occur in the late progression from epithelial dysplasia to invasive carcinoma in 50% of all HNSCC (Nawrotz H et al., 1994). In addition to LOH the function of wild-type TP53 is often inactivated by other mechanisms, for example by inactivation through the HPV E6 protein, or

MDM2 overexpression. Mutations of the TP53 gene can be found in 50% to 80% of HNSCC, and are associated with a poor survival prognosis (Boyle JO et al., 1993; Poeta ML et al., 2007).
Loss of the chromosomal region 3p is an early event in dysplasia and invasive HNSCC and is found in up to 70% of HNSCC. Yet, there is much controversy about the involved genes present in 3p and their impact on HNSCC development and has to be further examined (Hogg RP et al., 2002).

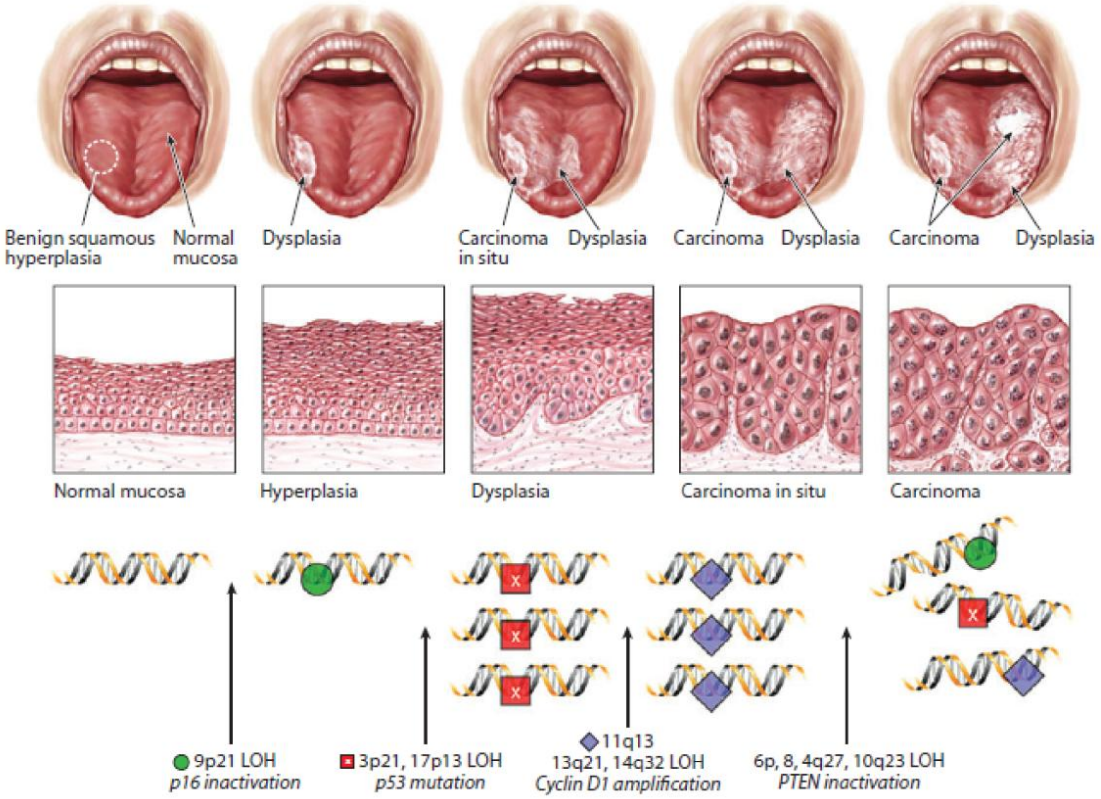


Figure 2: Genetic progression model for head and neck cancer. The accumulation of several genetical aberrations promotes the transformation from normal healthy mucosa through the development of hyperplasia, dysplasia, and carcinoma in situ, to invasive carcinoma. The accumulated genetic alterations include inactivation or mutations of tumor-suppressors (e.g. PTEN, p16, and p53) or overexpression of oncogenes (e.g. Cyclin D1). *Picture was taken from Pai SI, Westra WH, 2009.*

Despite the inactivation of tumor suppressors, the activation of oncogenes plays an important role in the development of HNSCC. The epidermal growth factor receptor (EGFR) has strongly been implicated in the pathogenesis of HNSCC. It is overexpressed in 90% of all head and neck cancers, compared to normal mucosa (Grandis JR et al., 2004) and is a negative predictor for

overall- and disease-free survival (Ang KK et al., 2002; Abhold EL et al., 2012) and radiotherapy resistance (Ang KK et al., 2004).
Amplification of 11q13 and overexpression of Cyclin D1, which enables G1-S transition, is seen in 30% to 60% of HNSCC and associated with increased rate of lymph node metastasis and overall poor prognosis (Perez B et al., 2012).
A link between HPV infection and HNSCC has been established recently. HPV DNA has been identified in 15% of HNSCC patients, of which HPV-16 is the most common subtype (95%) (D'Souza G et al., 2007). Patients with HPV-related HNSCC have a higher risk to develop oral- and orpharyngeal squamous cell carcinoma and usually are not smokers, are at a young age, and have a high lifetime number of heterosexual partners (Perez B et al., 2012). Surprisingly, the presence of HPV type 16 DNA is independently associated with a favorable prognosis including a lower disease specific mortality, compared to HPV-negative patients (Schwartz SR et al., 2001).
Other commonly deregulated pathways in head and neck cancer are activation of the PI3K pathway through PTEN and TGF-β receptor deletion (Bian Y et al., 2012), overexpression of cyclooxigenase-2 (COX-2), vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs), leading to increased metastasis and angiogenesis (Mineta H et al., 2000; Gallo O et al., 2001; Lim SC et al., 2003).

1.1.3 Treatment and Outcome

Even though most head and neck tumors are squamous cell carcinomas, recent insights reveal that HNSCC tumors in fact are unexpectedly heterogeneous depending on the localization and genetic composition of the tumor, hindering accurate prognostication, treatment planning and the identification of the causative cancer genes (Götte K et al., 2005). In addition primary tumor site, stage and respectability, as well as patient factors, including swallowing, airway considerations, and desire for organ preservation, require an appropriate management and the involvement of many specialists, including surgeons, medical oncologists, radiation oncologists, plastic surgeons, and dentists (Argiris A et al., 2008). Early-stage tumors are usually treated with surgery or radiotherapy (Stepnick D et al., 2010) and have a favorable prognosis, with survival curve rates ranging between 60% and 90%, depending on tumor site and extension of the disease. The recommended choice for the 60% of patients with advanced tumors at diagnosis is surgery combined with postoperative radiotherapy. The current standard for patients with an unresectable disease, or when organ preservation is desired, is concurrent cisplatin-based chemoradiation (Vermorken JB et al., 2010).

The quality of life for patients with HNSCC has increased in the recent years due to advanced surgical protocols, as well as the increased role of organ preservation- and reconstruction protocols, including free tissue transfer (Meyers LL et al., 2008). Other factors are the reduction of radiation-induced morbidity due to the use of new radiotherapeutic techniques, like intensity-modulated radiotherapy (Vergeer MR et al., 2008).
Thanks to the growing understanding of the genetical mechanisms involved the development of HNSCC, new therapies targeting specific components of the signal machinery have been developed and the use of biological agents has entered the field (Moral M et al., 2008). Most notably is the application of the epidermal growth factor receptor (EGFR) - specific antibody cetuximab combined with chemotherapy, that has been shown to induce antitumor effects (Fung C et al. 2010; Leemans CR et al., 2011). Despite advances in conventional therapy including surgery, chemotherapy, and radiation, the 5-year survival rate for patients with head and neck cancer is still among the lowest of the major cancers (Carvalho AL et al., 2005; Leemans CR et al., 2011). Only 40-50% of patients will survive for 5 years, due to of the frequent development of regional and distant metastasis, as well as secondary primary tumors, and most importantly, because of the development of locoregional recurrences (Goerner M et al., 2010; Thomas SM et al., 2009).

1.2 Recurrent tumors

1.2.1 Clinical significance of recurrent tumor development

The main cause for treatment failure of HNSCC is locoregional recurrence (Paleri V et al., 2008). The majority of more advanced HNSCC treated with a form of combined modality treatment, will eventually relapse, either locoregionally in 30% to 40% of patients, or form distant metastasis in 20% to 30% of patients (Marur S et al., 2008). The backbone of treatment for patients with recurrent or metastatic head and neck squamous cell carcinoma is chemotherapy combining different agents, like the epidermal growth factor receptor inhibitor cetuximab in combination with 5-fluorouracil and Cisplatin (Péron J et al., 2012). However, due to an aggressive phenotype and resistance against various therapies, the overall main survival for recurrent or metastatic tumors remains still less than 1 year. Only a small portion of patients

can successfully be treated, while for most of them palliative treatment is the only remaining option (Vermorken JB et al., 2010; Price KA et al., 2012). Regarding the success of treatment there is some evidence that HPV positive recurrent oropharynx tumors have a more favorable outcome than HPV negative tumors when treated with chemotherapy (Gillison ML et al., 2000).

1.2.2 Molecular mechanisms of recurrent tumor development

The molecular mechanisms that lie behind the development of recurrent head and neck cancer are not well examined yet. There is some evidence for an association between EGFR mediated Ras-PI3K signaling that confers radioresistance and has a negative impact on locoregional control as well as treatment outcome of head and neck cancer patients (Gupta AK et al., 2002). An important hallmark in the development of recurrent head and neck cancer is the process of local invasion and spread to regional lymph nodes, which is made possible by the highly invasive behavior of tumor cells and rich lymphatic drainage. Persistent invasion and migration into the surrounding tissue causes local dissemination, or penetration of lymphatics, which leads to the production of local recurrent tumors or distant metastasis (Kramer RH et al., 2005).
In clinical investigations head and neck tumors from patients with a high risk to develop recurrence had a 5-year locoregional control of 58%, compared to 88% of low risk tumors. Those high risk tumors were characterized by parameters including positive surgical margins, an increased number of N3 lymph nodes, strong extranodal spread, and perineural invasion, which all suggest a highly invasive growth for these high-risk tumors (Fagan JJ et al., 1998; Langendijk JA et al., 2005;). For carcinoma of the oral cavity it has been shown that vascular invasion, perineural invasion, extracapsular extension, positive margins and T classification inversely correlate with the 5-year locoregional control (Hinerman RW et al., 2004). The development of occult metastasis or nodal metastasis of squamous carcinoma of the oral tongue can be associated with a muscle infiltration depth over 4mm and an infiltrating-type invasion front as well as a weakly defined invasion front consisting of disseminated tumor islands (Byers RM et al., 1998; Sparano A et al., 2004; Lim SC et al., 2004).

Taken together, those experimental and clinical data strongly suggests that especially tumors with a highly invasive potential, consisting of disseminated tumor islands, which lost contact to the invasion front, tend to develop locoregional recurrences (Figure **3**). Thus, to achieve a better locoregional control and increase patient survival, it is of crucial importance to examine the exact molecular mechanisms that promote invasion of head and neck tumor cells.

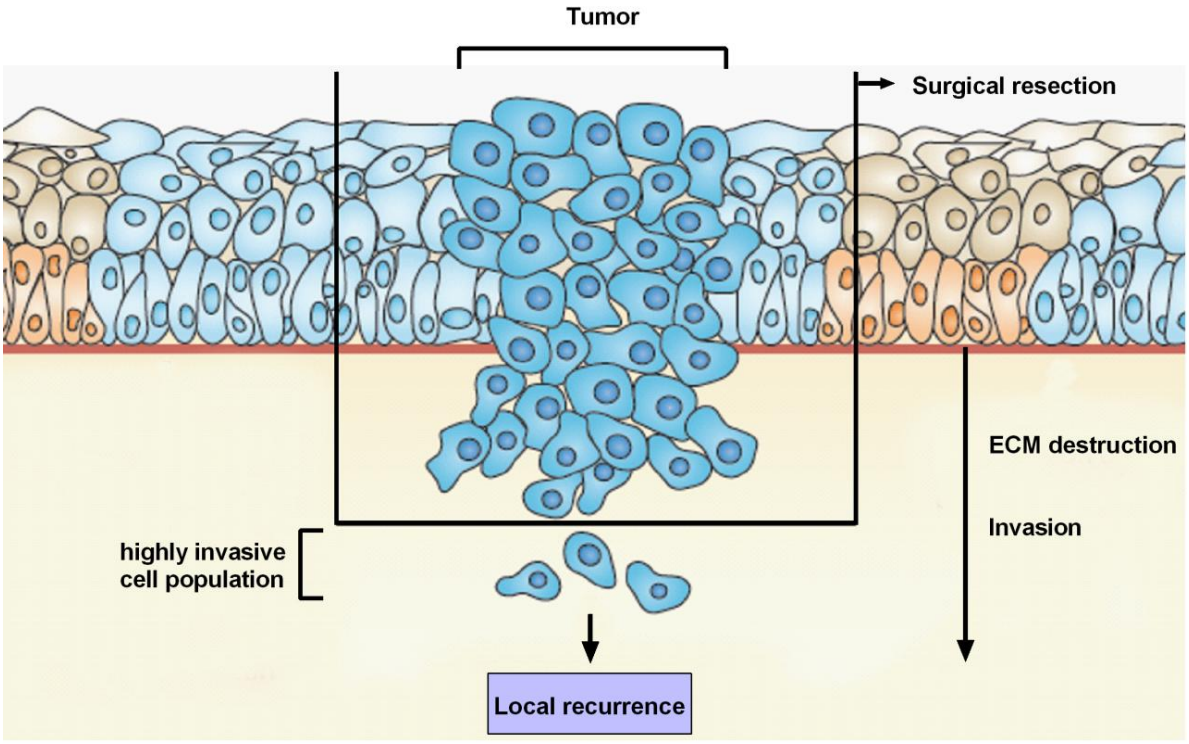


Figure 3: Invasion of tumor cells leads to local recurrence. Within one tumor, populations of highly invasive cells penetrate the mucosal membrane by destruction of the extracellular matrix and invade the surrounding mucosal tissue. After surgical resection, those highly invasive and aggressive cell populations remain inside the mucosal tissue out of which later the recurrent tumor arises. *Picture taken and modified from CR Leemans et al., 2011.*

1.3 The process of cancer cell invasion

1.3.1 Definition and clinical significance

The term cancer cell invasion describes the penetration of cancer cells into neighboring tissue beyond the borders of their primarily tissue from which they originate, which is achieved by degradation of the surrounding extracellular matrix (ECM). Under normal conditions during the events of embryonic development, immune response, or wound healing the process of invasion is a tightly coordinated genetic program, triggered by extracellular signals like growth factors or

chemokines. This process requires a well-tuned expression and activity of signal molecules, transcription factors and other gene products, so that when the cell reaches its designated destination, the invasion program is terminated and the cell can differentiate (Ozanne BW et al., 2006; Friedl P et al., 2011). In cancer cells these signal pathways, controlling the process of invasion, are often disturbed and invasion continues beyond the normal extend (Malliri et al., 1998). As a consequence, cancer cell invasion is a crucial event for the transformation of a locally growing tumor into a spreading and life threatening disease. By the means of enhanced and uncontrolled invasion tumor cells are also able to enter into the circulation system of blood vessels and lymph node channels, by which they reach distant organs and form secondary tumors, called metastasis. The formation of distant metastasis is an important prognostic factor for some cancers like breast cancer (Parker B et al., 2003), whereas in other cancer types, like head and neck cancer, the process of local invasion and metastasis to locoregional lymph nodes is an important prognostic factor and associated with decreased patient survival (Rivelli V et al., 2011).

1.3.2 Molecular mechanisms involved in the process of invasion

The biological mechanisms underlying the process of invasion have been well characterized in the recent years. It is the aberrant expression and activity of certain genes and signal pathways that leads to an invasive phenotype (Figure **4**). At the same time the function of those genes is not only restricted to the phenotype of invasion. Instead, there is a mutual relationship between invasion and other phenotypes like growth, differentiation, and survival, which can be regulated at the same time by one single gene (Mareel et al., 2002).

One important type of regulators of tumor cell invasion are adhesion molecules consisting of several protein families, including Cadherins and Integrins. The transmembrane glycoprotein epithelial (E)-Cadherin, which is a subclass of the Cadherin family, controls the maintenance of intracellular junctions in epithelial tissues by a calcium dependent manner (Kim SA et al., 2011). For breast cancer it has been shown that tumor cells with impaired E-Cadherin expression tend to grow more infiltrative (Oka H et al., 1993). In colon cancer loss of α-Catenin, an essential element of the E-Cadherin invasion suppressor complex, is directly linked to the acquisition of an invasive phenotype of non-invasive cancer cells, thus pointing out the function of E-Cadherin as a suppressor of tumor invasion and a promoter of differentiation (Vermeulen SJ et al., 1999; Strumane K et al., 2004). N-Cadherin, another adhesion molecule, promotes motility and invasion. In squamous carcinoma cells transfection of N-Cadherin cDNA causes a decrease of E-Cadherin expression and induces an invasive phenotype (Islam S et al., 1996).

Integrins mediate the interaction of tumor cells with the cytoskeleton structure of the ECM and are strongly implicated in invasion and metastasis of solid tumors (Desgrosellier JS et al., 2010). The membrane glycoprotein CD44 is widely expressed on lymphocytes and high expression of CD44 is associated with an increased capacity to produce metastasis in renal carcinoma (Bozzuto G et al., 2010).

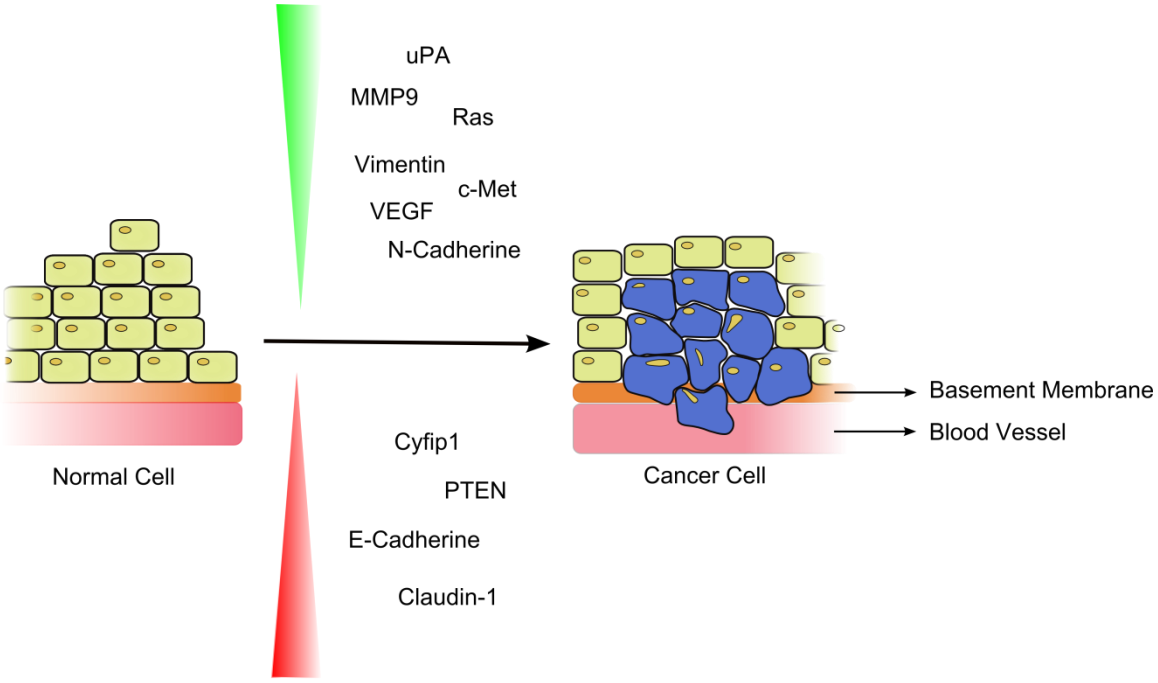


Figure 4: A schematic diagram of cancer cell invasion. The aberrant expression of genes, including the upregulation of oncogenes and downregulation of tumor suppressor genes leads to cancer cell invasion. By destruction of the ECM, cells are able to cross the basal membrane and penetrate blood vessels or the mucosal tissue. By this means tumor cells can form distant metastasis or recurrent and secondary tumors.

The degradation and remodeling of the extracellular matrix (ECM) is an essential step in the process of invasion. It is mainly achieved by two types of proteolytic enzymes, the plasminogen activator system components (uPA) and matrix metalloproteinases (MMPs) (Conese M et al., 1995; Stamenkovic I et al., 2000). MMPs are highly conserved metal atom-dependent endopeptidases, which are capable of breaking down most of the basal membrane and ECM components by degradation of fibrillar collagen, which leads to enhanced invasion and metastasis (Curran S et al., 2000). Transcription factors like the activator protein 1 (AP-1) or the Smad (mothers against decapentaplegic homolog) proteins are able to modulate the expression of invasion-promoting genes like MMPs or uPA and are associated with tumor cell invasion and epithelial mesenchymal transition (EMT) (Davies M et al., 2005). Other important regulators of cancer cell invasion, which can be activated through mutation and become oncogenes

promoting invasion both in early and late stage of cancer development, are c-Met, epidermal growth factor receptor (EGFR), Ras proteins, the phosphates and tensin homologue (PTEN), RhoA/C, Cdc42, Rac1, Vimentin, or the SRC gene (Irby RB et al., 1999; Campbell PM et al., 2004; Sahai E et al., 2005;). MMPs, transcription factors, and other genes responsible for tumor invasion, can be regulated by growth factors and cytokines through the Mitogen-activated protein kinases, which thus play a crucial role in cancer cell invasion (Kumar B et al., 2010; Huang S et al., 2000).

1.4 Stress- activated protein kinases (SAPKs)

1.4.1 The Mitogen-activated protein kinase (MAPK) signaling cascade

Mitogen-activated protein kinases (MAPKs) are evolutionary conserved signal transducing enzymes, which convert extracellular stimuli into a wide range of cellular responses, by connecting cell-surface receptors to critical regulatory targets within the cell. MAPKs play important roles in various cellular and multicellular processes like cell growth, differentiation, apoptosis, and migration (Roberts PJ et al., 2007). Aberrant activation of MAPK cascades is characteristic for many cancer types, even though their exact role in the development of cancer is complex and still confusing, due to several cross-talk reactions and feedback mechanisms (Engelberg et al., 2004).

Protein kinases covalently attach phosphates to the side chain of serine, threonine, or tyrosine residues of specific proteins inside cells and thus control their activity or interaction with other molecules (Chang L et al., 2001; Johnson GL et al., 2002). The activation mechanism of MAPKs includes a G-protein working upstream of a core module consisting of three sequentially acting kinases. In response to a extracellular stimuli a GTP-binding protein of the Ras/Rho family interacts with a serine/threonine MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates the MAPK through dual phosphorylation on threonine and tyrosine residues (Figure **5**) (Schaeffer HJ et al., 1999; Chen Z et al., 2001; Kyriakis JM et al., 2001). Such an arrangement provides signal amplification, and the possibility for regulatory interference, which allows the cells to precisely control the duration and amplitude of the signal strength (Kolch W et al., 2000). There are several distinct groups of

MAPKs that have been characterized. The three main types are extracellular signal-regulated kinase (ERK) 1/2, Jun N-terminal kinase (JNK)1/2 and the p38 isoforms α/β/γ/δ (Dhillon AS et al., 2007).

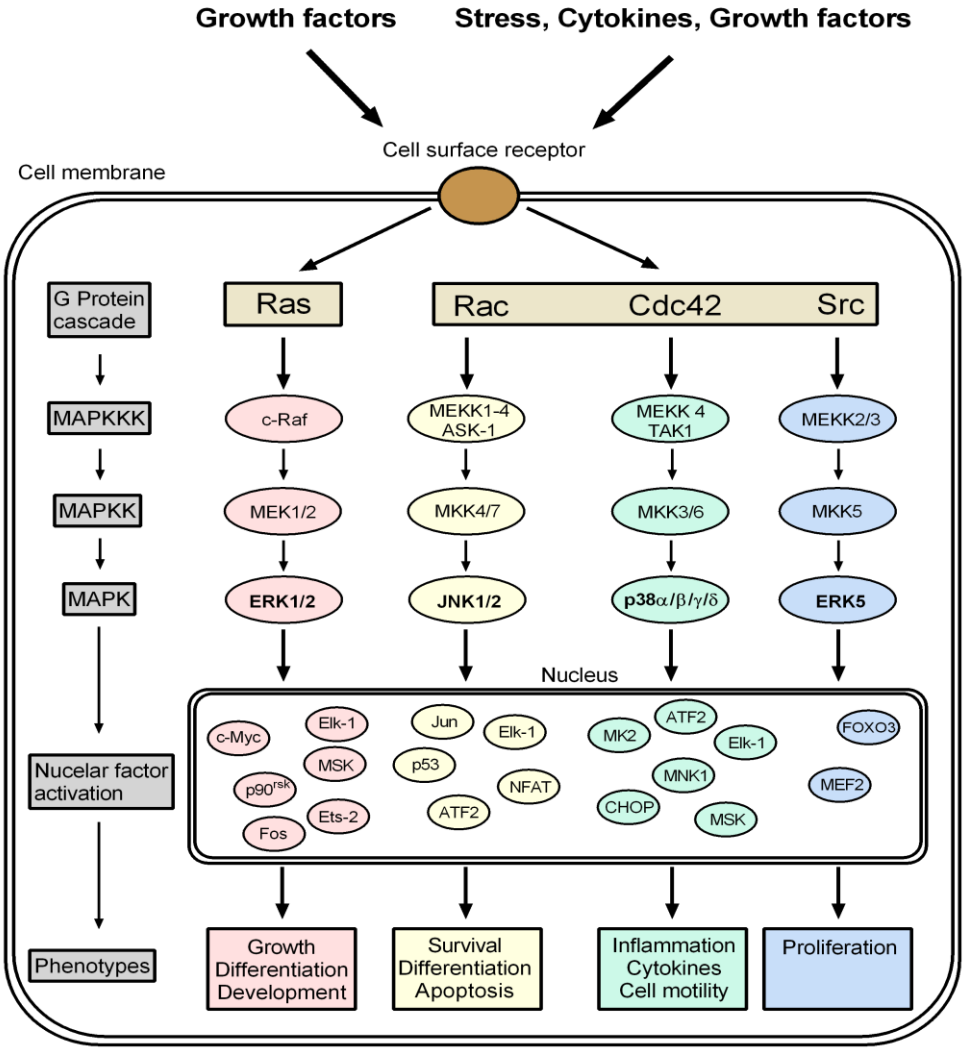


Figure 5: MAPK signaling in cancer cell. The diagram illustrates the various components of the most important MAPK signaling pathways that promote the malignant development of tumors. Though not indicated in the picture, there are numerous cross-talk reactions and feedback loops among all pathways.

1.4.2 Extracellular signal-regulated kinases (ERK)

ERK1/2 is a widely expressed 42-/44-kDa MAPK and mainly implicated in the regulation of cell proliferation, but is also involved differentiation, survival, and motility. The ERK pathway can be

activated mainly by growth factors and phorbol esters, but also by various cytokines or infections and carcinogens (Kohno M et al., 2006; McCubrey JA et al., 2007). The mammalian ERK1/2 module is a component of a three-kinase module that includes the MAPKKK c-Raf, B-Raf, or A-Raf, which are activated by the proto-oncogene Ras. The MAPKKK module activates the dual-specific MAPKK MEK1, and MEK2 which in turn phosphorylate ERK1/2 within a conserved Thr-Glu-Tyr motif inside of its activation loop (Crews CM et al., 1992; Marshall CJ et al., 1994; Kohno M et al., 2006). Activated ERK1/2 phosphorylates various downstream targets, including membrane proteins (Calnexin), nuclear substrates (Elk-1, c-Fos, c-Myc, FOXM1, STAT3), cytoskeletal proteins (Paxillin) and several mitogen kinases (MKs) (Babu GJ et al., 2000; Roux PP et al., 2004; Ng DC et al., 2011). Under normal physiological conditions the ERK MAP kinase module plays a central role in growth and survival of human cells. An auto-control mechanism associated with nuclear/cytoplasmic shuttling ensures the appropriate intensity and duration of ERK activity in response to growth factors and other extracellular stimuli (Lawlor MA et al., 2000; Costa M et al., 2006).

In cancer cells this fine-tuned mechanism is often disturbed (Pouysségur J et al., 2003). ERK is constitutively active in more than 36.2% of all tumors, which in most cases is due to the disorder and inappropriate activation of Raf or Ras (Avruch J et al., 1994; Hoshino R et al., 1999). In human lung cancer cells it has been shown that the ERK/MAPK pathway is crucial for cell migration and invasion of tumor cells upon growth factor stimulation (Lu Z et al., 2011). ERK1/2 also promotes proliferation of colorectal cancer cells (Kress TR et al., 2010), and confers resistance to apoptosis in prostate cancer cells (Rasola A et al., 2010). Inhibition of ERK in gene based cancer therapies often was reported to act anti-cancerous in experimental systems and patients (Sebolt-Leopold JS et al., 1999; Ligresti G et al., 2008; Bartholomeusz et al., 2012; Ding Z et al., 2012).

1.4.3 Stress-activated protein kinases (SAPK)

The stress activated protein kinases JNK and p38 are usually activated by stress stimuli like osmotic shock, UV/IR irradiation, or cytokine stimulation and they play an important role in the regulation of cellular proliferation, differentiation, motility and apoptosis (Malemund; 2007).
The JNKs were characterized as stress-activated protein kinases that bind and phosphorylate the DNA binding protein c-Jun, which is a component of the AP-1 transcription factor complex, and increase its transcriptional activity (Johnson GL et al., 2002). Further investigations show that the activation of c-Jun by JNK can be induced by UV light and is dependent on the oncogene Ha-

Ras (Dérijard B et al., 1994). Upon mutation of the JNK phosphorylation sites within the c-Jun protein ($Jun^{S63AS73A}$) in mice, skin tumor development after induction of Ras and c-Fos was significantly impaired (Behrens A et al., 2000), pointing out an important role for JNK as a mediator of Ras and AP-1-mediated oncogenesis.

The stress-activated MAPK p38 was originally identified as a kinase which is phosphorylated and activated by stimulation with endotoxic lipopolysaccharide cytokines (Han J et al., 1994) and mediates interleukin-1 induced pro-inflammatory activity (Lee JC et al., 1994). Beyond stress-response p38 is involved in other cellular functions like oogenesis, differentiation, and survival (Nebreda AL et al., 2000). Recently it has been established that, upon transduction of extracellular signals into the nucleus and other cellular components, p38 turns on target genes which are involved in pro-oncogenic processes (Ono K et al., 2000). Upon activation, p38 phosphorylates the transcription factor ATF-2 (Zayzafoon M et al., 2002) and further, p38 activity is essential for Anisomycin- and ultraviolet (UV)-stimulated induction of c-Jun and c-Fos (Hazzalin CA et al., 1996). p38 also leads to the phosphorylation and activation of small heat shock protein hsp27 after both stress or growth factor stimulation, resulting in remodeling of the actin cytoskeleton (Guay J et al., 1997). In prostate cancer cells it has been shown that Il-6-induced TGF-β stimulation, which leads to tumor progression, is mediated by multiple pathways including JNK, Ras and p38 (Park JI et al., 2003).

1.4.4 The role of p38 in invasive processes of head and neck cancer

The role of p38 in invasive processes of cancer cells has also been established. Using the p38 inhibitor SB203580 in human epithelial breast cancer cells, it has been shown that p38 is the key mediator of H-Ras induced cell motility and that it leads to an invasive phenotype by upregulation of matrix metalloproteinase 2 (MMP-2) (Kim MS et al., 2003; Shin I et al., 2005). In prostate cancer cells p38 and ERK promote cell invasion via regulation of the G protein-coupled P2Y purinoreceptor (Chen L et al., 2004).

p38 also exerts important functions in the tumor development and invasion of HNSCC. STAT3, which is normally overexpressed in head and neck cancer is and a strong promoter of malignant transformation via Il-6/8, displays a decreased activity upon inhibition of p38 (Riebe C et al., 2011). Inhibition of the two p38 isoforms p38α/β reduced the SCC cell number and invasion, while inhibition of p38α, but not p38δ, resulted in apoptotic cell death (Juntilla MR et al., 2007). In HSNCC cell lines MMP-10 driven invasion and metastasis is partially dependent on p38 activity (Deraz EM et al., 2011) and secretion of Il-8, which triggers angiogenesis, is significantly elevated after activation of p38 (Riebe C et al., 2007).

Exposure of HNSCC cells to Cisplatin and other platinum based compounds in an experimental setup markedly increased p38 activity, indicating an involvement of p38 in radiation-based therapy of HNSCC (Hérnandez LJ et al., 2003). In fact, p38α levels in the serum of HNSCC patients at cancer diagnosis were elevated significantly and correlated inversely with the success of radio-therapy, pointing out an important role of p38 as a suitable prognostic marker in the serum of HNSCC with respect to radio-therapy (Gill K et al., 2012).

Even though there is a large body of evidence for the implication of p38 in invasive processes of HNSCC cancer cells, there is only little information available about the exact mechanisms and the role of p38, as well as for other MAPKs, in the development of recurrent tumors. Due to the fact that p38, as well as other MAPKs, often display cross-talks with other MAPK components, and beside oncogenic effects also exert tumor-suppressing functions, it is more feasible to search for further downstream targets in order to target invasion more specifically.

1.5 The Forkhead box protein M1 (FOXM1)

1.5.1 Expression and physiological function of FOXM1

The Forkhead box protein M1 (FOXM1), also known as HFH-11/-3, MPP-2, Win, or Tridient (Laoukili J et al., 2007), is a transcription factor of the Forkhead family that share homology in their conserved winged-helix DNA-binding domain (Mardsen I et al., 1997). It was first identified by the three-dimensional structure of its winged-helix HNF-3/Forkhead DNA recognition motif, using X-ray crystallography (Clark KL et al., 1993). The human FOXM1 gene consists of 10 exons, spanning approximately 25kb on the 12p13-3 chromosome (Laoukili et al., 2006). Two exons are alternatively spliced, leading to the three different splice variants FOXM1-A, FOXM1-B, and FOXM1-C, of which only the isoforms B and C are transcriptionally active and regulate their target genes by two different mechanisms (Alves J et al., 2007). They both activate through binding to conventional FOXM1 binding sites 5'-AGATTGAGTA-3' (Yao KM et al., 1997), while FOXM1-C can additionally transactivate promoters by binding to the TATA boxes P1 (5’-TATAATGC-3’) and P2 (5’-TATAAAG-3’) (Wierstra I et al., 2006). FOXM1-A has no transcriptional activity, but since it has retained its DNA binding properties it is believed to have a dominant negative effect on transcriptional activation (Ye H et al., 1997).

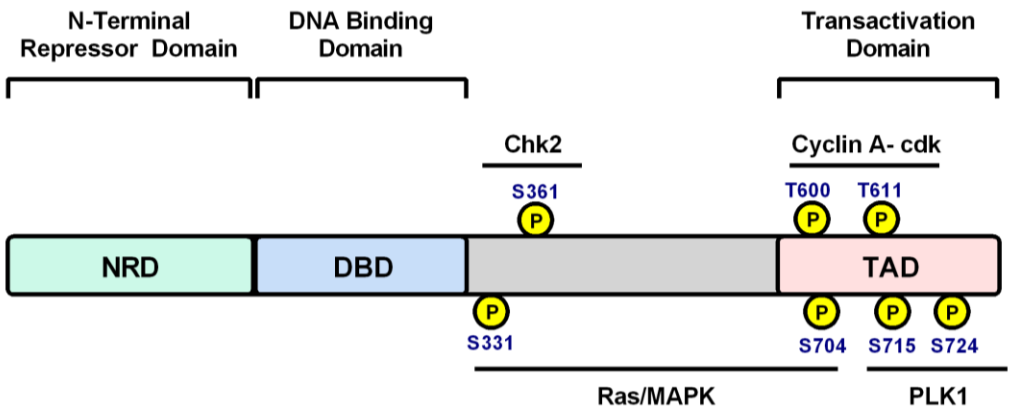


Figure 6: Functional domains within the FOXM1 protein. The graphic shows the most important components of the FOXM1 protein including its phosphorylation sites. Each domain has distinct functions regarding the DNA binding properties, expression, and transactivational activity of FOXM1. Picture was m*odified after Schwenen H et al., 2009.*

FOXM1 is a proliferation-specific gene whose expression is restricted to actively dividing cells of embryonic tissues (Korver W et al., 1997), or to highly proliferating adult tissues including the thymus and testis, whereas it is barely detectable in quiescent or terminally differentiated cells (Ye H et al., 1997). FOXM1 is a key regulator of the cell cycle, playing a critical role for G1/S transition, entry into mitosis (Wang IC et al., 2005), for the proper execution of mitosis and for chromosome stability (Laoukili J et al., 2005). FOXM1 deficient cells show severe defects in chromosome number and integrity. Depletion of FOXM1 in mouse embryos uncouples the S-phase from mitosis and leads to postnatal death as a result of the development of polyploid cardiomyocytes and hepatocytes (Korver W et al., 1998). In addition, FOXM1$^{-/-}$ mice suffer from respiratory failure after birth due to inhibited lung maturation (Kalin V et al., 2008). Despite the orchestration of the cell cycle FOXM1 regulates the expression of genes involved in DNA damage repair (Tan Y et al., 2007), tissue regeneration (Ye H et al., 1997), organogenesis (Korver W et al., 1998) and the process of aging (Laoukili et al., 2006).

1.5.2 Genetic regulation of FOXM1 expression and activity

The activity of FOXM1 is regulated through phosphorylation by various kinases at different stages of the cell cycle, which determines its localization and activation state. Initial phosphorylation at its C-terminus occurs at the late G1/S-phase by Cyclin E/cdk2 (Major ML et al., 2004), followed by Ras-MEK-ERK1/2 mediated phosphorylation just before G2/M-entry,

which translocates FOXM1 to the nucleus and stimulates its transactivating activity (Ma RY et al., 2005). Additional hyperphosphorylation during the G2/M phase involving the Polo-like kinase-1 (PLK-1) and Cyclin B/Cdk1 increases FOXM1 transcriptional activity further (Fu Z et al., 2008; Chen YJ et al., 2009) (Figure **7**). At the end of M-phase FOXM1 becomes degraded by the anaphase-promoting complex/ cyclosome APC/C E3 ubiquitin ligase complex and its adaptor Cdh1 (Park HJ et al., 2008). FOXM1 expression and activity during the cell cycle is negatively controlled by p53 or p19ARF (Kalinichenko VV et al., 2004; Gusarova GA et al., 2007; Pandit B et al., 2009).

FOXM1 itself controls the transcriptional activation of several target genes like the SCF ubiquitin ligase complex subunits Skp2 and Cks1, Kinase-interacting stathmin (KIS), and JNK1 during the G1/S phase (Petrovic V et al., 2007; Wang IC et al., 2008). At later stages of the cell cycle it regulates the transcription of a number of genes that are crucial for the G2/M progression, including Cyclin B, Cdc25B, Aurora B, PLK-1, Survivin, and CENP-A (Wonsey DR et al., 2005; Kim IM et al., 2005; Chen YJ et al., 2009). FOXM1 has also been shown to bind directly to genes involved in invasion, angiogenesis, and survival of tumors cells including c-myc, hsp70, MMP-2, VEGF, and c-Fos (Wierstra I et al., 2007; Wang IC et al., 2008; Ahmad A et al., 2010).

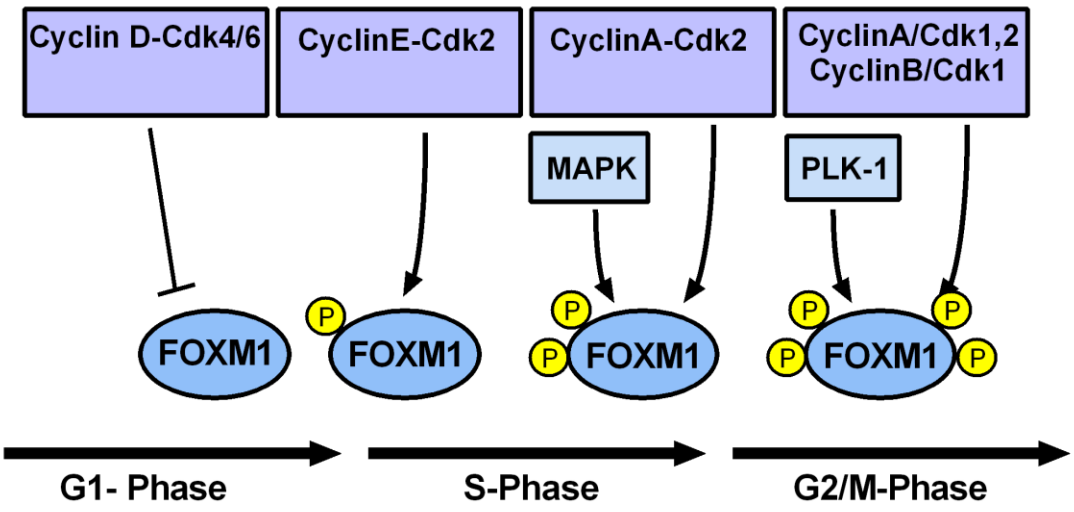


Figure 7: Regulation of FOXM1 during the cell cycle. The picture shows the various phosphorylation steps that regulate the activity of FOXM1 throughout the cell cycle. Within this process phosphorylation through cyclin dependent kinases and members of the Ras-MAPK pathway play an important role. *Modified after Lakoukili J et al., 2006*.

1.5.3 The role of FOXM1 in cancer progression

Regulating such a broad range of genes, FOXM1 is considered as a proto-oncogene which plays a central role in the initiation, progression, motility and development of many cancer types (Figure **8**). Its expression is upregulated in tumors of the liver, pancreas, colon, breast, lung, and prostate (Rayhaudhury P et al., 2011). In tumors the activity of FOXM1 is often deregulated by amplification of its chromosomal locus 12p13 (Weber RG et al., 1996; Sato Y et al., 2001) or by deregulation of its transcriptional activity through other genes or kinases like p38 or ERK (Teh MT et al., 2010; Behren A et al., 2010; Lok GT et al., 2011), leading to malignant phenotypes.

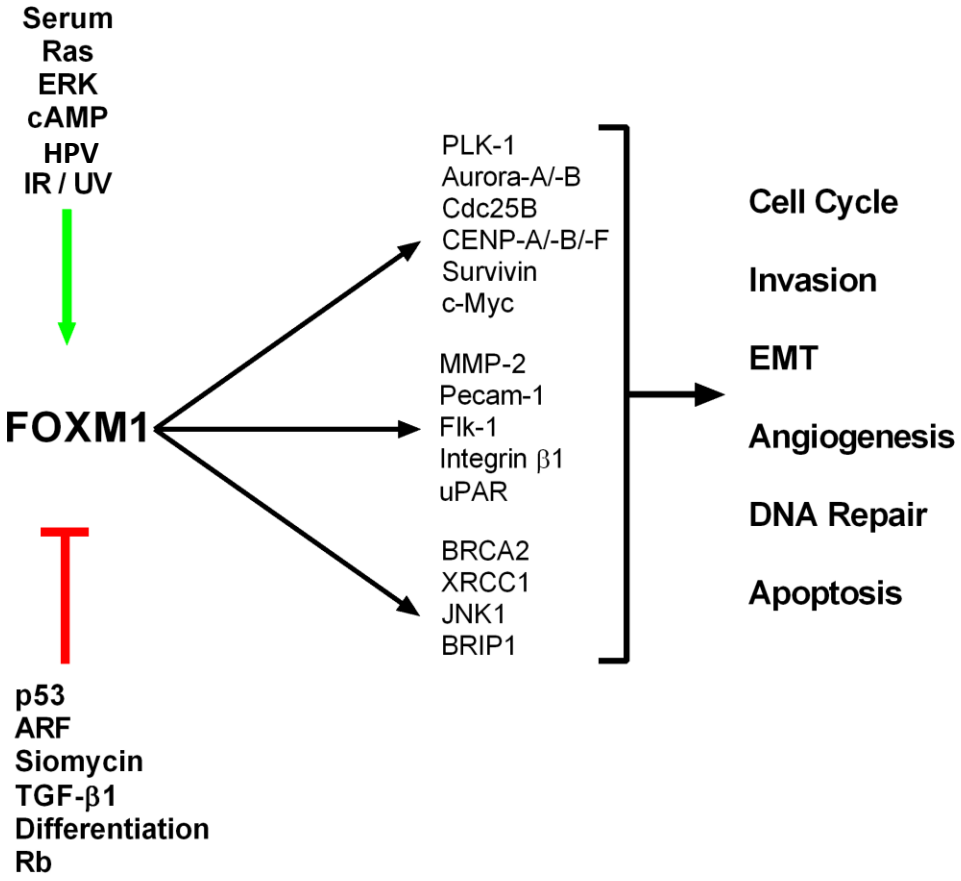


Figure 8: Involvement of FOXM1 in the gene regulatory network of human cancers. FOXM1 is a crucial player in the genetic network that regulates various tumor phenotypes. Genes that regulated FOXM1 are often mutated, or deleted in tumors, leading to an aberrant FOXM1 activation. A deregulated activation of FOXM1 in turn, has a huge impact on tumor development, since FOXM1 regulates a wide range of downstream genes, which are involved in the promotion of tumorgenesis.

FOXM1 has been shown to play an important role in stress-induced apoptosis during cancerogenesis (Park HJ et al., 2009; Halasi M et al., 2012), to promote angiogenesis via VEGF

(Zhang Y et al., 2008), tumor growth (Li D et al., 2013), and epithelial-mesenchymal transition (Bao B et al., 2011). In clinical investigations it was shown that elevated FOXM1 expression leads to resistance against chemotherapy in breast cancer (Kwok JM et al., 2010; Carr JR et al., 2010), promotes metastatic processes in prostate cancer (Chandran UR et al., 2007), is essential for the development of hepatocellular carcinoma (Kalinichenko VV et al., 2004), and correlates with poor prognosis of patients with various other cancers (He SY et al., 2012; Xue YJ et al., 2012). The link between FOXM1 and invasion has recently been established. Glioma cells that possess elevated levels FOXM1 have an increased MMP-2 expression which enhances invasion and thus contributes to tumor progression (Dai B et al., 2007). In osteosarcoma cells FOXM1 enhances MMP-2/-9 dependent invasion via upregulation of JNK1 expression (Wang IC et al., 2008), while in ovarian cancer the ERK/FOXM1 cascade significantly increases invasion (Chan DW et al., 2012).

1.5.4 The role of FOXM1 in head and neck cancer

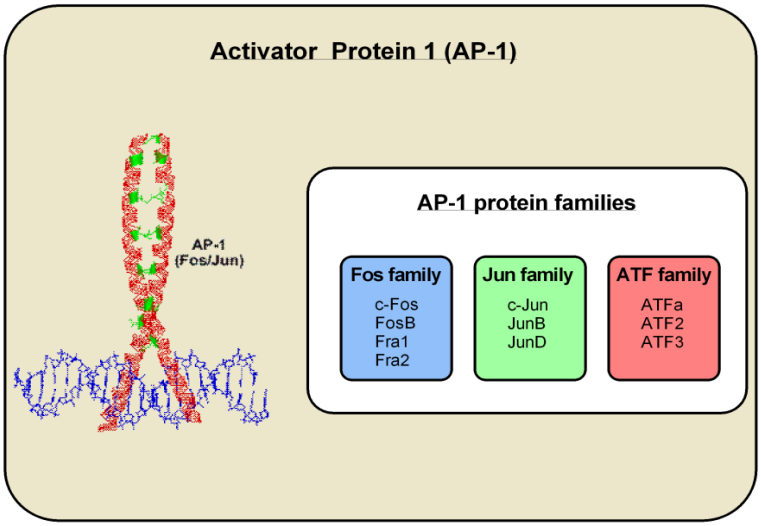
FOXM1 has been shown to be significantly upregulated in human HNSCC as well as in premalignant dysplastic lesion (Singh B et al., 2001), where it is mainly expressed in the proliferative epibasal layer of the epithelium (Gemenetzidis E et al., 2010). Further, FOXM1-mediated transformation of keratinocytes in HNSCC could be enhanced by nicotine, giving evidence for a striking role of FOXM1 in the early oncogenesis of HNSCC and as a potent marker of malignant conversion (Gemenetzidis E et al., 2009; Waseem A et al., 2010). A clinical relevance between FOXM1 and head and neck cancer has been established in laryngeal squamous cell carcinoma. Here, FOXM1 expression correlated with histological differentiation, T stage, lymph node metastasis, and inversely correlated with patient overall survival (Jiang LZ et al., 2011). There is also some evidence that FOXM1 is involved in invasive mechanisms of HNSCC cells, mainly via activity of MMP's (Chen CH et al., 2009; Ahmed M et al., 2012).

The regulation of such a wide spectrum of tumor-promoting phenotypes like growth or invasion indicates a central role for FOXM1 in the initiation and progression of head and neck cancer and the development of recurrent tumors. Nevertheless, the exact mechanisms of how FOXM1 and its downstream targets regulate invasion of HNSCC still remains unclear and has not been examined in detail.

1.6 Activator Protein 1 (AP-1)

1.6.1 The AP-1 protein members and their regulation

The Activator Protein 1 (AP-1) transcription factor is a dimeric complex that consists of protein members of the Fos (c-Fos, FosB, Fra-1, Fra-2), Jun (c-Jun, JunB, JunD) and the ATF family (Angel P and Karin, 1991) (Figure **9**). It was discovered as a 12-O-tetra-decanoylphorbol-13-acetate (TPA)-inducible transcription factor, which binds to the enhancers of viral SV40 gene (Wagner EF et al., 2001). AP-1 proteins dimerize through a leucine-zipper motif and contain a basic domain for interaction with the DNA backbone through recognizing TPA-responsive elements (TRE) within AP-1 inducible promoters (Angel P et al., 1991). The AP-1 protein is implicated in a variety of biological processes including the proliferation, survival, differentiation, and transformation of cells and is induced by growth factors, cytokines, TPA, oncoproteins and physical or chemical stress (Lee W et al., 1987; Jochum W et al., 2001; Chang L et al., 2001; Shaulin E and Karin M, 2002).



Picture 9: The Activator Protein-1. The picture gives an overview of the main AP-1 protein families. The main components of the AP-1 complex are members of the Fos, Jun, and ATF family. *Picture modifyed from Dipak P. Ramji et al., 2002.*

MAPKs play an import role in the activation of the AP-1 complex by enhancing its activity through increased expression or phosphorylation of their distinct substrates (Chang L et al., 2001). ERK has been shown to increase the expression of the AP-1 members Fra-1 and Fra-2, leading to enhanced proliferation and DNA synthesis (Treinies I et al., 1999). ERK also accounts for the mitogen-induced transcription of c-Fos via of the ternary complex factor (TCF) members

Elk-1, and SRF by recruiting them to the serum response element (SRE) of c-Fos (Gille H et al., 1992; Marais R et al., 1993; Monje P et al., 2005).
DNA-damaging agents are also able to induce the expression of AP-1 members (Devary Y et al., 1991). This type of AP-1 activation is mediated through pro-inflammatory cytokines and genotoxic stress involving the two SAPKs JNK and p38. Upon UV-irradiation JNK1 and JNK2 are both translocated to the nucleus where they phosphorylate c-Jun as well as Elk-1 and stimulate its transcriptional activity, which in turn leads to increased expression of c-Fos (Cavigelli M et al., 1995). JNK has also been shown to activate ATF-2 after treatment of cells with pro-inflammatory cytokines or UV irradiation (Gupta S et al., 1995).
p38 has been demonstrated to mediate UV-induced AP-1 transactivation and c-Fos expression in human keratinocytes (Silvers AL et al., 2003), and also leads to increased c-Jun gene transcription after lipopolysaccharide (LPS) stimulation (Han J et al., 1997). p38 regulates AP-1 activity also through phosphorylation. In response to UV light p38 associates with c-Fos and phosphorylates the transactivation domain, initiating its translocation to the nucleus, which finally leads to enhanced AP-1 driven gene expression (Tanos T et al., 2005).

1.6.2 The role of AP-1 in tumor development

Deregulated expression of the AP-1 complex is a crucial factor in tumor development and progression. Transfection of the c-Fos oncogene into murine papilloma cells leads to a malignant conversion, involving the activation of Ha-Ras (Greenhalgh DA et al., 1988), while c-Fos knockout mice were not able to develop Ha-Ras induced tumors (Saez E et al., 1995). In transgenic mice, that overexpress c-Fos, normal cell growth was disturbed, leading to the development of osteosarcomas (Grigoriadis AE et al., 1993). Another AP-1 member c-Jun plays a predominant role in the development of skin and liver tumors. Transactivation-mutant c-Jun mice displayed a dramatic inhibition of papilloma induction (Young MR et al., 1999), while primary hepatocytes lacking c-Jun had a decreased number and size of tumors due to an elevated p53 expression and increased apoptosis (Eferl R et al., 2003). AP-1 is also critical for invasion of cancer cells. In breast cancer tumors members of the Fos family (Fra-1, Fra-2, c-Fos) stimulated invasion of MCF7 cells via the upregulation of MMPs and components of the urokinase plasminogen activator (uPA) system (Milde-Langosch K et al., 2004). The role of AP-1 in head and neck cancer has recently been established. In premalignant and malignant HNSCC cells stimulation with tobacco carcinogens upregulated AP-1 expression, which enhanced angiogenesis and invasion through increased Il-8 and VEGF expression (Swenson WG et al., 2011).

1.7 Urokinase Plasminogen Activator (uPA)

1.7.1 Synthesis and function of the Urokinase Plasminogen Activator

The processes of organogenesis during embryonic development, inflammatory reactions, as well as malignant invasion and metastasis of tumors require cells to transcross the boundaries of their normal tissue and to migrate into other compartments of the body (Vassalli et al., 1985). This process involves the coordinated destruction of the extracellular matrix (ECM) and the breakup of cell-cell and cell-ECM contacts. Several protease systems including a board range of proteolytic enzymes with different activities and substrate specifities are capable to degradate ECM components, which pose a physical barrier for the migrating cells, and thus allow tumor cell movement and invasion (Pöllänen et al., 1990).

Plasmin is a protease which facilitates the destruction of the ECM, by degredating several ECM components like fibronectin, laminin (Liotta LA et al., 1981), and converting other proteases (Salo T et al., 1982; Tryggvason K et al., 1987). Plasminogen activators are highly specific serine proteases that are capable of converting the zymogene form of plasminogen into the active serine protease plasmin. In mammals there are two main types of plasminogen activators; the tissue-type plasminogen activator (tPA), which is has important functions in vascular fibrinolysis, and the urinary-type plasminogen activator (uPA), which is more important for tissue remodeling and cell motility (Sidenus N et al., 2003). After transcription of the uPA gene, its 2.5 kb mRNA (Verde P et al., 1984) is translated into the inactive, 50 kDa single chain zymogene form of uPA (pro-uPA), which is composed of three distinct regions (Kasai S et al., 1985). It is then secreted into the extracellular space and converted into the active two chain form (20 kDa + 30 kDa) by catalytic activity of plasmin (Nielsen LS et al., 1982). Within the extracellular space uPA is almost exclusively localized at cell-cell contact sites and focal adhesion contacts within membrane-rich fractions (Pöllänen J et al., 1988).

1.7.2 Members of the Urokinase Plasminogen Activator system

uPA binds with high affinity to the glycosylphosphatidyl inositol (GPI) anchored glycoprotein uPA receptor (uPAR) and thereby alters its activation. The association of uPA to the uPAR accelerates the kinetics of plasmin conversion (Ellis V et al. 1991). Furthermore, due to its polarized

expression on the leading front of the cell-ECM or cell-cell contacts, uPAR can direct the uPA protein to specific compartments of the cell surface where invasion is required (Estreicher A et al., 1990). uPA/uPAR interactions are also important for the regulation of cell adhesion properties via Vitronectin (Hoyer-Hansen G et al., 1997) and invasion (Fazioli F et al., 1997). In some cell types the uPA/uPAR mediated increase of invasiveness is not due to enhanced proteolytics activity of uPA, but through uPA induced signal transduction via uPAR, involving the activation of tyrosine kinases (Resnati M et al., 1996).

Beside uPA and uPAR, the plasminogen activator inhibitor (PAI-1), a 52 kDa glycoprotein, plays an important role in the biology of the uPA system. It is relatively specific for plasminogen activators and forms 1:1 complexes with the active form (two chains) of uPA and blocks its active site (Wun TC et al., 1987). PAI-1 alone, and in cooperation with uPA, also exerts tumor growth promoting functions like angiogenesis and migration of endothelial cells in breast tumors (Bajou K et al., 2002) (Figure **10**).

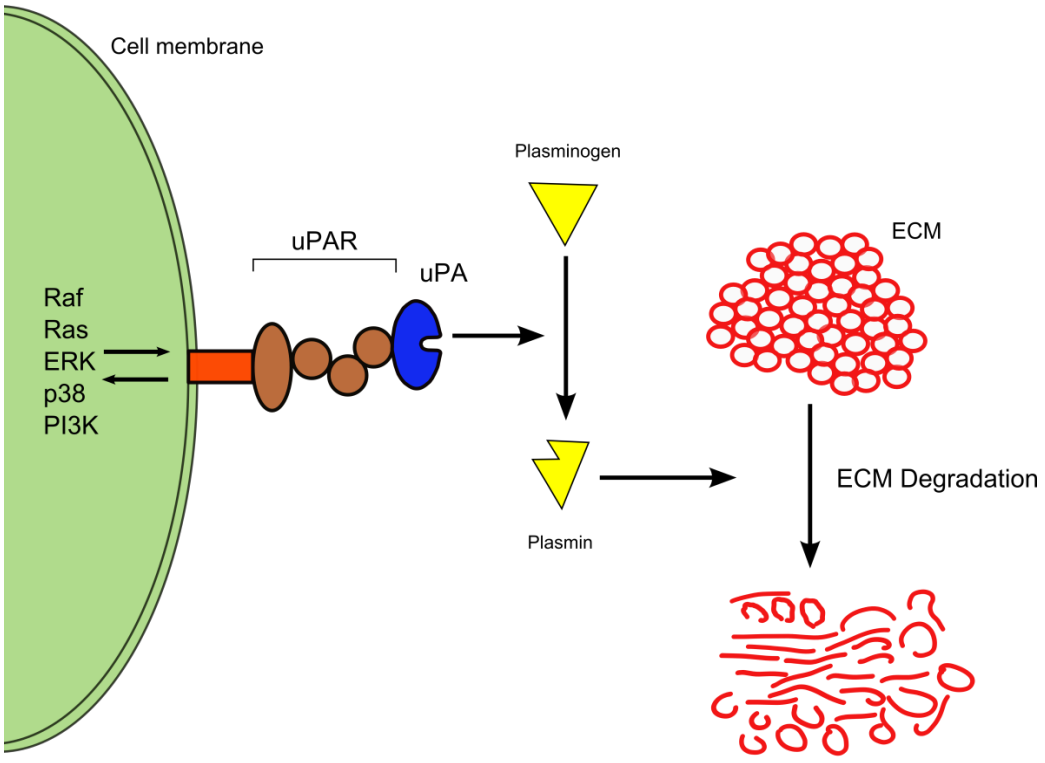


Figure 10: The Urokinase Activator system. Receptor-bound uPA facilitates conversion of zymogenic Plasminogen into active Plasmin, which in turn degradates the extracellular matrix, making tumor cell migration and invasion possible. uPA expression and activity is enhanced by Raf-RAS-MAPK signaling, while at the same time receptor bound uPA can trigger the activation of MAPK signaling.

1.7.3 Implications of uPA in cancer progression

uPA is implicated in the progression and aggressiveness of various cancer types (Minoo P et al., 2010; Huang S et al., 2000). There is some evidence that uPA expression is induced by growth factor-mediated activation of the Ras-MAPK pathway (Bell SM et al., 1993; Pepper MS et al., 1992). In pancreatic cancer cells ERK1/2 and p38 mediate HGF-induced uPA expression (Lee KH et al., 2003), while sustained ERK1/2 activity is necessary for Ha-Ras-mediated stimulation of the uPA promoter in ovary adenocarcinoma (Lengyel E et al., 1995). Further, it had been shown that p38 signaling enhances uPA expression (Shin BA et al., 2003) and that uPA is involved in p38-mediated migration of endothelial cells and invasion of breast cancer cells (Montero L et al., 1999; Huang S et al., 2000; Yu J et al., 2004). uPA has also been shown to be regulated by the AP-1 protein, since AP-1 binding elements within the uPA promoter are necessary to induce phorbol ester and hepatocyte growth factor-induced expression of uPA (Nerlov C et al., 1992; Ried S et al., 1999).

The role of uPA in the development of HNSCC has been examined in various studies. uPAR has been demonstrated to be upregulated in HNSCC compared with non-malignant tissue (Schmidt et al., 2000), while uPA levels and activity were also elevated in tumors from HNSCC patients compared to control samples (Petruzzelli GJ et al., 1993). In oral squamous cell carcinoma it has been demonstrated that uPA was upregulated in tumor tissue and lead to an invasive phenotype (Clayman G et al., 1993). With respect to those findings uPA might exert important functions in the development of recurrent head and neck tumors.

1.8 Aims of this study

The development of recurrent tumors is the main cause of treatment failure in head and neck cancer patients. The process of invasion has a significant impact on the formation of recurrent tumors and tumor metastasis. It is known that SAPKs can promote tumor invasion and also FOXM1 has been associated with cancer cell invasion in various tumors. However, the exact mechanism of how FOXM1 regulates invasion has not been examined yet. Since both, SAPKs and FOXM1, trigger a variety of phenotypes, they are not suitable targets for adjuvant cancer therapy. Therefore, identification of targets acting downstream of SAPKs and FOXM1 and regulating tumor cell invasion is necessary in order to block cancer cell invasion efficiently.

The aim of this thesis is to investigate the exact mechanism of how FOXM1 regulates invasion in HNSCC cells and to find a general mechanism for FOXM1-mediated regulation of invasion. To achieve this goal three HNSCC lines, FaDu, SCC-25, and CAL-27, will be used to analyze the effects of pharmacological inhibition of p38 SAPK signaling and siRNA-mediated FOXM1 reduction on invasive behaviour of tumor cells in Matrigel-coated Boyden chambers. Reporter gene assays using full length and truncated versions of uPA promoter will help to study the role of FOXM1 in uPA gene regulation. Bioinformatic analysis of the publically available gene expression datasets will be employed to analyze the correlation between FOXM1 and uPA expression. To assess genome-wide nucleotide sequences of FOXM1 binding regions and to study binding of FOXM1 and c-Fos to the uPA promoter, the available FOXM1 ChIP-seq data will be analyzed and chromatin immunoprecipitation assays will be performed. The long-term goal of this study is to understand mechanisms responsible for recurrent tumor development and to identify new potential targets for adjuvant cancer therapy.

2 Material and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used in this study were of analytical grade. They were purchased from Carl Roth (Karlsruhe, Germany), Invitrogen (Karlsruhe, Germany), and Sigma (Munich, Germany).

2.1.2 Oligonucleotides

Name	Sequence/Company
FOXM1	HS_FOXM1_1_SG QuantiTect (QT00000140)
GAPDH	5'-CGCTCTCTGCTCCTCCTGTT-‘3 5'-CCATGGTGTCTGAGCGATGT-‘3
FOXM1	FOXM1 Hs01073586_m1 (Applied Biosystems)
uPA	PLAU Hs01547054_m1 (Applied Biosystems)
GAPDH	TaqMan GAPDH Control Reagents 402869 (Applied Biosystems)
ChIP Primer (B10)	5’-TCAGAGCCAACCTTGCTACTTCC-‘3
ChIP Primer (B11)	5’-CTTCAGAGCCAACCTTGCTACTTC-‘3
ChIP Primer (B12)	5’-GAGAGACTTCTGTGCTTGCTGAGC-‘3
ChIP Primer (B7)	5’-GGAGACTGGAGGACAAAATAA-‘3
ChIP Primer (F19)	5’-AACCTGGGAGTTTCGGGGTAA-‘3
ChIP Primer (F4)	5’-GGTTCAAAATGACCCCAAGCC-‘3

2.1.3 Inhibitors

Name	Company
uPA Inhibitor II, UK122	Calbiochem (672152-5MG)
P38 Inhibitor, SB203580	Sigma-Aldrich (S8307)
Polo-like Kinase Inhibitor II, BTO-1	Santa Cruz (sc-204206)
JNK Inhibitor, SP600125	Sigma-Aldrich (S5567)
Mitomycin C from S. caespitosus	Sigma-Aldrich (M0503)
Siomycin A from S. sioyaensis	Sigma-Aldrich (S6076)
Phorbol 12-myristate 13-acetate	Sigma-Aldrich (P1585)
Anisomycin	Sigma-Aldrich (A9789)

2.1.4 siRNA

Name	Sequence/Company
FOXM1 siRNA (h)	Santa Cruz (sc-43769)
FOXM1 siRNA (m)	Santa Cruz (sc-44877)
PLK-1 siRNA (m)	Santa Cruz (sc-43769)
c-Fos siRNA (h)	Santa Cruz (sc29221)
Control siRNA-A	Santa Cruz (sc-37007)
Neg. si Allstars siRNA AF	Qiagen (1027284)

2.1.5 Plasmids

Name	Characteristic trait	Reference/Company
pCAT3 Basic vector	TRE Basic vector	Promega (Germany)
5x TRE CAT	Ap-1 5xTRE reporter	Lengyel E et al., 1995
uPA^{2345}-CAT	wild-type uPA-CAT reporter	Verde et al., 1988
uPA^{2106}-CAT	uPA del.(2106)-CAT reporter	Verde et al., 1988
uPA^{1963}-CAT	uPA del.(1963)-CAT reporter	Verde et al., 1988
uPA^{1870}-CAT	uPA del.(1870)-CAT reporter	Verde et al., 1988
uPA^{1570}-CAT	uPA del.(1570)-CAT reporter	Verde et al., 1988
uPA^{660}-CAT	uPA del. (660)-CAT reporter	Verde et al., 1988
FOXM1 cDNA	FOXM1 expression plasmid	Origene (NM_021953)

2.1.6 Cell lines and bacterial strains

Name	Type	Supplier
NIH3T3	murine wild-type NIH-3T3 fibroblasts	Cell lines service (Heidelberg, Germany)
NIH3T3-*Ha-Ras*^{EJ}	NIH3T3 expressing mutated *Ha-ras*	Cell lines service (Heidelberg, Germany)
NIH3T3-*MKK3b(E)*	NIH3T3 expressing *MKK3*	Behren et al., 2009
SCC-4	human squamous cell carcinoma from the tongue	ATCC
SCC-25	human oral squamous carcinoma of the tongue	ATCC
SCC-9	human squamous carcinoma of the tongue	ATCC
SCC7	murine epithelial squamous cell carcinoma.	ATCC
FaDu	human hypopharyngeal carcinoma	ATCC
Cal-27	human oral adenosquamous carcinoma cell line	ATCC
A549	human adenocarcinomic alveolar basal epithelial cells	University of Cincinnati
E. coli NEB5alpha	chemically competent bacteria	Stratagene, La Jolla, USA

2.1.7 Antibodies

Name	Company	Species	Dilution
FOXM1 (C-20)	Santa Cruz (sc-502)	Rabbit polyclonal	1:2000
FOXM1 (K-19)	Santa Cruz (sc-500)	Rabbit polyclonal	1:1000
uPA	American Diagnostica (3689)	Mouse monoclonal	1:200
uPA (C-20)	Santa Cruz (sc-6830)	Goat polyclonal	1:200
uPA (H-140)	Santa Cruz (sc-14019)	Rabbit polyclonal	1:500
PAI-1 (H-135)	Santa Cruz (sc-8979)	Rabbit polyclonal	1:200
c-Fos	Abcam (ab7963)	Rabbit Polyclonal	1:500
p-c-Fos (T232)	Abcam (ab17933)	Rabbit polyclonal	1:500
p38	Cell Signaling (#9212S)	Rabbit Polyclonal	1:1000

p-p38 (Thr180/Tyr182)	Cell Signaling (#9211S)	Rabbit Polyclonal	1:200
JNK	Cell Signaling (#9252S)	Rabbit Polyclonal	1:500
p-JNK (Thr183/Tyr185)	Cell Signaling (#9251S)	Rabbit Polyclonal	1:250
β-actin (l-19)	Santa Cruz (sc-1616)	Rabbit polyclonal	1:1000
β-actin	Abcam (sb 8226)	Mouse monoclonal	1:5000
αTubulin (E 19)	Santa Cruz (sc-12462)	Goat polyclonal	1:2000
p-ATF2 (Thr71)	Cell Signaling (#9221)	Rabbit polyclonal	1:500
p-ATF2 (F1)	Santa Cruz (sc-8398)	Mouse monoclonal	1:500
c-Jun	Abcam (ab5795)	Rabbit polyclonal	1:1000
p-c-Jun (T93)	Abcam (ab28854)	Rabbit Polyclonal	1:250
PLK-1	Cell Signaling (#4535)	Rabbit polyclonal	1:500
PLK-1 (H-152)	Santa Cruz (sc-5585)	Rabbit polyclonal	1:1000
p-PLK-1 (Thr210)	Cell Signaling (#5472)	Rabbit Polyclonal	1:500
Cleaved-Caspase 3 (Asp175)	Cell Signaling (#9661)	Rabbit polyclonal	1:250
Goat Anti-Rabbit IgG H&L	Merck (401315)	Goat monoclonal	1:5000
Goat Anti-Mouse IgG H&L	Merck (401215)	Goat monoclonal	1:5000

2.2 Methods - Cell biology

2.2.1 Cell Culture Media and Supplements

Name	Company
DMEM	PAA, Cölbe, Germany
MEM	PAA, Cölbe, Germany
PBS	PAA, Cölbe, Germany
Antibiotic/Antimycotic	Invitrogen, Karlsruhe
Fetal Bovine Serum Standard Quality	PAA, Cölbe, Germany
L-Glutamine	Invitrogen, Karlsruhe
Trypsin	PAA, Cölbe, Germany
Optimem	Invitrogen, Karlsruhe
DMSO	PAA, Cölbe, Germany

2.2.2 Cell lines

Human HNSCC cell lines were purchased from ATCC. Cells were maintained in Dulbecco's modified Eagle's medium (SCC-25) or minimum essential medium (FaDu, CAL-27) supplemented with 10% fetal bovine serum (Invitrogen, Germany), 2mM L-Glutamine (Invitrogen, Germany) and antibiotics (50µg/ml Penicillin-Streptomycin, Invitrogen, Germany) in a humidified atmosphere of 6% CO_2 at 37°C. Wild-type NIH3T3 and NIH3T3 cells expressing mutated *Ha-Ras* were purchased from CLS (Cell-Lines-Service, Heidelberg, Germany) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. NIH3T3 cells expressing *MKK3b(E)* were constructed by transfection of NIH3T3 cells with linearized vector DNA and subsequent treatment with growth media (DMEM supplemented with 10% fetal calf serum) containing 750µg/ml G418. Following two weeks of treatment with selection media, the surviving cell colonies were harvested and pooled. These cell lines were continuously grown in selection media up to passage 15. The cell line SCC7 was cultured as described (Behren et al., 2010).

2.2.3 Transient transfections

In all cases cells were trypsinized 24 hours before transfection and seeded at a density of 2 x 10^5 cells per 6-well or 10cm dish. Transfection of siRNA into HNSCC cell lines was carried out using HiPerFect Transfection Reagent (Qiagen) or Lipofectamine™ 2000 (Invitrogen), while for transfections with expression plasmids Lipofectamine™ 2000 was used. All solutions and vectors were diluted Opti-MEM® I reduced Serum Media (Invitrogen). Prior to transfection, cell media was exchanged with antibiotic-free media containing 0.5% FCS, which was again replaced with normal growth media 4-6 hours after transfection. All transfections were performed according to manufacturers' optimized protocols at different doses as indicated for 48 hours before functional assays were carried out. Cell lines treated with transfection reagent alone or scrambled siRNA were included as mock controls. Transfection efficiency was measured by transfection with GFP-fused reporter plasmids.

2.2.4 Cell growth rate measurement

For determining the growth rate HNSCC cell lines cells were trypsinized and viable cells were identified by staining with trypan blue (Sigma-Aldrich, Germany). For quantification the number of viable and unstained cells was counted using a Neubauer counting chamber. Samples were counted in triplicate over a period of five days. Cell number $\pm$ standard deviation (s.d) versus days was plotted.

2.2.5 Invasion assays

For invasion assays 80.000 of the indicated cells were plated out in 500µl serum-free medium (SFM) in Matrigel-coated Boyden chambers (Beckton Dickinson, USA). The Boyden chambers were placed in 24-well companion plates with DMEM+ 10% fetal calf serum and incubated for 24-36 hours. The media contained the inhibitors UK122, SB203580, or dimethylsulfoxide (DMSO), or nothing. After the invasion process cells were stained with DMEM/MTT solution. Cells remaining inside the Matrigel were removed using cotton swabs, and the membrane now containing only the infiltrated cells was cut out. Cotton swabs as well as membranes were placed in DMSO and the solution was densitometrically analyzed at 562nm against DMSO. The percentage of invasive cells was calculated as percentage optical density of membrane-anchoraged cells versus overall optical density.

2.2.6 Freezing and thawing of cells

For freezing, cells were grown in cell T_{-75} culture flasks to approximately 90% cell density, washed twice in PBS and trypsinized. The cell suspension was centrifuged at 3000 rpm for five minutes and the pellet subsequently resuspended in freezing media (regular cell culture media containing 20% DMSO and 20% FBS). The cell suspension was then transferred to cryo-vials. Those were placed inside a cell freezer (Nunc, Germany) to allow a constant cooling rate of 1°C per minute and finally stored at -80°C or liquid nitrogen.

Frozen cells were thawed in a 37°C water bath for 2 minutes and transferred to petri dishes containing the appropriate culture medium. After 12-18 hours medium was exchanged to remove the rest of DMSO from the media.

2.2.7 Nuclear staining

For nuclear staining prior to microscopic investigation, cells were incubated with Hoechst-3342 Dye (Sigma-Aldrich, Munich, Germany) for 30 minutes at 30°C, 5% CO_2.

2.3 Methods - Biochemistry

2.3.1 Preparation of whole cell lysates

RIPA buffer
50 mM Tris-HCL, pH 7.4
1% NP-40
0.25% Na- deoxycholate
150 mM NaCl
1mM EDTA
add 100ml bidest. H_2O
prior to use, phosohatase- and protease inhibitor cocktail (Invitrogen, Germany) is added

Whole cell lysates were prepared by rinsing the cells twice in ice-cold PBS buffer. Cells were scrapped off in 1.5 ml ice-cold PBS into a 1.5ml Eppendorf tube and pelleted at 3000 rpm for five minutes at 4°C. The resulting pellet was resuspended in RIPA buffer, containing PMSF and a protease inhibitor cocktail. The suspension was kept on ice for 15-20 minutes with short vortexing each 5 minutes and was then centrifuged for 10 minutes at 13.000 rpm (4°C). The supernatant containing the protein solution was transferred into a new 1.5ml Eppendorf tube and stored at -20°C for short-term use.

2.3.2 Cell fractionation

Cell fractionation was done using the NE-PER® Nuclear and Cytoplasmatic Extraction Reagents Kit (Thermo Scientific, Germany). The volume ration of CERI:CERII:NER reagents was 200:11:100µl. Cells were washed twice in PBS and scraped off in 1ml PBS containing protease inhibitors. The cell solution was pelleted for 10 minutes at 3000 rpm at 4°C. Ice-cold CERI was added to the cell pellet (100µl per 10µl packed cell volume), vortexed at the highest setting for 15 second until the pellet was fully suspended and put on ice for 10 minutes. Ice-cold CERII was added to the tube, vortexed for 5 seconds at highest settings and incubated on ice for 1 minute. Cells were again vortexed for 5 seconds at highest settings and centrifuged at maximum speed (13.000 rpm, 4°C) for 5 minutes in a microcentrifuge. The supernatant, containing the cytoplasmatic extract, was immediately transferred to a new, pre-chilled, tube. The insoluble pellet was suspended in ice-cold NER buffer and vortexed at the highest setting for 15 seconds. The sample was then placed on ice for 40 minutes including vortexing for 15 seconds at the highest setting every 10 minutes. After the incubation the samples were centrifuged for 15 minutes at maximum speed (13.000 rpm, 4°C). The supernatant, containing the nuclear extract, was transferred to a new, pre-chilled, tube. Both cytoplasmatic, and nuclear extracts were stored at -80°C for further use.

2.3.3 Protein concentration measurement

Protein concentration was determined using the DC Protein Assay (Bio-Rad, Germany). For this purpose, 20µl of reagent S were added to 1ml of reagent A (=A1). 25µl A1 were mixed with 5µl protein lysate on a 96-well plate and subsequently 200 µl of reagent B was added to the mixture. The 96-well plate was incubated for 15 minutes at room temperature, prior to measuring the absorption at 595 nm.

2.3.4 Immunoprecipitation

For immunoprecipitation, Protein A Agarose (Pierce, Darmstadt, Germany) was used. Protein lysates were equalized in protein content and 30 µl of the 50% Agarose A slurry was added to 500µl of cell lysate. The solution was precleared for 30 minutes at 4°C under rotation. The solution was centrifuged for 5 minutes at 2500 rpm at 4°C. Subsequently the supernatant was

transferred to a new microcentrifuge tube. The Agarose pellet containing unspecific bound protein was discharged and 1µg of antibody was added to the solution and incubated over night at 4°C under gentle rotation. After the overnight incubation 37 µl of the Protein A Agarose slurry were added to the solution and incubated for 4 hours at 4°C under rotation. Immunoprecipitates were pelleted at 2500 rpm at 4°C for 5 minutes. The pellets were washed 4x with RIPA buffer. After the final wash the supernatant was discharged and the pellet resuspended in 60µl 2x sample buffer, boiled for 10 minutes and finally Agarose beads were pelleted by centrifugation. Samples were then analyzed using SDS-PAGE.

2.3.5 CAT-ELISA

Cells were seeded into 6-well plates and after 24 hours transfected with a chloramphenicol acetyltransferase (CAT) reporter construct fused to the wild-type or deleted fragments of the human urokinase promoter, or a 5xTRE-AP1 responsive element, together with the recommended amount of the desired expression plasmid or siRNA. For negative control, cells were transfected with the empty pCAT3 Basic vector. 48 hours after transfection cells were lysed and equalized for their protein amount and CAT activity was measured using the Colorimetric enzyme immunoassay for the quantitative determination of chloramphenicol acetyltransferase (CAT-ELISA) from Roche. The absorption was determined at 492nm using an Anthos 2010 multiplate reader (Anthos Mikrosysteme GmbH, Krefeld, Germany). All experiments were carried out as triplicates.

2.3.6 Western Blot

Solutions

<u>10X SDS Running Buffer</u>
144.0g Glycine
30.2g Tris
10.0g SDS
ad 1L H_2O
for 1X SDS Running Buffer the 10X stock was diluted 1/10 in bidest. water

1x Transfer Buffer
100ml 10X SDS Running Buffer
200ml Methanol
700ml H_2O

Washing Buffer (PBS-T)
1x PBS
0.005% Tween 20

Blocking Buffer
1x PBS-T
5% skim milk

Sample buffer (4X)

0.25M	Tris/HCL 6.9
8%	SDS
40%	Glycerol
20%	Mercaptoethanol
0,002%	Bromphenole blue

SDS PAGE

SDS PAGE was performed according to Laemmli (1970). Solutions needed were stored at 4°C until further use. Whole cell lysates were prepared by adding suitable amount of RIPA lysis buffer supplemented with a cocktail of protease and phosphatase inhibitors (Invitrogen, Germany) to the cell pellet. Equal amounts of proteins were suspended in SDS sample buffer and heated for 5 minutes at 95°C. The samples were put on ice for 5 minutes and then centrifuged for 10 minutes at maximum speed. 10-50µg protein lysate were loaded on a 10-12% SDS-Polyacrylamide Gel and run in a Gel running chamber (Bio-Rad, Munich, Germany) at 100 Volts for approximately 90 minutes. For protein standard the Precision Plus Protein™ ALL Blue Standard (#161-0343, Biorad, United-States) was used. Prior to use, the marker was heated at 95°C for 3 minutes and transferred immediately to ice after heating. 5µl marker was added to the SDS- Gel.

for one 10% gel	Resolving Gel (ml)	Stacking Gel (ml)
30% Acrylamide	5.000	1.000
1M Tris-HCL pH8	6.000	1.250
10% SDS	0.200	0.100
H_2O	3.800	7.600
APS	0.100	0.075
TEMED	0.010	0.015

Protein transfer

After the SDS PAGE, proteins were transferred from the gel to an Immobilon-P PVDF membrane (Milipore, Schwalbach, Germany) using a MiniProtean II Wet Blot Chamber (Bio-Rad, Munich, Germany). Prior to the transfer, filter papers and membranes were cut, equaling approximately the size of the gel and the membrane was activated in methanol for 10 seconds and subsequently in water for 10 seconds before being equilibrated in transfer buffer (0.2M Glycin, 0.025M Tris, 0.1% SDS). For blotting, sponges and filter papers were soaked in transfer buffer and everything was set up according to manufacturer's recommendation. Proteins were transferred for 60 minutes at 100 Volts, while the running chamber was kept cool by placing it into a 4°C room and the usage of ice-blocks.

Immunodetection of proteins

After the electrotransfer, membranes were blocked for 1 hour at room temperature in 5% blocking buffer (5% skim milk in PBST + 0.005% Tween 20). After blocking, the membrane was incubated with the primary antibody, which was diluted at the appropriate concentration in blocking solution, over night at 4°C under slow rotation.

After the incubation unbound antibody was removed from the membrane by washing it 3x 10 minutes in washing solution (PBST + 0.005% Tween 20). The membrane was then incubated for 1 hour at RT with the secondary antibody, diluted in blocking solution at the appropriate concentrations ranging from 1:4000 to 1:30.000. Unbound antibody was again removed by washing the membrane 3x10 minutes in washing buffer. The membrane was incubated with ECL Plus detection solution (GE Healthcare, Munich, Germany), which is based on chemoluminescent detection, for 5 minutes at room temperature. After the detection solution was squeezed out, Hyperfilm ECL films (GE Healthcare, Munich, Germany) were exposed to the membrane under red light in a darkroom for the appropriate time and subsequently developed in a HyperProcessor (GE Healthcare, Munich, Germany).

2.3.7 Immunohistostaining

Reagents

Peroxidase solution:
1:100 dilution of 30% H_2O_2 solution in water

Blocking solution:
0.1% BSA/PBS
0.2% Tween 20
10.0% Serum

Staining procedure for cryo sections

Frozen slides were taken out from -80°C and air dried for 30 minutes. Tissue slides were fixed using ice-cold (-20°C) acetone for 10 minutes and rinsed three times in PBS (5 minutes each time). The tissue area was encircled using a hydrophobic PAP pen and washed twice in PBS. To block endogenous peroxidase activity, slides were incubated for 10 minutes in 0.3% H_2O_2/H_2O under light protection and washed twice in PBS. To prevent unspecific labeling, tissue slides were incubated in blocking buffer for 30 minutes and subsequently washed twice in PBS. The primary antibody was incubated over night at 4°C in the appropriate dilution. After the incubation time, slides were washed three times in PBS and incubated with biotinylated secondary antibody of the same species as the primary antibody (1:200) for 30 minutes at room temperature and afterwards washed three times in PBS. The HRP-conjugated avidin-biotin complex (VECTASTAIN-ABC System) reagent (Vector Labs, United Kingdom), which was prepared exactly 45 minutes before use, was incubated for 30 minutes at room temperature under light protection. After washing three times in PBS, the antibody color was revealed using DAB Peroxidase substrate kit (Vector Labs, United Kingdom), until the desired color intensity was reached. The staining procedure was stopped by immersing the slides into tap water. Tissue slides were subsequently counterstained in haematoxylin solution for 1-2 minutes and washed briefly in distilled water. Cover slips were fixed using Mowiol solution and slides were examined under the light microscope.

Staining procedure for paraffin sections

For paraffin sections, samples were deparaffinised by incubating them twice in Xylol for 10 minutes. For hydration samples were washed subsequently in EtOH 95%, 90%, 80%, 70%, 50%, 30% and rinsed twice in water for 2 minutes each. For antigen retrieval, slides were immersed in a water bath containing sodium citrate pH 6.0 at 95°C for 20 minutes. Subsequently, the slides were removed from the water bath and placed at room temperature allowing them to cool for 20 minutes. The tissue area was encircled using a hydrophobic PAP pen and the following steps were performed as for cryo sections.

2.3.8 uPA activity Assay

Cells were cultured in a six-well plate and transfected or stimulated with the indicated constructs, plasmids, and solutions. 48 hours after transfection/ stimulation the uPA activity in the 1:20-1:100 diluted supernatant was measured using the IMUBIND® uPA ELISA (No. 894) from American Diagnostica according to manufacturers' protocols.

2.4 Methods - Molecular Biology

2.4.1 RNA Isolation

Total RNA from cultured cells was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

2.4.2 Quantification of RNA and DNA samples

The amount of DNA and RNA present in the samples was determined using a NanoDrop Biophotometer (PeqLab, Biotechnologie, Erlangen, Germany).

2.4.3 Reverse Transcription (RT)- PCR

For reverse transcription the amount of RNA samples to compare was equalized with RNase-free water. RT-PCR was carried out using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol using the supplied reagents.

2.4.4 Quantitative End-point PCR

Quantitative End-point PCR was carried out as described using the below depicted protocol and cycle numbers as appropriate for the respective gene.

Reagent	Amount in µl
10x Buffer	2.5 µl
10mM dNTPs	1 µl
25mM $MgCl_2$	1 µl
Taq Polymerase	1 µl
10mM forward Primer	1 µl
10mM reverse Primer	1 µl
H_2O	15.5 µl
DNA (2µg)	2 µl
Total	**25 µl**

Step	Temperature in °C	Time (minutes)	
1	94	2:00	
2	94	0:30	25-36 cycles
3	AT*	0:30	
4	72	0:45	
5	72	4:00	
6	4	∞	

*= annealing temperature

2.4.5 RQ-PCR

For RQ-PCR, cDNA was prepared from the total RNA extracts (1µg) by using the High Capacity RNA-to-cDNA Kit from Applied Biosystems. The amount of each gene was determined using the TaqMan® Gene Expression Assay from Applied Biosystems. All reactions were run in triplicate and the number of copies of each gene was normalized with the expression of the housekeeping gene GAPDH.

2.4.6 DNA Amplification

DNA was amplified using a Bio-Rad Mini Thermocycler (Bio-Rad, Munich, Germany) and Taq Polymerase (New England Biolabs, Frankfurt/Main, Germany) with supplied buffer according to the manufactures' recommendations and dNTPs purchased from Fermentas. Amplification was carried out according to the protocol described below:

Step	Temperature in °C	Time (minutes)	
1	94	5:00	
2	94	0:30	
3	AT*	0:30	25-36 cycles
4	72	0:30	
5	72	3:00	
6	4	∞	

*= annealing temperature

2.4.7 Agarose Gel Electrophoresis

Gel electrophoresis was carried out in horizontal electrophoresis chambers (Bio-Rad, Munich, Germany). 1% Agarose was prepared by dissolving Ultrapure Agarose (Invitrogen, Karlsruhe, Germany) in 1x Tris-acetate-EDTA (TAE) buffer and adding 10µl of ethidium bromide (1mg/ml) to each 150ml agarose solution. 10x loading buffer was added to the DNA samples for subsequent electrophoresis at 150V for 30 minutes depending on DNA size and gel concentration. After gel electrophoresis, DNA fragments were visualized under UV light (302 nm) and photographed.

2.4.8 ChIP-Assay

Cell isolation and crosslinking

Cells were cultured until reaching a density of 60%. Histones were crosslinked to the DNA by adding 37% Formaldehyde to 15ml of culture medium to a final concentration of 1% and incubation for 10 minutes under slow agitation. Crosslinking was stopped by adding Glycine to a final concentration of 0.125M and incubating for 5 minutes under slow agitation. Cells were washed 3x with PBS and scraped into a 1.5ml microcentrifuge with 1.5ml ice-cold PBS, supplemented with a protease inhibitor cocktail. Cells were pelleted at 2000 rpm for 5 minutes at 4°C. The cell pellet was then resuspended in 500µl RIPA buffer and incubated on ice for 15 minutes. Cells were lysed by causing mechanical disruption (15 strokes with a needle) and aliquoted into 2ml graduated canonical tubes (Fisherbrant, Houston, USA).

Sonification

Cell lysates were sonified in water using a sonificator machine (Misonix Inc., USA) with the following settings:

Sample	Amplitude	Pulse ON (sek.)	Pulse OFF (sek.)	Total ON (min.)
Control	0	0	0	0
Sample	30	30	30	08:00

For checking the efficiency of sonification 150µl H_2O, 8µl NaCl, 1µl 20µg/µl Proteinase K and 1µl of RNAse A were added to the sonified cell lysates and incubated over night at 65°C for crosslinking reversal. After the incubation, DNA was isolated using the DNA Isolation Kit (Qiagen, Düsseldorf, Germany). 10µl DNA were mixed with 1x DNA loading buffer and loaded on a 1% agarose gel.

Immunoprecipitation

Protein samples were centrifuged for 10 minutes at 13.000 rpm at 4°C. 100µl of the sonificated cell supernatant was diluted in 900µl of ChIP dilution buffer. The supernatant was precleared using 37µl Salmon Sperm DNA/Protein A Agarose Slurry-50% for 30 minutes at 4°C under

rotation. The pellet was centrifuged briefly at 2000 rpm at 4°C and discharged. 2µg antibody was added to the supernatant and incubated over night at 4°C under slow agitation.
After the overnight incubation, 30µl Salmon Sperm DNA/Protein A Agarose Slurry-50% were added to the protein-antibody solution and incubated for 4 hours at 4°C under slow agitation. The agarose was pelleted at 2000 rpm for 5 minutes at 4°C and the supernatant containing unbound, non-specific DNA was carefully removed. The Protein A agarose/ antibody/ histone complex was washed for 5 minutes with 0.5ml of the following buffers in the order as given below under agitation:

1. Low Salt Immune Complex Wash buffer, 1x wash, 4°C
2. High Salt Immune Complex Wash buffer, 1x wash, 4°C
3. LiCl Salt Immune Complex Wash buffer, 1x wash, 4°C
4. 1x TE Buffer, 2x wash, RT

100µl TE buffer containing 200mM NaCl, 0.1mg/ml Proteinase K was added to the Protein A agarose/ antibody/ histone complex and incubated at 65°C overnight. The next day the solution was centrifuged for 5 minutes at 13.000 rpm and the pellet was discharged. DNA was recovered by using the Qiagen purification kit and eluted in 50µl elution buffer.

PCR reaction

PCR was performed using 2.5µl of DNA and included 1/10 of the starting material as a positive loading control. The following PCR settings have been used

Master-Mix:

Reagent	Amount in µl
10x Buffer	2.5 µl
10mM dNTPs	1 µl
25mM $MgCl_2$	1 µl
Primer forward	1 µl
Primer reverse	1 µl
Taq Polymerase	1 µl
H_2O	15.5 µl
DNA	2 µl
Total	**25 µl**

PCR machine settings:

Step	Temperature in °C	Time (in minutes)
1	94	2 min
2	94	30 sec
3	AT*	45 sec (25-38x)
4	72	30 sec
5	72	5 min
6	4	∞

*= annealing temperature

2.4.9 Transformation of Bacteria

Preparation of chemically competent cells

LB medium:
10g Bacto-Tryptone
5g Bacto-yeast extract
10g NaCl
for LB-Agar 15g agar was added
LB media was adjusted to pH 7.5 with NaOH and sterilized by autoclaving

TFB I:

Reagent	Stock solution	In 200 ml
30 mM potassium acetate	0.5 M	12 ml
50 mM mangane chloride	1.0 M	10 ml
100 mM rubidium chloride	0.5 M	40 ml
10 mM calcium chloride	0.5 M	04 ml
15% glycerol		30 ml
H_2O bidest.		104ml

Adjust to pH 5.8, Store at 4°C

TFB II:

Reagent	Stock solution	In 200 ml
10 mM NaMOPS	0.1 M	5.0 ml
75 mM calcium chloride	0.5 M	7.5 ml
10 mM rubidium chloride	0.1 M	1.0 ml
15% glycerol		7.5 ml
H_2O bidest.		29 ml

Adjust to pH 5.8, Store at 4°C

2ml LB medium were inoculated with a single *E.coli* NEB5α- colony and incubated overnight on a rotary shaker (200 rpm). Two 5ml tubes with LB were each inoculated with 50µl of the overnight culture at 37°C and 200 rpm. At an OD^{600} of 0.8 each culture was added to a new flask containing 100ml pre-warmed LB-media. This main culture was incubated on a rotary shaker until it reached an OD^{600} of 0.5, subsequently transferred to sterile and chilled 50ml centrifuge tubes and kept on ice for 5 minutes. After centrifuging at 4000 rpm at 4°C for 5 minutes, the supernatant was decanted and tubes were placed back on ice. 10ml of TFBI was added to each of the tubes and cells were resuspended before centrifuging under the previously mentioned conditions. The supernatant was again decanted, the cells were carefully resuspended in 2ml TFBII and aliquoted into 50ml fractions which were shock-frozen in liquid nitrogen and stored at -80°C until further use.

Transformation of cells

E.coli NEB5α cells were thawed on ice. 0.8 µl β-mercaptoethanol was added to 100µl of bacteria suspension. The tubes were swirled gently and incubated on ice for 10 minutes with swirling every 2 minutes. Subsequently 0.1-50ng DNA was added to one cell aliquot. Tubes were swirled gently and incubated on ice for 30 minutes. Next, the tubes were heat-pulsed in a 42°C water bath for exactly 45 seconds and incubated on ice for 2 minutes. 0.9ml pre-warmed LB media was added and incubated for 1 hour at 37°C under shaking at 225-250 rpm. 100µl of the bacteria solution was plated on LB agar containing the desired selection-antibiotic.

2.4.10 Plasmid Purification

Plasmid purification was carried out using either the QIAprep Spin Miniprep Kit or the QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

2.4.11 Isolation of genomic DNA from cells

200 µl Phenol/Chloroform is added to 200µl of sample, vortexed and centrifuged for 5 minutes at maximum speed. The upper aqueous phase, containing the DNA, is taken out into a new Eppendorf microcentrifuge tube, mixed with 30µl of 2M sodium acetate (pH 5.2) and two volumes of 100% Ethanol. Samples were incubated for one hour at -20°C and centrifuged for 15-30 minutes at 4°C at maximum speed. After centrifugation the pellet was washed twice in 70% Ethanol and air-dried. The pellet was finally resuspended in an appropriate amount of water.

2.4.12 Bioinformatics

Genomic positions of FOXM1 ChIP-Seq peaks from the human ECC-1, SK-N-SH, MCF7, GM12878 and MDA-MB231 cell lines were retrieved from the ENCODE TF Binding track (http://genome.ucsc.edu/ENCODE/index.html) and GEO database (GSE40762 dataset). Intersection of FOXM1 peaks from the replicate experiments was calculated using GALAXY server. Overlap larger than 200 nucleotides was used as a threshold. Web-based CentDist program was used to identify the enriched TRANSFAC motifs within FOXM1 ChIP-Seq peaks and to plot their distribution. Gene expression datasets including lung (GSE19188 and GSE14814), cervix (GSE7803), esophagus (GSE20347) and oral (GSE30784 and GSE31056) tumor samples and the respective normal tissue samples were downloaded from GEO database and analyzed using web-based O-Miner program (Cancer Bioinformatics Group at the Barts Cancer Institute) or Bioconductor tools (http://www.bioconductor.org/). Statistical calculation and plotting was done using R (http://www.R-project.org/).

2.5 Statistics

The experimental results presented in the figures are representatives of at least three or more observations. For each experimental data point the SEM from triplicate experiments was calculated as noted in the legends and is shown as error bars using the Microsoft Excel or Graph Pad Prism program. The significance of the *in-vitro* data was determined using the Students *t* test (2-tailed). P values of <0.05 were considered statistically significant.

3 Results

3.1 Regulation of FOXM1 expression by stress activated protein kinases

3.1.1 Regulation of FOXM1 by p38 and Ha-*Ras* in mouse fibroblasts

Ha-Ras has been shown to trigger invasion via the activation of downstream mitogen-activated protein kinases (MAPK) and stress-activated protein kinases (SAPK) (Behren et al., 2010). Fibroblasts, the major cell type in the stroma surrounding the tumor tissue, play an important role in cancer progression (Tyan et al., 2011). To determine the influence of p38 and Ha-Ras on FOXM1 expression in mouse fibroblasts, protein samples from wild-type NIH3T3 cells with constitutively activated and overexpressed Ha-Ras^{EJ} or MKK3^{act} (Figure **11**) were examined for their FOXM1 expression.

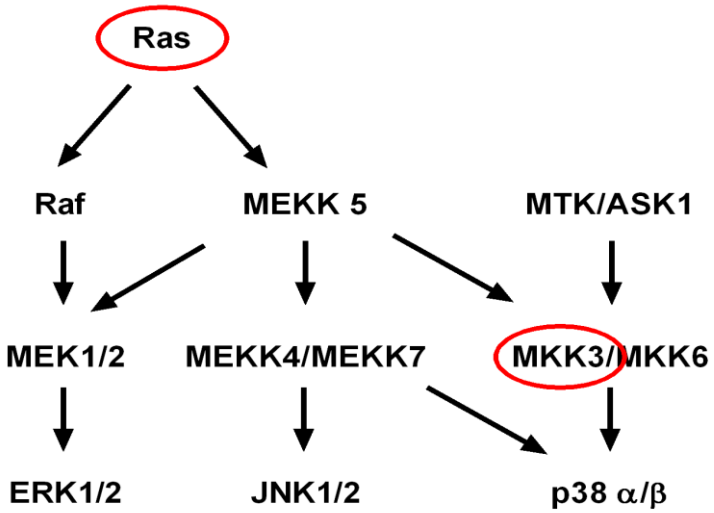


Figure 11: Scheme for Ha-Ras^{EJ}/-MKK3^{act} - activation in NIH3T3 mouse fibroblasts. Ras and MKK3 (red circles) were constitutively activated by overexpression in mouse fibroblasts, where they trigger further downstream kinases (*after Rincon M et al., 2000*).

After isolation of whole cell protein lysates western blot analysis revealed that Ha-Ras^{EJ}-overexpression significantly increases FOXM1 protein expression and elevates level of phospho-p38. MKK3^{act}-expressing mouse fibroblasts had elevated FOXM1 protein levels as well, though the increase was weaker compared to the Ha-Ras^{EJ}-transfected cells (Figure **12**).

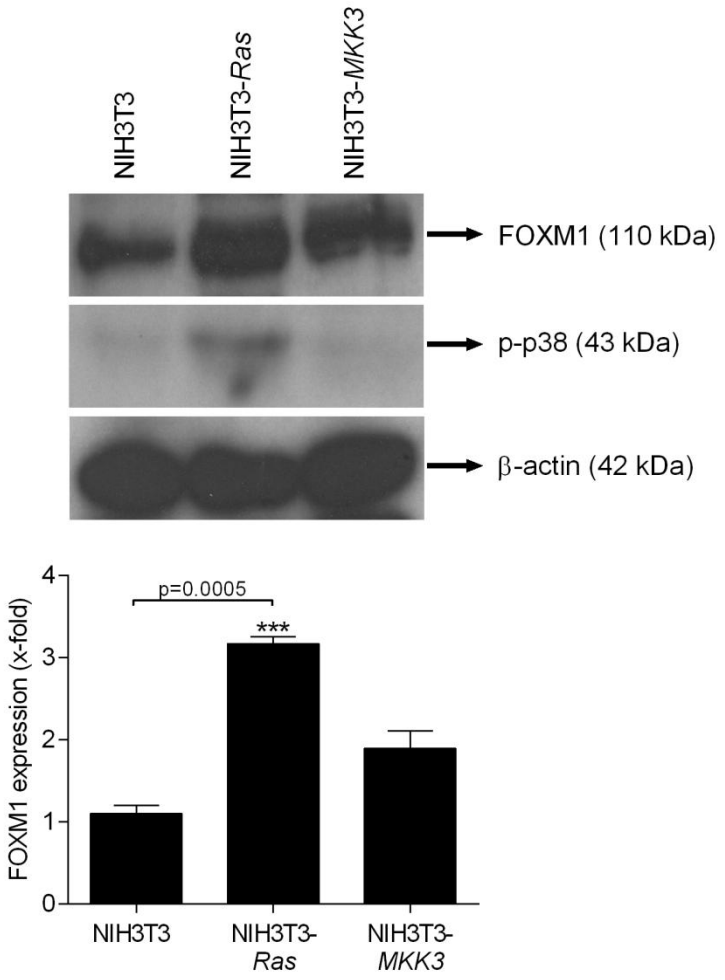


Figure 12: p38 and Ha-Ras regulate FOXM1 expression in mouse fibroblasts. Whole protein lysates were isolated from Ha-Ras^{EJ}- and MKK3^{act}-activated NIH3T3 mouse fibroblasts and analyzed for FOXM1 protein expression and p38 phosphorylation levels. FOXM1 protein expression was normalized to the β-actin expression levels. Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

In order to ensure that the significant, Ha-Ras^{EJ}-mediated, elevation of FOXM1 protein level also was dependent on p38, the activity of p38 was blocked in Ha-Ras^{EJ} expressing mouse

fibroblasts, using the chemical p38-inhibitor SB203580. Subsequently, total mRNA was isolated and FOXM1 expression levels were measured by performing a real-time qPCR. As expected, FOXM1 mRNA levels were 2-fold elevated in Ha-Ras^{EJ} expressing mouse fibroblasts compared to wild-type NIH3T3 cells. This increase of FOXM1 mRNA levels in Ha-Ras^{EJ}-expressing mouse fibroblasts could be blocked significantly using the p38 inhibitor SB203580 (Figure **13**). These data show that in mouse fibroblasts Ha-Ras and, to a less extent, p38 increase FOXM1 expression. Furthermore, Ha-Ras induced FOXM1 expression depends on the activity of p38.

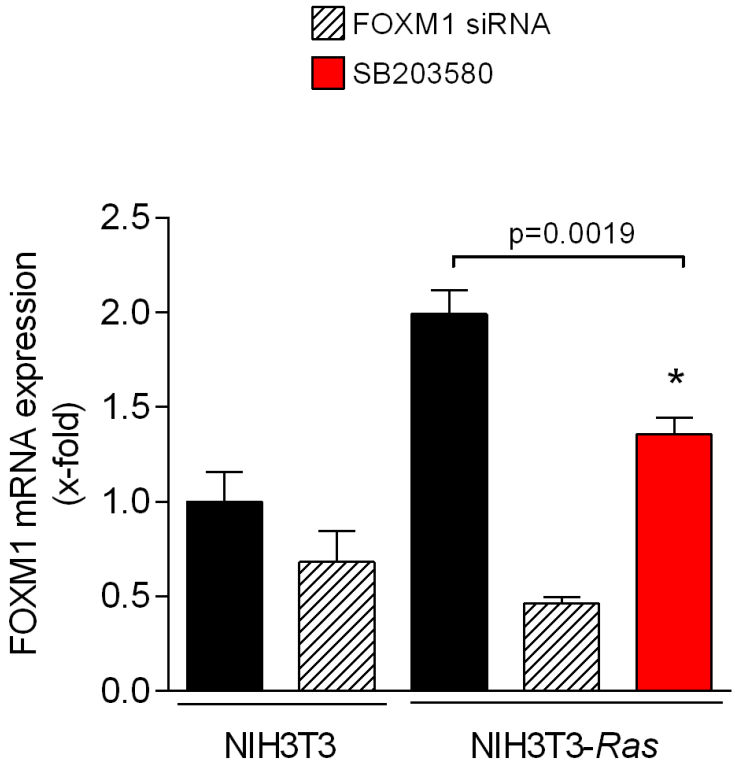


Figure 13: Ha-Ras induced FOXM1 expression is partially dependent on p38. Wild-type NIH3T3 and Ha-Ras^{EJ} activated NIH3T3 cells were incubated with SB203580 or treated with FOXM1 siRNA. mRNA was isolated and FOXM1 expression levels were quantified in a real-time qPCR. mRNA levels were normalized using the housekeeping gene GAPDH. Statistics were done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

3.1.2 The role of FOXM1 in p38-mediated *in vitro* invasion of mouse fibroblasts

It is well established that p38 is able to induce *in vitro* invasion of mouse fibroblasts (Behren et al., 2005). As shown in Figure 1, p38 regulates FOXM1 expression in mouse fibroblasts. Therefore, we aimed to examine if FOXM1 is involved in p38-induced *in vitro* invasion of fibroblast cells. The invasion of NIH3T3 and NIH3T3-MKK3^{act} cells was analyzed using Matrigel-coated Boyden chambers. As expected, NIH3T3-MKK3^{act} cells displayed a 1.5-fold higher invasion rate compared to wild-type NIH3T3 cells. Subsequently, FOXM1 was blocked in NIH3T3-MKK3^{act} cells by incubating them with different concentrations of the FOXM1 inhibitor Siomycin A, during the invasion process. Upon inhibition of FOXM1, the invasion of NIH3T3-MKK3^{act} cells was significantly decreased, almost to the levels of wild-type NIH3T3 cells (Figure **14**). This demonstrates that in NIH3T3 mouse fibroblasts FOXM1 is involved in p38-mediated *in vitro* invasion.

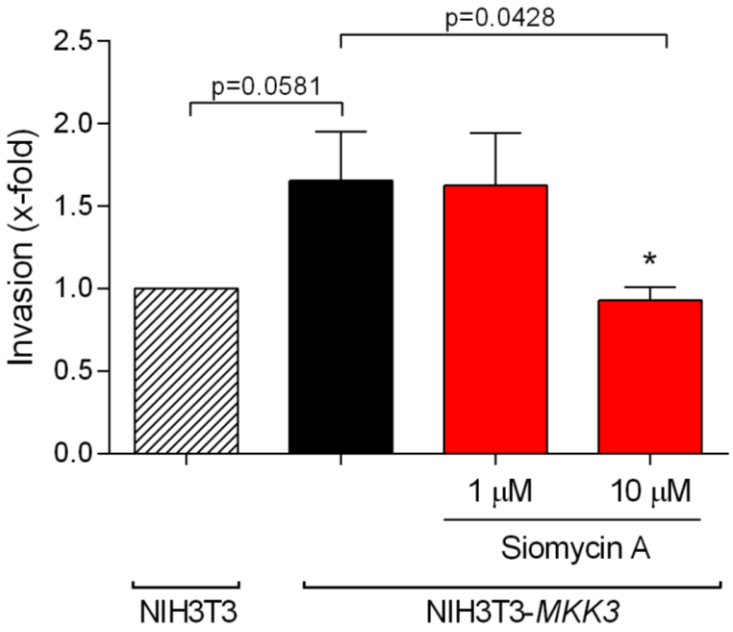


Figure 14: FOXM1 is necessary for p38-mediated *in vitro* invasion. Invasion of wild-type NIH3T3 and NIH3T3-MKK3^{act} cells was measured using Matrigel-coated Boyden chambers. FOXM1 expression in NIH3T3-MKK3^{act} cells was blocked by incubation with different concentrations of Siomycin A, prior to the invasion assay. Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

3.1.3 Regulation of FOXM1 expression by SAPKs in the epithelial SCC7 cell line

To show that FOXM1 is regulated by stress-activated protein kinases (SAPK) in the epithelial SCC7 tumor cell line, which later was used for generation of the animal model for oral cancer, experiments were performed using reversible, ATP-competitive inhibitors of SAPKs. The inhibitors SB203580 and SP600125 already have been shown to block the activity of p38 (Young et al., 1997) and JNK (Brydon et al., 2001). To ensure, that these inhibitors effectively target the respective kinases in our experiments, western blots were performed to examine their influence on phosphorylation of the SAPK downstream targets c-Jun and ATF-2. To examine the effectiveness of the p38 inhibitor SB203580, which in further experiments would be used in the mouse model of oral cancer, SCC7 cells were treated with the p38 inhibitor SB203580 (10µM) for 1 hour and subsequently incubated with 10µg/µl of the SAPK activator Anisomycin for 15 minutes. As western blots results show, Anisomycin-induced p38 activity in SCC7 cells could be efficiently blocked by SB203580 (Figure **15a**).

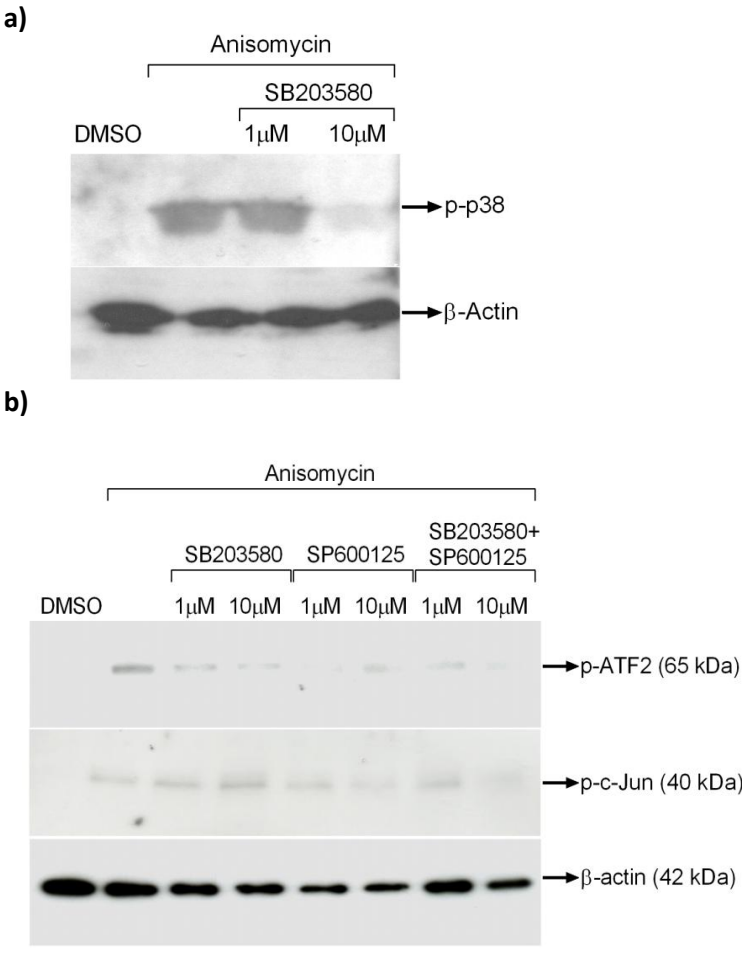


Figure 15: The SAPK inhibitors influence the activity of p38/ JNK and their downstream targets. SCC7 cells were treated with 1µM and 10µM of SB203580 for one hour and subsequently incubated for 15 minutes with Anisomycin (10µg/µl). Protein lysates were isolated and levels of phosphorylated p38 were detected in a western blot. β-actin expression indicates equal loading (a). SCC7 cells were treated with 1µM and 10µM of SB203580 and SP600125 respectively for one hour and subsequently incubated for 15 minutes with Anisomycin (10µg/µl). Protein lysates were isolated and the phosphorylation status of c-Jun and ATF-2 was detected in a western blot (b).

To further analyze the influence of the SAPK inhibitors on downstream targets of p38 and also JNK, SCC7 cells were incubated with SB203580 and SP600125 for 1 hour at different concentrations (1µM, 10µM). The cells were subsequently incubated with Anisomycin for 15 minutes and the effect on phosphorylation of the p38/JNK downstream targets ATF2 and c-Jun was measured in a western blot. SB203580 efficiently blocked phosphorylation of the p38 downstream target ATF-2, while SP600125 inhibited the phosphorylation of c-Jun upon stress induction (Figure **15b**). Thus, both SAPK inhibitors block p38/JNK and their downstream targets efficiently and can be used for functional analysis of the two kinases.

Since the specificity of the SAPK inhibitors was ensured, they were applied on epithelial SCC7 mouse tumor cell line to examine if SAPKs have an influence on the expression of FOXM1. SCC7 cells were incubated with 10µM of SB203580 and SP600125 for 24h and 48h. Whole cell lysates were isolated and FOXM1 protein levels were measured in a western blot. FOXM1 protein levels were significantly decreased after stimulation with both inhibitors. Upon p38 inhibition, FOXM1 levels were 40% decreased after already 24 hours and 50% after 48 hours as compared to the vehicle treated samples. Using the JNK inhibitor, no changes in FOXM1 expression could be observed after 24 hours. After 48 hours FOXM1 levels were decreased for 60% (Figure **16**).

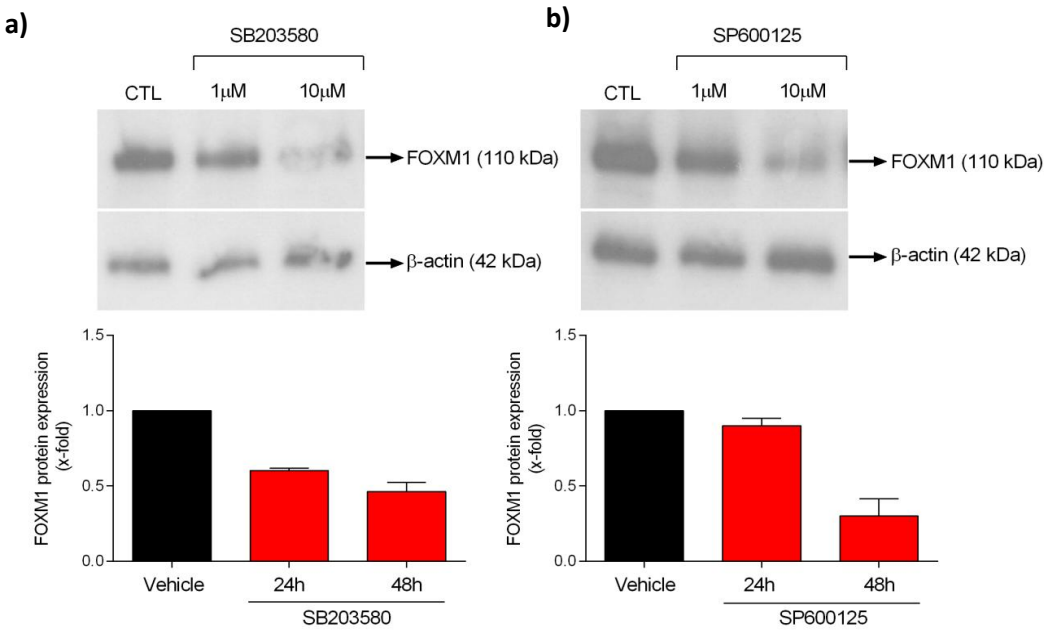


Figure 16: p38 and JNK regulate FOXM1 protein expression in SCC7 cells. SCC7 cells were treated with 10µM of SB203580 (a) and SP600125 (b) for 24 and 48 hours, respectively. Subsequently, protein lysates were isolated and FOXM1 protein expression was detected in a western blot. Results from three different western blots were quantified and plotted as bars. Bars show mean values ± SD from three independent experiments in triplicates.

To confirm the results on mRNA level, total mRNA from SCC7 cells, treated with both SAPK inhibitors for 48 hours, was collected and analyzed for FOXM1 expression in a semi-quantitative PCR reaction. FOXM1 mRNA levels were slightly decreased after 48 hours for both inhibitors, indicating that in SCC7 cell FOXM1 is regulated by SAPKs rather through protein stabilization than mRNA transcription (Figure **17**).

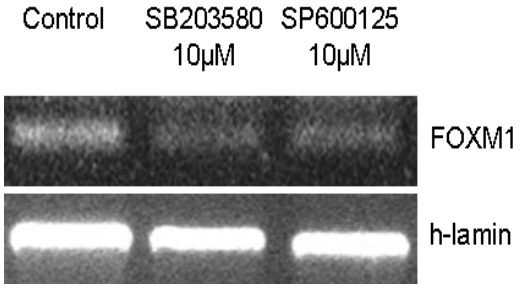
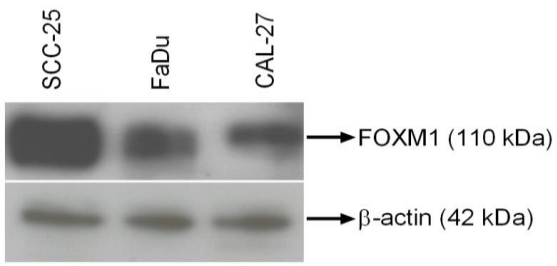


Figure 17: p38 and JNK regulate FOXM1 mRNA expression in SCC7 cells. SCC7 cells were treated with 10µM of SB203580 and SP600125 for 48 hours, respectively. Subsequently, mRNA was isolated and FOXM1 mRNA expression was detected in a PCR reaction. mRNA levels were equalized for h-lamin expression.

3.1.4 Regulation of FOXM1 expression by SAPKs in human HNSCC cells

In order to confirm the regulation of FOXM1 by SAPKs in human tumor cell lines, three human head and neck squamous cell carcinoma (HNSCC) cell lines (SCC-25, FaDu, CAL-27) were tested. Analysis of FOXM1 protein expression in these cell lines by western blot revealed that FOXM1 is differentially expressed on the protein level. Real-time qPCR analysis confirmed the differences observed on the protein levels also on the mRNA level (Figure **18**).

a)



b)

Cell line	Mean Ct. value	Cell line vs. Cell line	Ct.- difference	Devariation
FaDu	24.721	FaDu : CAL-27	-0.165	0.407
CAL-27	24.886	FaDu : SCC-25	1.445	0.087
SCC-25	23.275	CAL-27 : SCC-25	1.6211	0.087

Figure 18: FOXM1 is differentially expressed in human HNSCC cell lines. Protein and mRNA samples from three human HNSCC cell lines were isolated and examined for FOXM1 expression by western blotting (a) and real-time qPCR (b).

To test if FOXM1 expression correlates with the expression and phosphorylation status of p38 and JNK in the three human HNSCC cell lines, western blots for p38, JNK and their activated, phosphorylated forms were performed and compared with FOXM1 expression. p38 and JNK displayed differences in their activation status in the three HNSCC cell lines. SCC-25, which is featured by the highest FOXM1 expression, had high level of activated p38 and almost no detectable phospho-JNK. The two other cell lines, FaDu and CAL-27, which both expressed lower levels of FOXM1 had much lower p38 activity, but higher JNK activity. The expression levels of total p38 and total JNK were similar among all three cell lines (Figure **19a**).

The activity of SAPKs is rather determined by their phosphorylation status than by their expression levels (Wagner E F et al., 2009). Our gained data show that in human HNSCC cell lines FOXM1 expression directly correlates with the phosphorylation status and thus with the activity of p38, while the correlation with active JNK is inverse (Figure **19b**).

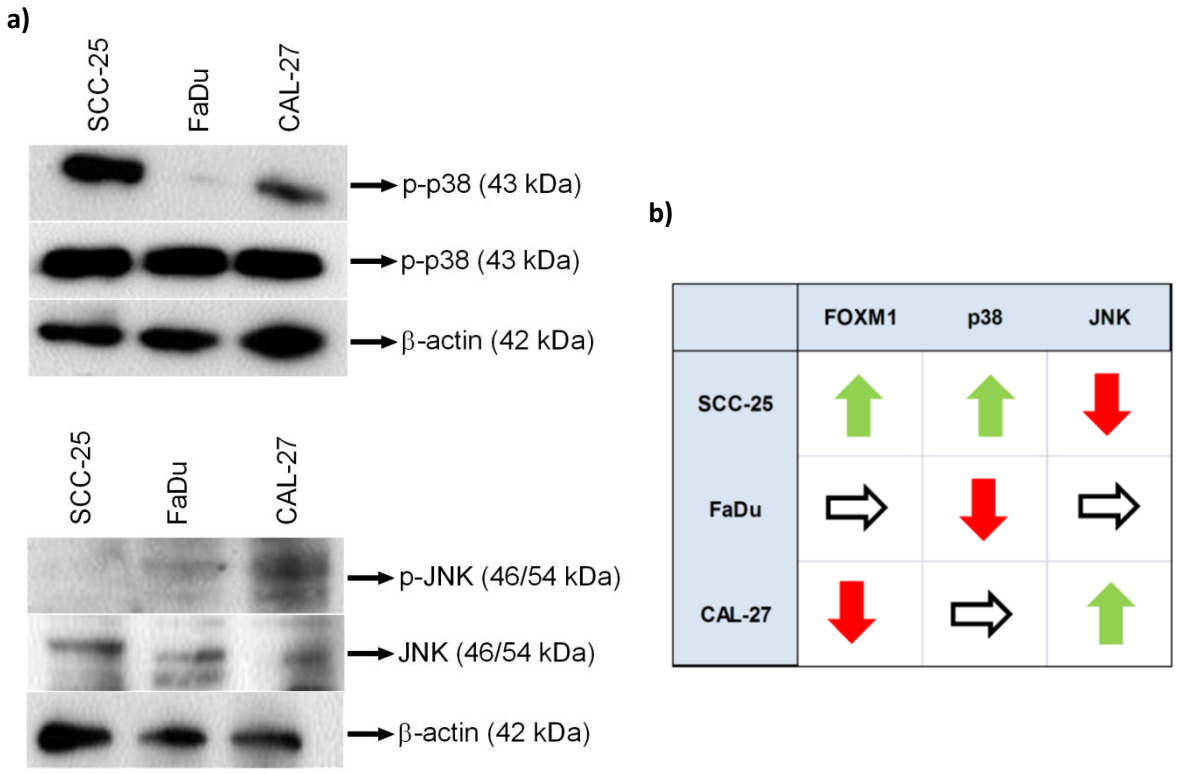


Figure 19: Expression and activity of SAPKs in human HNSCC cells. Protein samples from three human HNSCC cell lines were prepared and analyzed for the expression and phosphorylation status of the SAPKs p38 and JNK in a western blot experiment (a). Comparing the western blot data of FOXM1 expression and SAPK phosphorylation status in HNSCC cells, the expression of FOXM1 positively correlates with activity of p38 and negatively correlates with the activity of JNK (b).

To test in a functional analysis that FOXM1 expression indeed is dependent on p38, human HNSCC cell lines expressing detectable levels of FOXM1 were treated with the p38 inhibitor SB203580 for 24 and 48 hours, respectively. mRNA and protein samples were subsequently isolated and analyzed for FOXM1 expression by western blotting and real-time qPCR. Western blot data shows that inhibition of p38 in human HNSCC cells has no influence on the FOXM1 protein expression after 24h. After 48h, however, FOXM1 levels were decreased significantly in both cell lines tested in the experiment (Figure **20**).

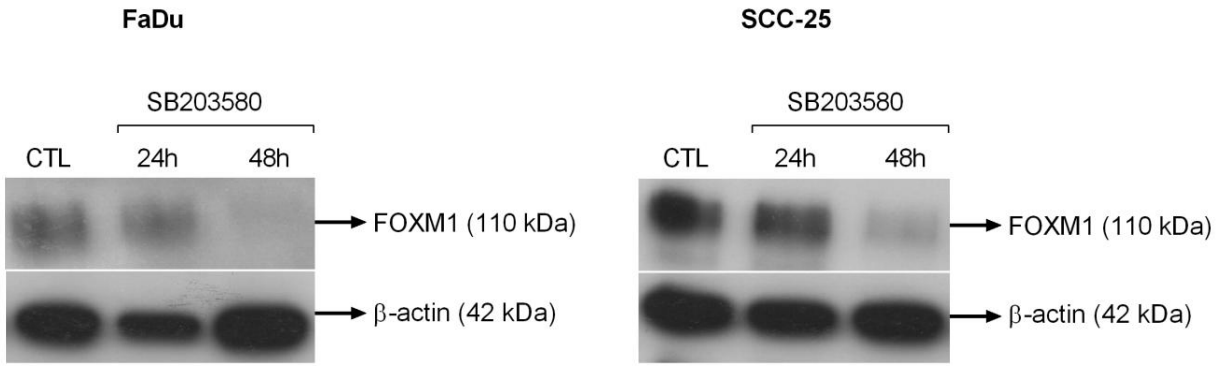


Figure 20: Influence of p38 on FOXM1 protein levels in human HNSCC cells. SCC-25 and FaDu cells were treated with the p38 inhibitor SB203580 for 24 and 48 hours. Protein samples were isolated and FOXM1 expression was measured in a western blot. β-actin expression served as loading control.

Results from the real-time qPCR analysis show that FOXM1 expression is also reduced on mRNA levels upon inhibition of p38 after 48 hours. In SCC-25 cells FOXM1 mRNA level was almost 40% lower compared to the DMSO-treated control cells. In FaDu cells FOXM1 mRNA level was significantly reduced down to 40% of the control expression level after 48 hours (Figure **21**). The effects observed in the real-time qPCR analysis are not as significant as on protein level. Similar to the SCC7 mouse tumor cells, these data suggest that expression of FOXM1 is rather regulated through protein stabilization than on the mRNA level.

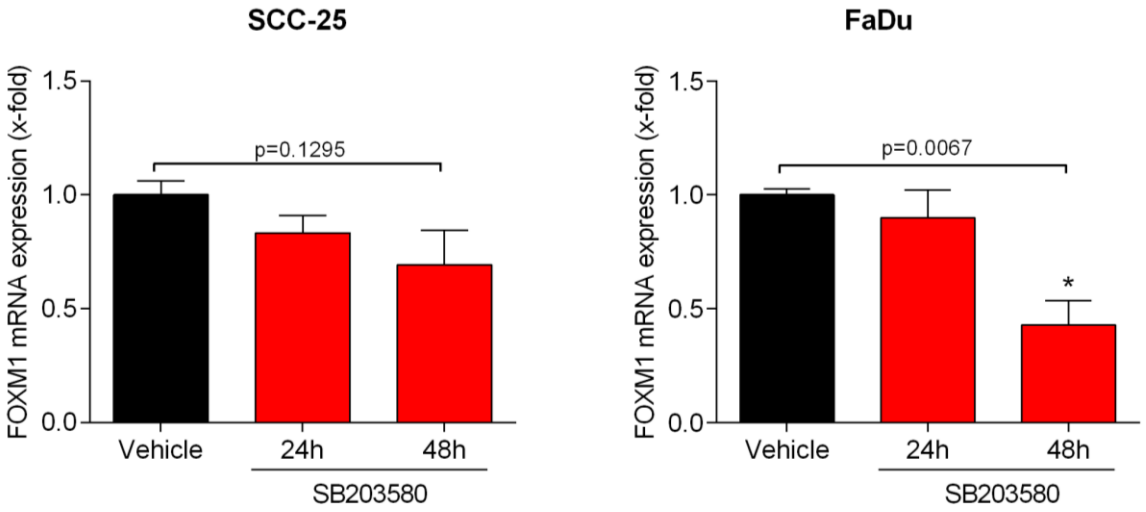


Figure 21: Influence of p38 on FOXM1 mRNA levels in human HNSCC cells. SCC-25 and FaDu cells were treated with the p38 inhibitor SB203580 for 24 and 48 hours. mRNA samples were isolated and FOXM1 protein expression was measured in a real-time qPCR. Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

The gained data indicate that FOXM1 expression correlates with the activity of p38 in human HNSCC cell lines and that p38 partially mediates the induction of FOXM1 expression by oncogenic Ha-Ras. Further, functional analysis reveals that FOXM1 expression is regulated by p38 in mouse SCC7 cells and human HNSCC cell lines on protein and, less significant, on mRNA levels. FOXM1 is further involved in p38-mediated invasion of mouse fibroblasts. After the establishment of a regulatory link between p38 and FOXM1 expression in human HNSCC cells it has to be examined if FOXM1 has the ability to regulate invasion of squamous head and neck cancer cells and thus might contribute to the development of recurrent tumors.

3.2 Influence of FOXM1 on invasion of HNSCC cells

3.2.1 Correlation of FOXM1 expression with invasiveness of HNSCC

It has recently been demonstrated that p38 can induce invasion of head and neck squamous carcinoma cells (Juntilla et al., 2007). Since we have now shown that FOXM1 is a downstream target of p38 in head and neck squamous cancer cells, we aimed to examine if FOXM1 plays a role in invasive processes of those cells.

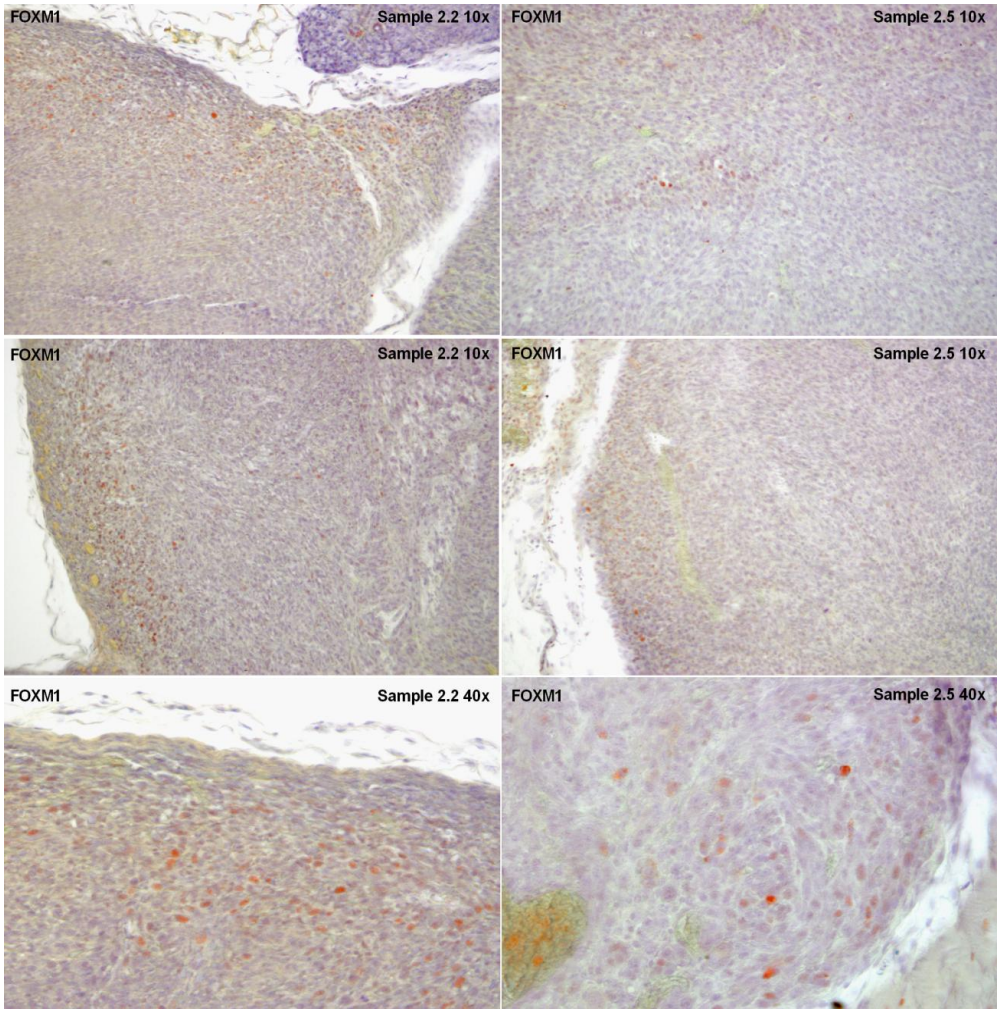


Figure 22: FOXM1 is expressed at the edge of SCC7 induced tumors. Tumors grown from the transplanted SCC7 cells were harvested and analyzed by immunohistochemical staining of paraffin sections using antibodies directed against FOXM1.

Areas of high invasion are usually found within the edge of a tumor, where high expression patterns of invasive markers like MMP-2 or MMP-9 are detected (Guo P et al., 2007). Immunohistochemical staining of SCC7-induced mouse tumors for FOXM1 protein expression shows that it is mainly expressed at the edge area of the tumor and a predominantly nuclear location of FOXM1 indicates that it is in an active state (Figure **22**). Those preliminary results obtained from the SCC7 tumor samples were a first indication that FOXM1 might be involved in invasive processes of HNSCC cells.

Next, we analyzed in human HNSCC cell lines if FOXM1 correlates with such phenotypic features as cell proliferation and invasion. The invasive potential was quantified by the use of Matrigel-coated Boyden chambers. Since all cell lines have different growth rates, prior to the invasion assay they were put under growth arrest by brief incubation with Mitomycin C, which efficiently induced growth arrest (Figure **23**).

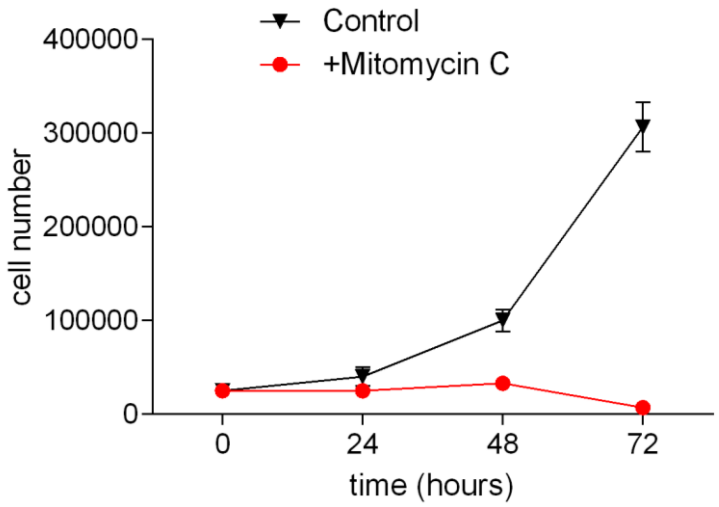


Figure 23: Mitomycin C induces growth arrest in HNSCC cells. HNSCC cells were incubated with Mitomycin C for 15 minutes, prior to the cell growth assay. Cell growth was measured using trypan blue dye exclusion and MTT reduction assay. Bars show mean values ± SD from three independent experiments in triplicates.

To determine the proliferation rate of the HNSCC cell lines, the growth rate was measured over a period of 5 days by counting the number of living cells under the microscope. The results show that the cell lines differ in their growth properties. While SCC-25 has a relatively slow growth rate, CAL-27 and FaDu proliferate much faster (Figure **24b**).

In vitro invasion assays using Matrigel-coated Boyden chambers revealed that the HNSCC cell lines differ significantly in their capacities to invade the Matrigel matrix. While the cell line SCC-25 displayed the highest invasion rate, FaDu and CAL-27 invade the Matrigel matrix to a lesser degree (Figure **24c**). Taken together, comparison of FOXM1 expression with the proliferation

and invasion rates of the HNSCC cell lines shows that FOXM1 expression rather correlates with invasion than with proliferation of human HNSCC cells (Figure **24a**).

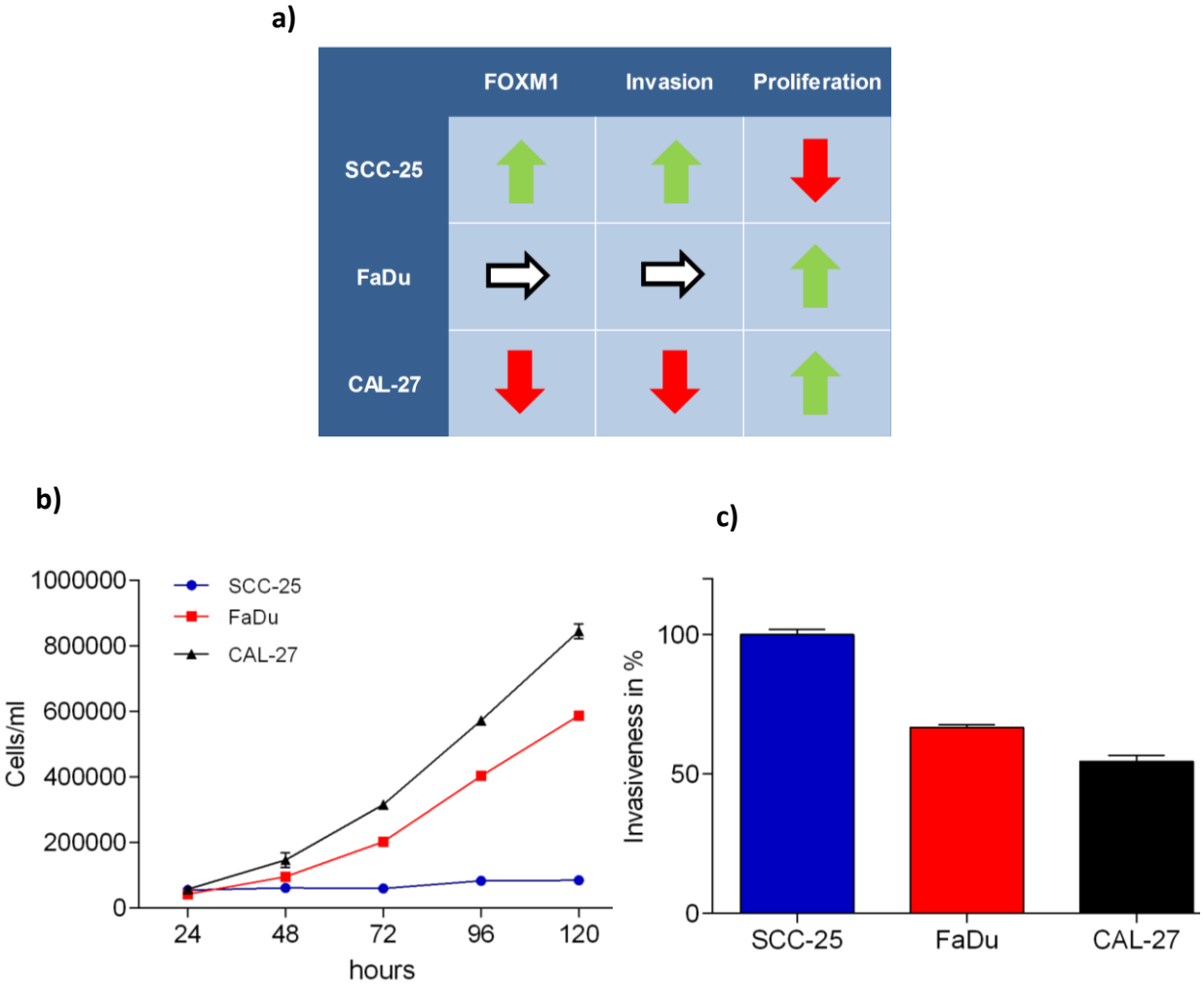


Figure 24: FOXM1 expression correlates with invasiveness of human HNSCC cells. The HNSCC cells differ in their ability to grow and to invade. Analysis of growth and invasive properties of human HNSCC cells revealed that FOXM1 expression rather correlates with the invasiveness of human HNSCC cell lines than with their proliferative capacities (a). Cell growth was measured using trypan blue dye exclusion and MTT reduction assay over a period of five days (b). The *in vitro* invasiveness of HNSCC cell lines was measured using Matrigel-coated Boyden chambers. Prior to the assay, cell growth was arrested by incubation for 15 minutes with Mitomycin C (c). Bars show mean values ± SD from three independent experiments in triplicates.

To further confirm that FOXM1 correlates with invasion of HNSCC cells, *in vitro* invasion assays in Matrigel-coated Boyden chambers with all three cell lines were performed. After separation of the invasive cell population from the non-invasive cells, mRNA was isolated and analyzed for FOXM1 expression. FOXM1 mRNA levels were significantly upregulated in invasive FaDu and

CAL-27 cells compared to the non-invasive cell populations. Invasive SCC-25 cells expressed also more FOXM1 mRNA than their non-invasive counterparts, but this difference was not significant (Figure **25**).

Those data clearly show that FOXM1 expression in HNSCC cells correlates with invasion and that FOXM1 expression is elevated in invasive cell populations compared to their non-invasive counterparts. This indicates that FOXM1 might directly regulate invasion of HNSCC cells.

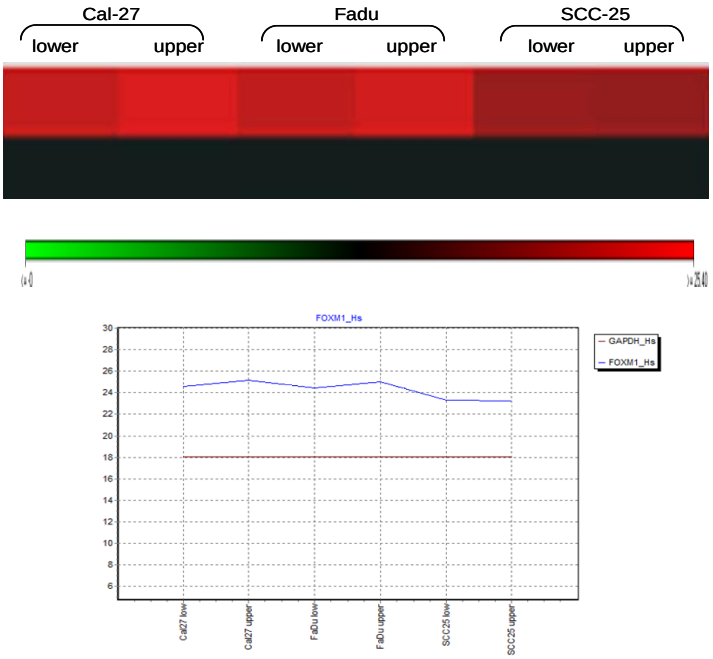


Figure 25: FOXM1 mRNA level is elevated in invasive HNSCC cells compared to their non-invasive counterparts. Invasive and non-invasive cells were separated in Matrigel-coated Boyden chambers. Subsequently, mRNA was isolated and examined for FOXM1 expression. mRNA amount was normalized to GAPDH expression. Results are displayed as a heat map picture (experiment was performed in collaboration with Dr. Peter Hofner).

3.2.2 FOXM1 directly regulates invasion of HNSCC cells

It has been shown that FOXM1 regulates invasion of diverse human cancer cell lines derived from osteosarcoma (Wang et al., 2008), glioma (Dai et al., 2007), ovary (Chan et al., 2012), cervix (He et al., 2012), colorectum (Chu et al., 2012), pancreas (Huang et al., 2012), breast (Ahmad et al., 2010), and thyroid (Ahmed et al., 2012; Bellelli et al., 2012) tumors. To test in a functional analysis that FOXM1 regulates invasion of HNSCC cells, FOXM1 expression was blocked using the FOXM1 inhibitor Siomycin A, which has been shown to downregulate FOXM1 expression and transcriptional activity (Radhakrishnan et al., 2006). HNSCC cells were incubated with Siomycin A for 36 hours, then protein lysates were isolated and examined for the expression of FOXM1 and the phosphorylation status of its downstream target PLK-1 (Fu Z et al.,

2008). At concentrations of 10µM Siomycin A reduced FOXM1 expression significantly, leading also to a declined phosphorylation of the FOXM1 downstream target PLK-1 (Figure **26**).

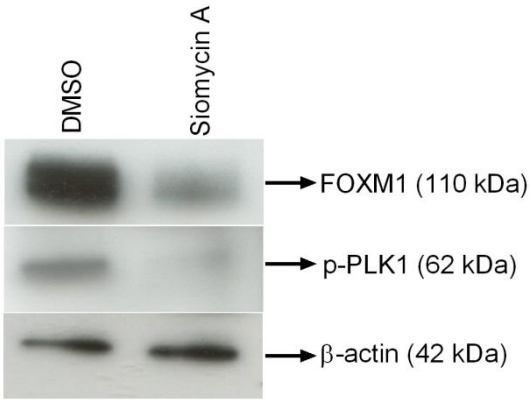


Figure 26: Siomycin A reduces FOXM1 protein expression. HNSCC cells were incubated with 10µM of Siomycin A for 36 hours. Protein lysates were isolated and protein expression levels of FOXM1 and the phosphorylation status of its known downstream target PLK-1 was examined by western blot. β-actin expression served as loading control.

After it was shown that Siomycin A is able to efficiently reduce FOXM1 protein level, the effect of this down regulation on HNSCC invasion was examined. FaDu cells were incubated with Siomycin A in different concentrations and *in vitro* invasion was measured in Matrigel-coated Boyden chambers. Siomycin A was able to reduce invasion in a concentration dependent manner. Already 1µM and 10µM reduced the invasion of FaDu cells. 20µM of Siomycin A reduced *in vitro* invasion of FaDu cells significantly (Figure **27**).

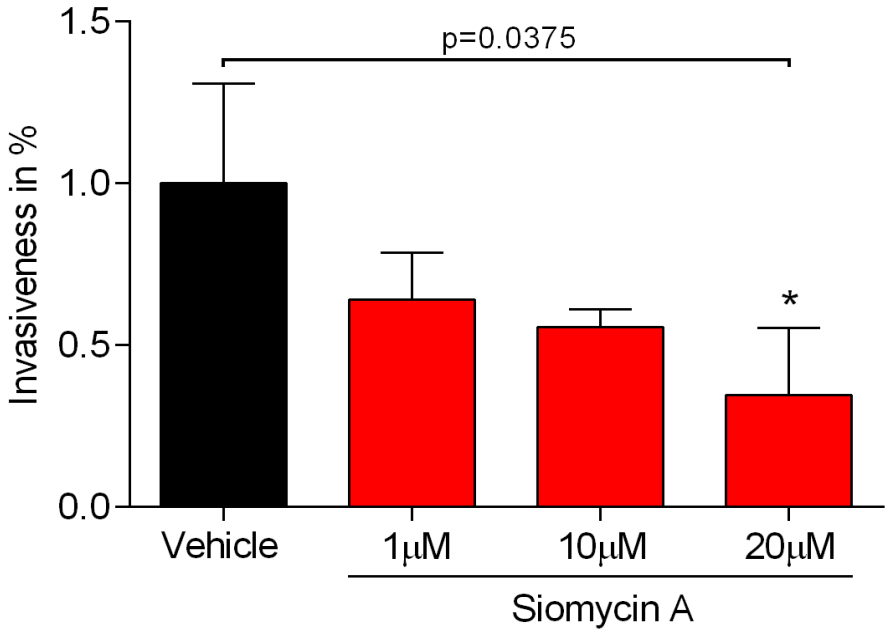


Figure 27: Siomycin A reduces invasion of HNSCC cells. FaDu cells were incubated with Siomycin A in the indicated concentrations for 36 hours. Cells were trypsinized and *in vitro* invasion assays using Matrigel-coated Boyden chambers were performed as previously described. Statistics were done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

Since Siomycin A is an antibiotic thiazole compound it had to be assured that it acts specifically on FOXM1 without causing side effects, which might adulterate the results of the *in vitro* invasion assays. Furthermore, it has already been described that Siomycin A can induce apoptosis through FOXM1 depletion (Uppoor et al., 2009). Therefore, western blots were performed, to test the pro-apoptotic effect of Siomycin A and FOXM1 siRNA by detection of cleaved Caspase-3, which is a widely used marker for apoptosis (Nicholson DW et al., 1995).
Siomycin A treatment has induced apoptosis in HNSCC cells, which was detected by elevated levels of cleaved Caspase-3. Treatment with FOXM1 siRNA did not induce cleaved Caspase-3 activity. As a positive control for ongoing apoptosis, cells were also treated for 48 hours with Mitomycin C, which induced cleaved Caspase-3 activity clearly (Figure **28**). Those data indicate that Siomycin A might not be suitable for performing invasion assays, since its apoptotic effects might interfere with the results of the *in vitro* invasion assays. Reducing FOXM1 expression via specific siRNA would be a better approach to analyze the effects for FOXM1 on invasion of HNSCC cells.

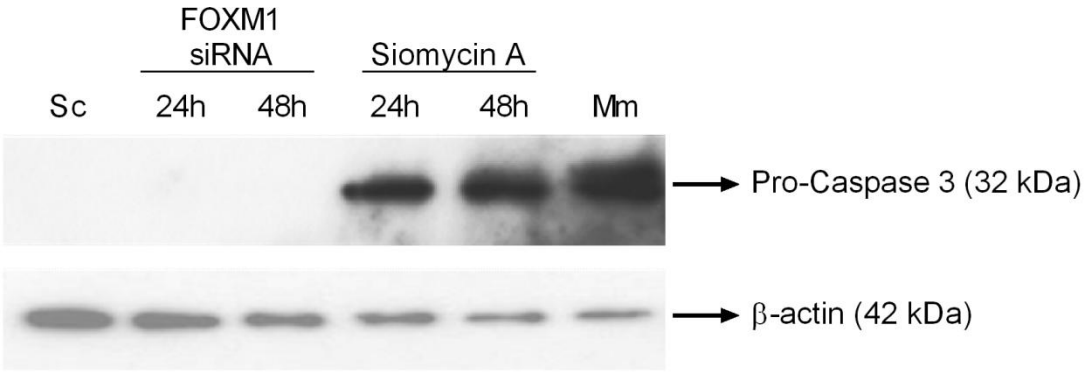


Figure 28: Siomycin A induces apoptosis in HNSCC cells. FaDu cells were treated with FOXM1 siRNA, Siomycin A (10µM) and Mitomycin C (10µg/µl) at the indicated time points. Protein lysates were equalized for β-actin expression and apoptosis was measured in a western blot by detection of cleaved Caspase-3. Sc= scrambled control, Mm=Mitomycin C.

Transient knockdown of FOXM1 expression in all three cell lines was performed by using specific siRNA. Upon transfection with siRNA, *in vitro* invasion assays were performed using Matrigel-coated Boyden chambers. Invasion was decreased in all three cell lines that were depleted in their FOXM1 expression. Whereas invasiveness of SCC-25 ($p=0.0121$) and FaDu ($p=0.0180$) cells was decreased significantly, invasion of CAL-27 cells, which express FOXM1 at very low level, was also decreased, but less significantly (Figure **29**).

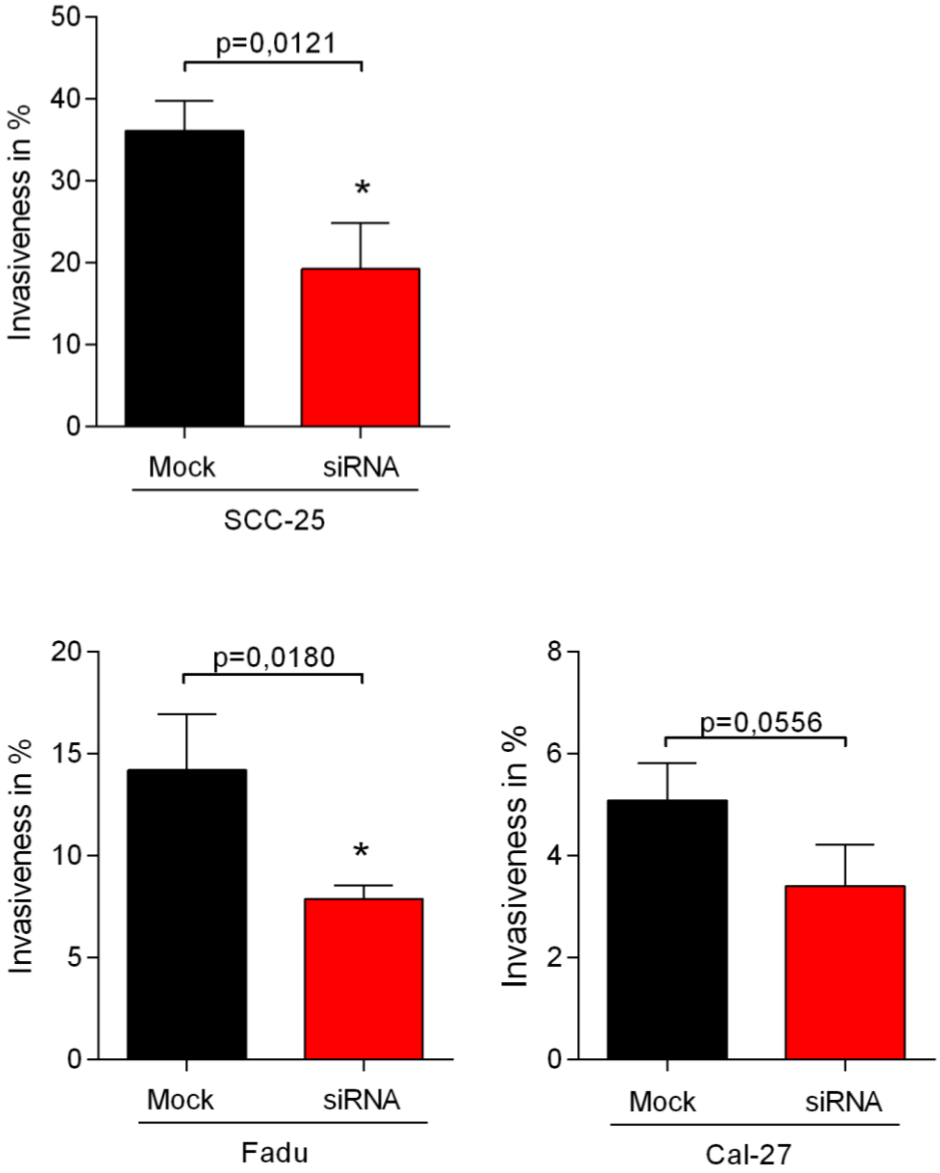


Figure 29: FOXM1 depletion via siRNA reduces invasion of HNSCC cells. The HNSCC cell lines SCC-25, FaDu and CAL-27 were transfected with FOXM1 siRNA and subsequently analyzed for their *in vitro* invasion using Matrigel-coated Boyden chambers. Statistics were done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

To confirm that the observed effects were FOXM1 specific, a rescue experiment, using a FOXM1 expression plasmid was performed. For this purpose FaDu cells were co-transfected with FOXM1 siRNA and FOXM1 cDNA and subsequently examined for their *in vitro* invasion properties in Matrigel-coated Boyden chambers.

As expected, FOXM1 siRNA reduced *in vitro* invasion of FaDu cells significantly. Re-expression of FOXM1 via an expression plasmid restored the invasive phenotype of FaDu cells. Those results indicate that the observed effects of FOXM1 knockdown on invasion are FOXM1-specific, since re-expression of FOXM1 restores invasion of FOXM1 depleted cells (Figure **30**).

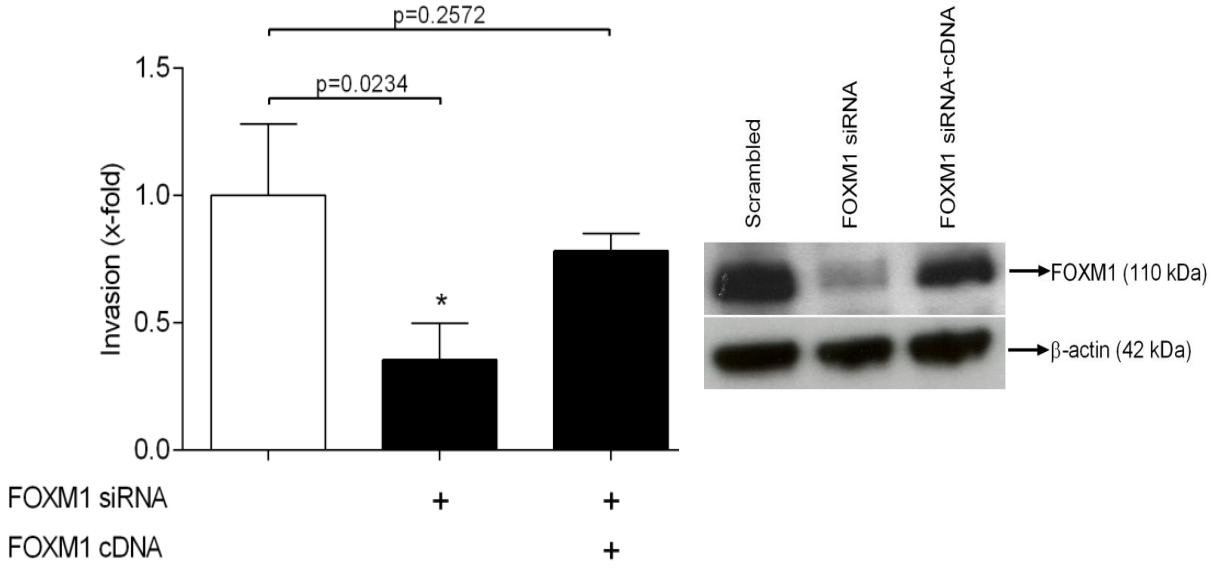


Figure 30: Decrease of invasion after FOXM1 knockdown can be rescued by re-expression of FOXM1. The HNSCC cell line FaDu was transiently transfected either with FOXM1 siRNA or FOXM1 siRNA plus FOXM1 expression plasmid. 48 hours after transfection cells were trypsinized and *in vitro* invasion was examined in Matrigel-coated Boyden chambers. Statistics were done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

Taken together, these data clearly show that FOXM1 plays a major role in the invasion of HNSCC cells. FOXM1 expression levels correlate with an invasive phenotype of human HNSCC cancer cells and FOXM1 is upregulated in invasive cell populations compared to non-invasive ones. Further functional analysis revealed that alteration of FOXM1 expression levels directly influences invasion of human HNSCC cells. Yet, the exact mechanism of how FOXM1 regulates invasion remains unclear. As a transcription factor FOXM1 can be directly involved in the invasive process as regulator of genes, for example encoding proteolytic enzymes, which in turn have the ability to directly modulate cell invasion.

3.3 FOXM1-regulated invasion via uPA

3.3.1 uPA as a potential downstream target of FOXM1

The serine protease urokinase plasminogen activator (uPA) has been implicated in the process of invasion and metastasis of a variety of human cancers (Dano K et al., 2005). uPA is also known to be activated by p38, an upstream activator of FOXM1 (Montero L et al., 1999). Furthermore, uPA promoter region contains several Forkhead-specific recognition sequences, which FOXM1 can potentially bind (Yao KM et al., 1997) (Figure **31a**). Thus uPA represents a potential transcriptional downstream target for FOXM1-mediated invasion. Bioinformatic analysis of the publically available FOXM1 ChIP-Seq data confirmed FOXM1 binding within the uPA promoter. However, the exact position of FOXM1 binding peak differs from the ones predicted based on location of the Forkhead-specific recognition (Figure **31b**).

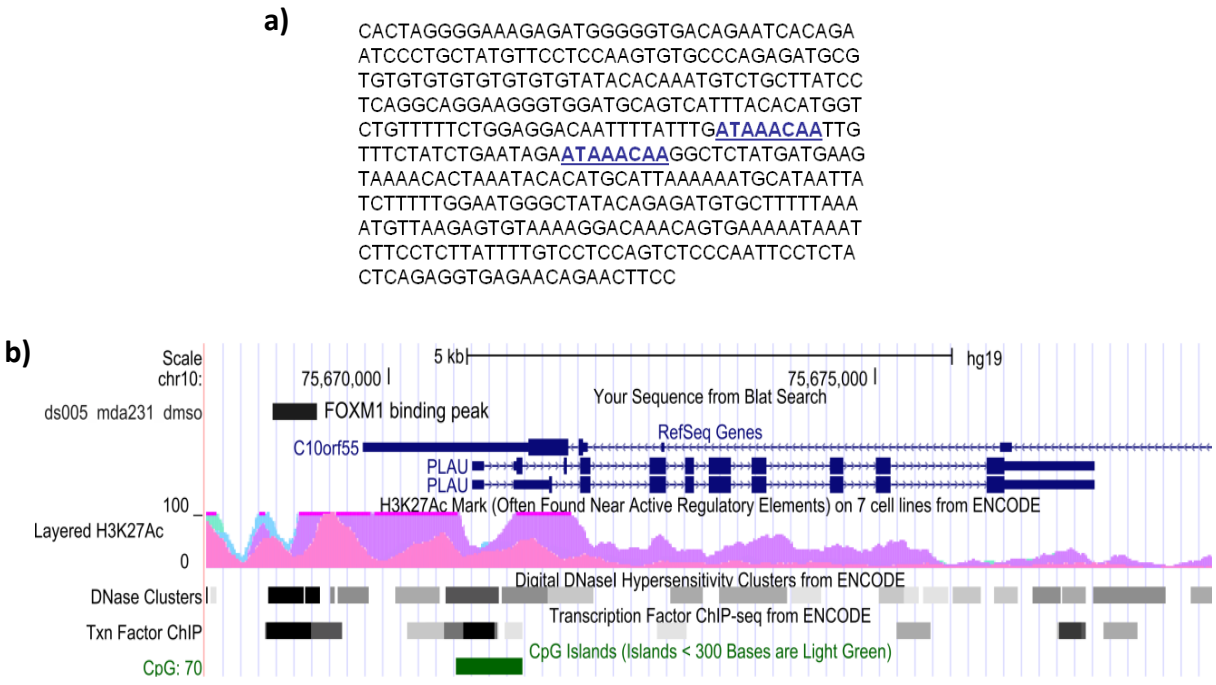


Figure 31: FOXM1 binding sites within the uPA promoter. uPA promoter region contains two ATAAACAA sequence motifs specific for Forkhead family members (a). Snapshot from the UCSC Genome Browser, demonstrating FOXM1 binding peak upstream of the uPA transcription start site in the MDA-MB231 breast cancer cell line (ChIP-seq study GSE40762) (b).

First analysis of the HNSCC cell lines for uPA expression shows that uPA, like FOXM1, is upregulated in invasive HNSCC cells. The highly invasive cell line SCC-25 expresses the highest amounts of uPA, while the less invasive cell lines FaDu and CAL-27 express significantly lower levels of uPA. Thus in our examined HNSCC cells the expression of uPA correlates with FOXM1 expression and *in vitro* invasion (Figure **32**).

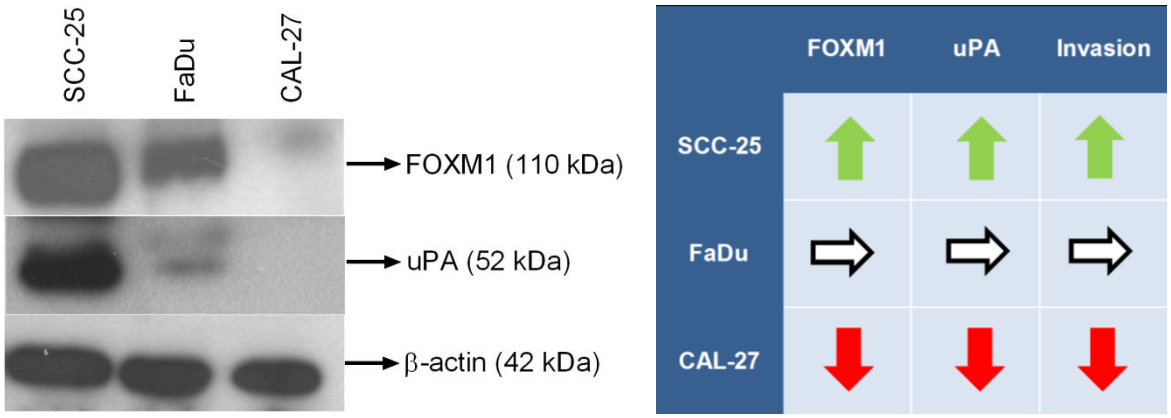


Figure 32: uPA expression correlates with FOXM1 expression and invasiveness of HNSCC cell lines. Protein lysates of human HNSCC cell lines were examined for uPA and FOXM1 protein expression by western blot and compared with their invasiveness. β-actin expression served as a loading control.

3.3.2 FOXM1 transactivates the uPA promoter in a Ras-dependent manner

To test if FOXM1 has the ability to transactivate the uPA promoter, we used the uPA wild-type promoter (uPA^{-2345}), placed in front of a chloramphenicol acetyltransferase (CAT) reporter gene (Figure **33**). This construct was transfected into HNSCC cells together with a FOXM1 expression plasmid. The CAT signal was measured by an ELISA assay.

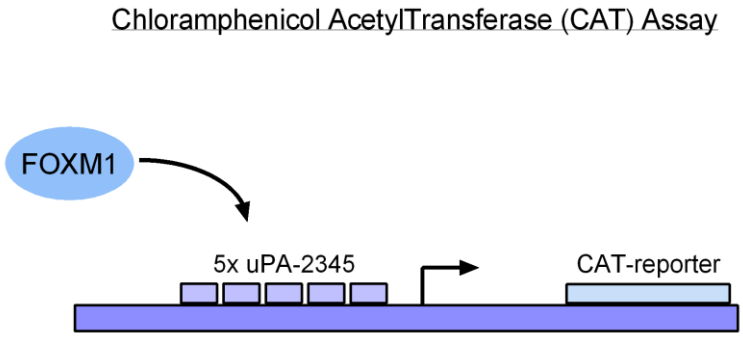


Figure 33: uPA promoter reporter constructs. The uPA wild-type promoter (2345 base pairs long) is fused to a chloramphenicol acetyltransferase (CAT) reporter. The activity of the uPA promoter was quantified by measurement of the CAT-signal using an ELISA assay.

FaDu cells transfected with the uPA^{-2345} promoter reporter construct displayed a basal uPA promoter activity, which was over 2-fold higher than the negative CAT-Basic transfected control. CAL-27 cells showed a lower basal uPA promoter activity, in line with the fact that they express almost no uPA protein. Upon co-transfection with a FOXM1 expression plasmid the activity of the uPA promoter reporter was elevated up to 5-fold in FaDu cells and 3.5-fold in CAL-27 cells compared to control samples (Figure **34**). This indicates that FOXM1 is able to transactivate the uPA promoter in HNSCC cells.

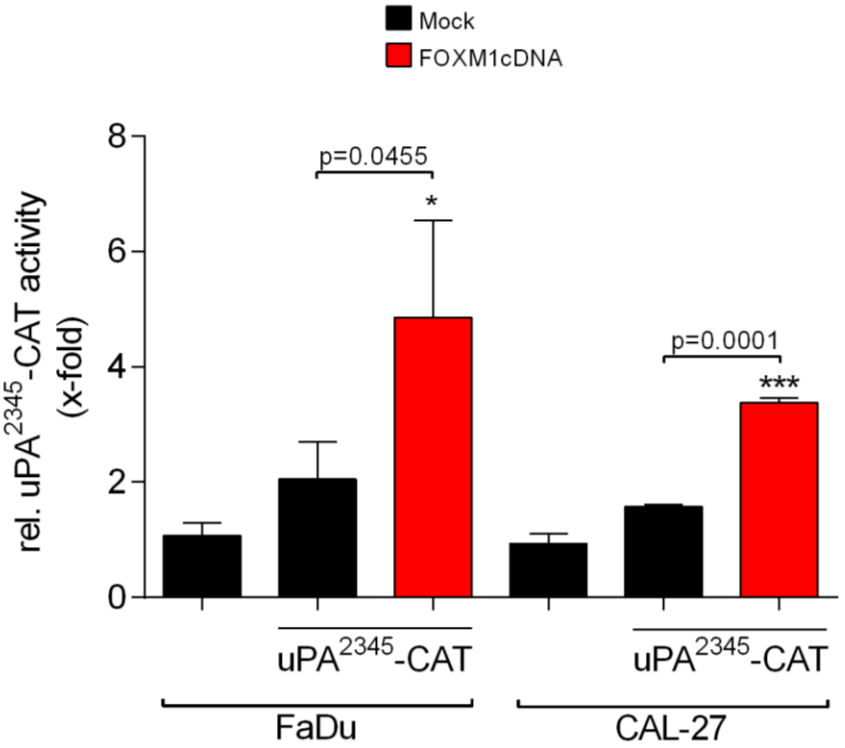


Figure 34: FOXM1 transactivates the uPA promoter in HNSCC cells. FaDu and CAL-27 cells were transfected with the uPA wild-type promoter fused to a CAT reporter (uPA^{-2345}-CAT) or co-transfected with the uPA^{-2345}-CAT reporter and a FOXM1 expression plasmid. Transfection with the CAT-Basic vector served as a negative control. CAT activity was measured using the CAT-Elisa Kit (Roche, Germany). Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

Ha- and K-Ras signaling plays an important role in HNSCC. Especially *K-Ras* is often overexpressed in HNSCC cell lines, for example in FaDu cells (Hoa et al., 2002). In order to examine if Ras plays a role in FOXM1 mediated transactivation of the uPA promoter, we repeated the wild-type uPA^{-2345} promoter-CAT assays in NIH3T3 wildtype fibroblasts and NIH3T3 cells with activated Ras. NIH3T3 wild-type cells almost showed no basal uPA promoter activity.

Additional transfection with the FOXM1 expression plasmid increased the uPA promoter activity up to 4-fold (Figure **35a**). In NIH3T3 Ha-Ras^{EJ} cells, however, the basal uPA promoter activity was 5-fold increased compared to negative control. Upon transfection with the FOXM1 expression plasmid this activity was further increased to over 10-fold (Figure **35b**). Those data show that an activated Ras background is necessary for FOXM1 to transactivate the uPA promoter more efficiently.

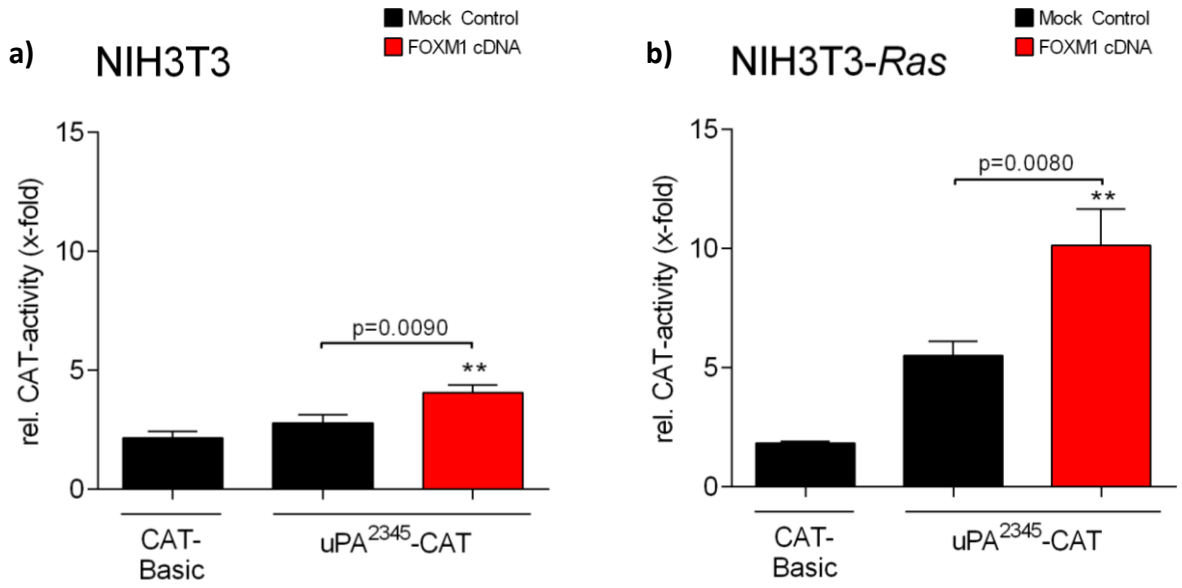


Figure 35: An activated Ras-background is necessary for FOXM1 to transactivate the uPA promoter efficiently. NIH3T3 wild-type mouse fibroblasts (a) and NIH3T3 cells with activated Ras protein (b) were transfected with the uPA^{-2345}-CAT reporter alone or co-transfected with a FOXM1 expression plasmid. CAT activity was measured using the CAT-Elisa Kit (Roche, Germany). Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

3.3.3 FOXM1 regulates uPA expression

High uPA expression is associated with increased tumor cell invasion and EMT, as for example in breast cancer (Jo M et al., 2009; Li XF, Oncogene 2009). To check if FOXM1 regulates uPA expression, FOXM1 protein levels in HNSCC cells were downregulated by using siRNA knockdown. Protein lysates were isolated and uPA expression was examined in a western blot. The western blot data shows that the siRNA successfully decreased FOXM1 protein levels. Upon

inhibition of FOXM1 the expression of uPA was also clearly decreased (Figure **36**). This demonstrates that in HNSCC cells FOXM1 regulates uPA expression on protein level.

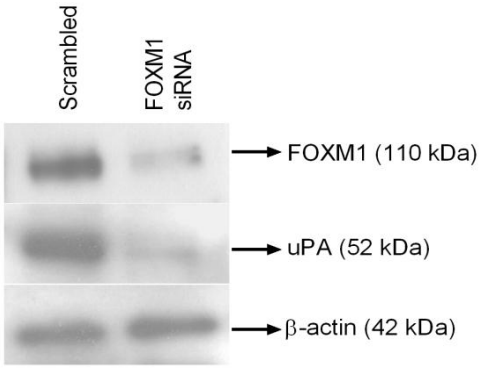


Figure 36: FOXM1 regulates uPA protein level. FaDu cells were transfected with FOXM1 siRNA. 48 hours after transfection protein lysates were isolated and uPA protein expression was measured in a western blot. Protein amounts were equalized using antibodies directed against β-actin.

In order to analyze if FOXM1 regulates uPA transcription levels, mRNA from FOXM1 siRNA and FOXM1 cDNA transfected HNSCC cells was isolated and analyzed for uPA expression. FOXM1 knockdown decreased uPA mRNA levels to almost 2-fold compared to the scrambled siRNA-treated control (Figure **37a**). Conversely, expression of extopic FOXM1 increased uPA mRNA levels to almost 3-fold (Figure **37b**). These data show that FOXM1 plays an important role in the transcriptional regulation of uPA. Altering FOXM1 levels influences uPA expression on mRNA and protein levels, which might contribute to the proteolytic uPA activity and invasion of HNSCC cells.

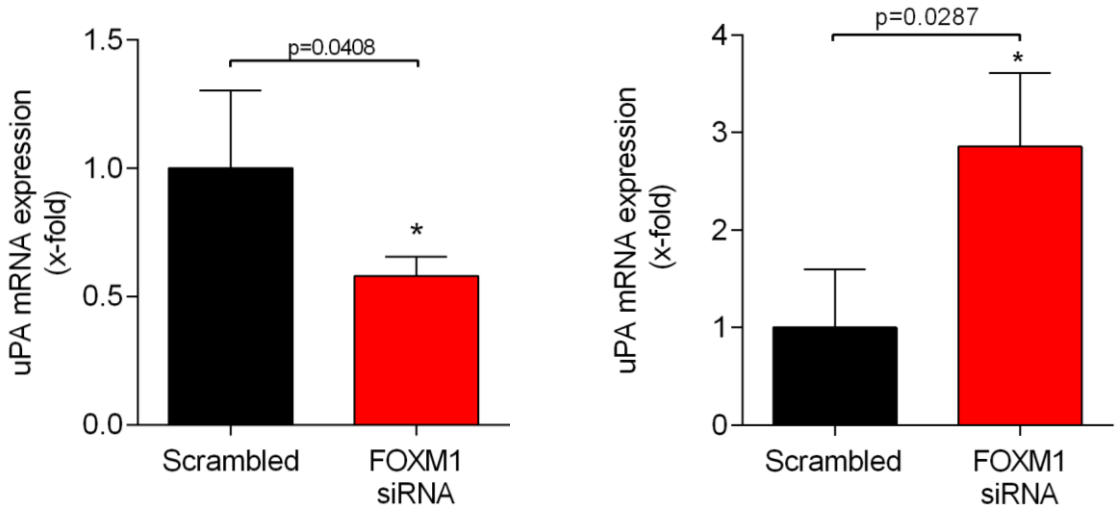


Figure 37: FOXM1 regulates uPA expression on transcription level. FaDu cells were transfected with FOXM1 siRNA (a) and a FOXM1 expression plasmid (b), respectively. Subsequently, mRNA was isolated and uPA mRNA expression was measured by real-time qPCR. Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

3.3.4 FOXM1 regulates invasion via increased uPA activity

uPA is secreted from cells and converts plasminogen to plasmin. Increased uPA proteolytic activity outside of the cells can promote cancer cell invasion and EMT (Shi X et al., 2008). To examine if the regulation of uPA by FOXM1 has an impact on uPA proteolytic activity and on cancer cell invasion, we first analyzed the influence of FOXM1 on activity of uPA secreted by HNSCC cells. For this purpose FaDu cells were treated with FOXM1 siRNA, p38 inhibitor (SB203580), and uPA inhibitor (UK122). 48 hours after stimulation/transfection cell supernatants were collected and uPA proteolytic activity was measured in an uPA activity ELISA. UK122, which served as a positive control for uPA inhibition, decreased uPA activity to under 20% compared to DMSO treated control. Inhibition of FOXM1 and p38 decreased uPA activity to 40% (Figure **38**). This data indicates that FOXM1 as well as p38, which acts upstream of FOXM1, regulate uPA, secreted by HNSCC cells. Since FOXM1 regulates the expression and activity uPA, it is likely that FOXM1 mediated *in vitro* invasion of HNSCC cells depends on uPA activity.

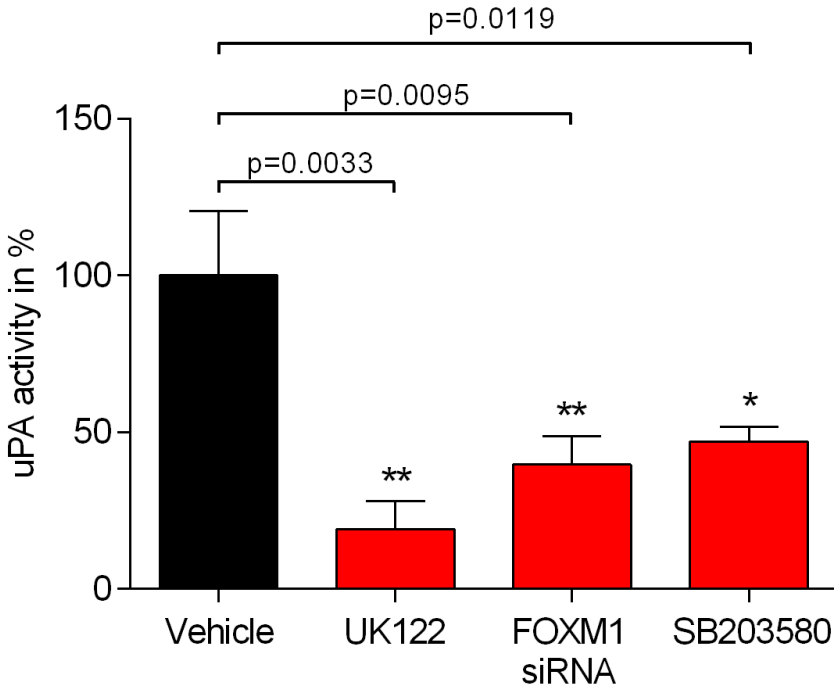


Figure 38: FOXM1 regulates the proteolytic activity of uPA in the supernatant of HNSCC cells. FaDu cells were transfected with FOXM1 siRNA, or incubated with the uPA inhibitor UK122 (100µM), or the p38 inhibitor SB203580 (10µM). 48 hours after transfection cell supernatants were collected and proteolytic activity of uPA was measured using ELISA test (American Diagnostica, USA). Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

To show that uPA, the downstream target of FOXM1, mediates FOXM1-induced invasion, *in vitro* invasion assays, using Matrigel-coated Boyden chambers, were performed. Prior to the assay, cells were transfected with the FOXM1 expression plasmid and the uPA inhibitor UK122, respectively.

Transfection with the FOXM1 expression plasmid increased invasion of FaDu cells significantly as expected. After simultaneous inhibition of uPA, using the uPA inhibitor UK122, transfection with the FOXM1 expression plasmid did not increased invasion of FaDu cell significantly (Figure **39**). This clearly shows that uPA is an important factor in FOXM1-mediated invasion of HNSCC cells. Without active uPA FOXM1 is no more able to increase invasion of HNSCC cells significantly.

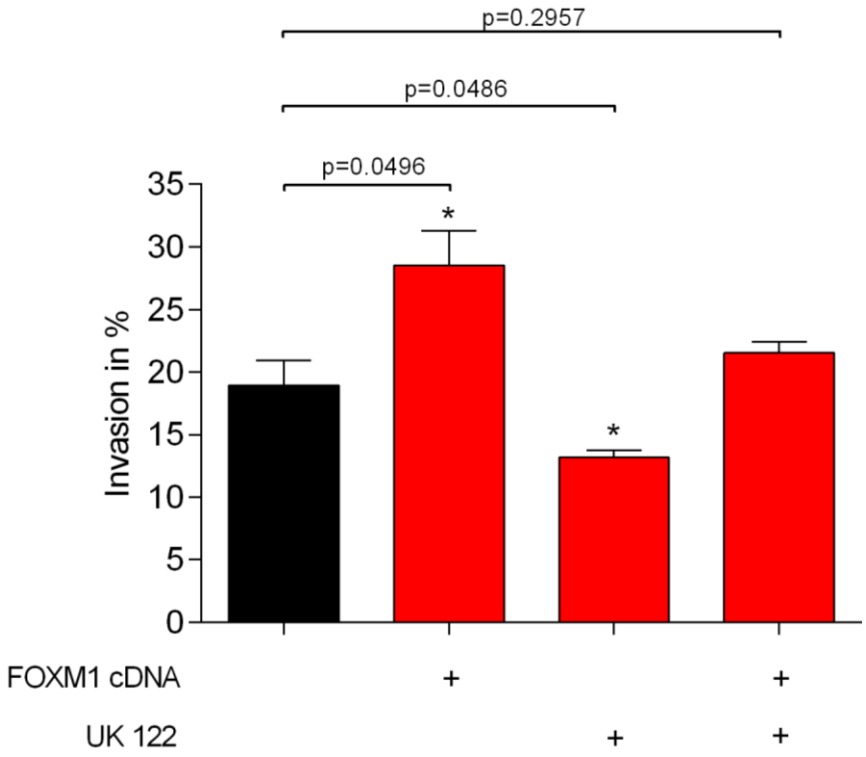


Figure 39: FOXM1 mediated invasion of HNSCC cells is dependent on uPA.
FaDu cells were transfected with FOXM1 siRNA and a FOXM1 expression plasmid and *in vitro* invasiveness was measured using Matrigel-coated Boyden chambers. As a control for uPA activity, prior to the invasion assay cells were incubated with the uPA inhibitor UK122 (10µM). Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

Taken together, these data show that FOXM1 transactivates the uPA promoter in HNSCC cells, which in turn leads to an increased uPA mRNA and protein expression. To regulate uPA expression efficiently, FOXM1 requires an active Ras background. All these regulatory mechanisms increase uPA proteolytic activity in the surroundings of the tumor cells, which finally elevates *in vitro* invasion of HNSCC cancer cells.

3.4 Regulation of uPA by FOXM1 via an AP-1 dependent mechanism

3.4.1 FOXM1 activates the AP-1 transcription factor

The activator protein 1 transcription factor (AP-1) as well as p38 are known regulators of uPA expression (Cuevas BD et al., 2005; Han Q et al., 2002). The uPA promoter possesses potential FOXM1 binding sites as well as AP-1 recognition motifs (Figure **40**), suggesting that FOXM1 can either directly bind to the uPA promoter and enhance its expression or regulate it indirectly via modulation of AP-1.

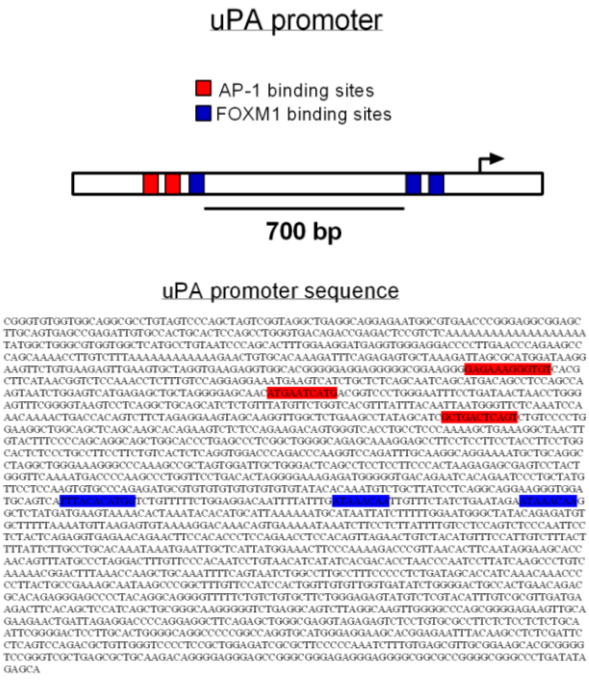


Figure 40: Transcription factor binding sites in the uPA promoter. The uPA promoter contains ATAAACAA sequence motifs, specific for Forkhead family members (blue) as well as AP-1 recognition sites (red).

Bioinformatic analysis of the publically available FOXM1 ChIP-Seq data from 5 different cancer cell lines shows that within the FOXM1 binding peaks, AP-1 consensus motifs are significantly enriched. The distribution of those sequences in FOXM1 bound DNA is not random, but is most dominant within the center of the FOXM1 binding peaks, where the binding of FOXM1 is expected to occur (Figure **41**). This non-random distribution of the AP-1 recognition motif within FOXM1 binding peaks indicates that FOXM1 and AP-1 might act together.

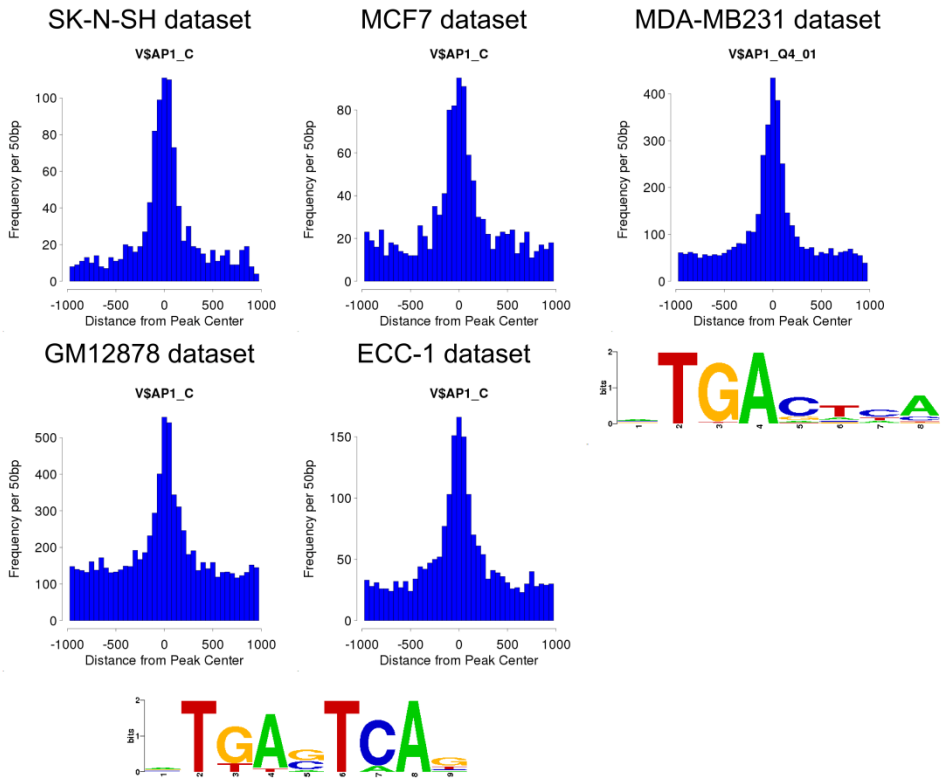


Figure 41: Frequency graph of the AP-1 recognition motif in the FOXM1 ChIP-seq peaks from five tumor cell lines. FOXM1 ChIP-seq peaks were analyzed for TF motif enrichment using CentDist program. The graphs show that AP-1 recognition motif occurs more frequent near the center of the FOXM1 binding peaks from the SK-N-SH, MCF7, GM12878, ECC- 1 and MDA-MB231 cell lines. The sequence logos of the TRANSFAC V$AP1_C and V$AP1_Q4_01 are shown below the frequency graph.

To test if FOXM1 *per se* is able to activate AP-1, a 5xAP-1 responsive element (5x-TRE) was fused to a CAT-reporter gene. HNSCC cells were transfected with the CAT-reporter plasmid and FOXM1 expression plasmid or FOXM1-siRNA, respectively. CAT activity was measured using a

CAT ELISA assay from Roche (Mannheim, Germany). Transfection with FOXM1 siRNA reduced 5xTRE-CAT signal significantly to 60% as compared to control. Ectopic expression of FOXM1 lead to a significant increase of AP-1 activity, thus showing that FOXM1 has the ability to increase the activity of AP-1 (Figure **42**).

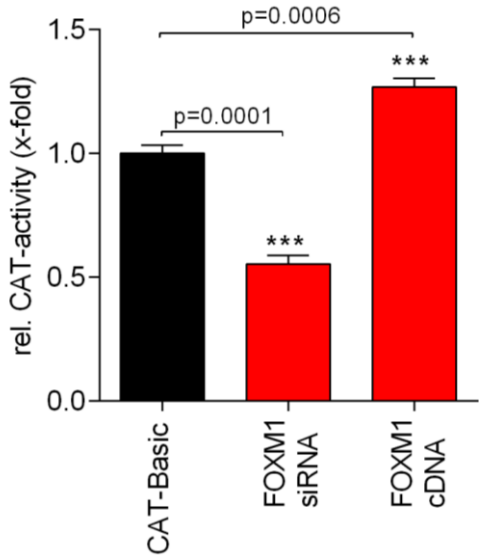


Figure 42: FOXM1 regulates the activity of AP-1. HNSCC cells were transfected with a 5xTRE-CAT reporter or co-transfected with the 5xTRE-CAT reporter and FOXM1 siRNA or a FOXM1 expression plasmid, respectively. 48 hours after transfection cell lysates were collected and CAT activity was measured using the CAT-Elisa Kit (Roche, Germany). Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

Phorbol 12-myristate 13-acetate (PMA) is a known activator of AP-1 (Roebuck KA et al., 1996). Prior to the CAT ELISA, cells were stimulated with 10nM of PMA for 12 hours and transfected with FOXM1 siRNA respectively. Stimulation with PMA increased AP-1 activity to 2.2-fold, as expected. Simultaneous inhibition of FOXM1 via siRNA was able to prevent PMA-mediated induction of AP-1 activity significantly, even though only to a low extent (1.8- fold) (Figure **43**).

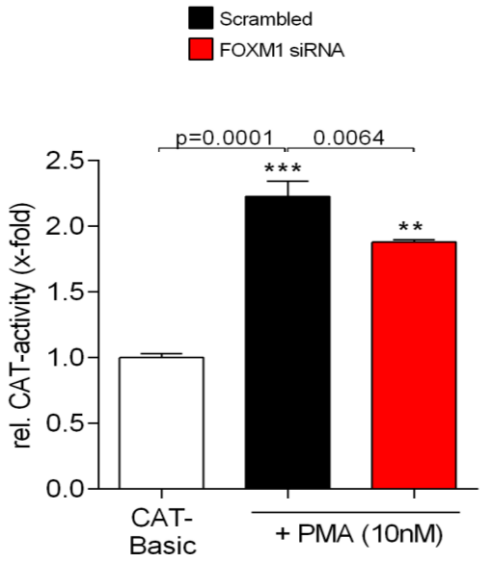


Figure 43: FOXM1 is involved in PMA mediated activation of AP-1. HNSCC cells were transfected with a 5xTRE-CAT reporter or co-transfected with the 5xTRE-CAT reporter and FOXM1 siRNA. Transfection with the CAT-Basic reporter served as negative control. 12 hours prior to the assay, cells were stimulated with PMA (10nM). After 48 hours post transfection, cell lysates were collected and CAT activity was measured using the CAT-Elisa Kit (Roche, Germany). Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

The AP-1 transcription factor is a heterodimer composed of several proteins. The two main members are proteins from the Jun and Fos families. Phosphorylation of those two molecules is necessary to stimulate AP-1 activity and transactivation of AP-1 responsive genes (Monje P et al., 2003).

FOXM1 levels were altered using siRNA and cDNA transfection and the influence on the expression and phosphorylation status of c-Fos and c-Jun was analyzed in a western blot. Overexpression or depletion of FOXM1 has no effect on the expression levels of c-Fos and c-Jun. Phosphorylation levels of c-Fos and c-Jun, however, were clearly increased upon induction of FOXM1 (Figure **44**). These results demonstrate that FOXM1 activates the AP-1 complex rather through phosphorylation than through elevated expression of the two main AP-1 members, c-Fos and c-Jun.

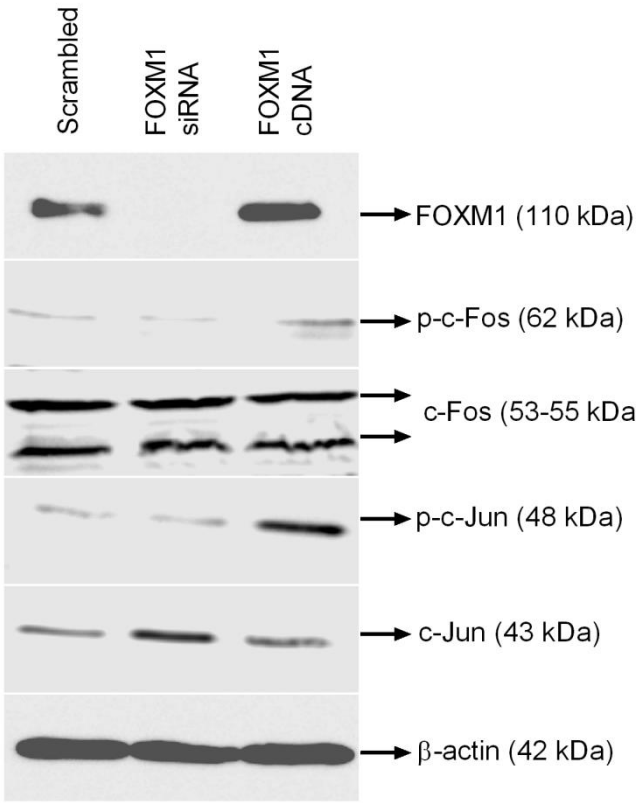


Figure 44: FOXM1 regulates phosphorylation of AP-1 members. A549 cells were transfected with FOXM1 siRNA and cDNA, respectively. Protein lysates were isolated and examined for expression levels of c-Fos, c-Jun, FOXM1 as well as for the phosphorylation status of c-jun and c-fos. Expression levels of the housekeeping gene β-actin served as a loading control.

To test the role of AP-1 in FOXM1 mediated invasion, *in vitro* invasion assays using Matrigel-coated Boyden chambers were performed. Transfection of FaDu cells with FOXM1 expression plasmid increased invasion significantly, as expected. Co-transfection with siRNA against one of the AP1 members (c-Fos) reverted this effect, indicating that AP-1 is necessary for FOXM1 mediated invasion (Figure **45**).

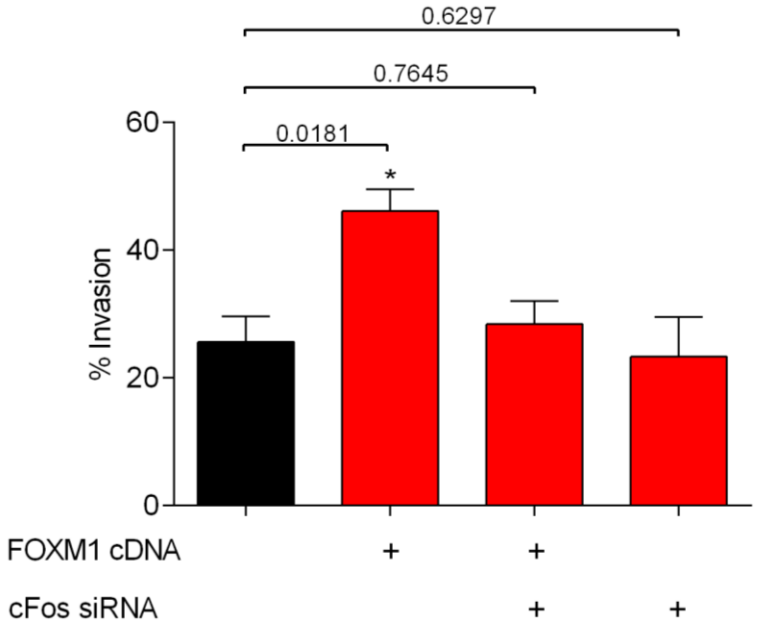


Figure 45: FOXM1 regulates invasion via AP-1. FaDu cells were co-transfected with FOXM1 cDNA plus c-Fos siRNA or with c-Fos siRNA and FOXM1 cDNA, respectively. 48 hours after transfection, invasion assays were performed using Matrigel-coated Boyden chambers. Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates.

3.4.2 Regulation of uPA via FOXM1 is mediated by AP-1

To check if the AP-1 recognition sequence within the uPA promoter is necessary for FOXM1-mediated activation, deletion mutants of the wildtype uPA^{-2345} promoter lacking the AP-1 binding sites were obtained (Figure **46a**).

The wild type uPA^{-2345}-CAT and the uPA^{-1870}-CAT reporter constructs were transfected into FaDu cells and FOXM1 was overexpressed using a FOXM1 expression plasmid. The intensity of the CAT signal was measured using a CAT-reporter Assay from Roche (Germany). FaDu cells transfected with the uPA^{-2345}-CAT promoter displayed a basal uPA promoter activity of 5-fold as compared to the CAT-Basic transfected samples. This CAT-activity could further be elevated by FOXM1 overexpression. The uPA^{-1870}-CAT deletion construct displayed a lower basal uPA

promoter activity. This basal activity could not be further increased by FOXM1 overexpression (Figure **46b**). The gained data shows that the AP-1 binding within the uPA gene is necessary for FOXM1 mediated transactivation of the uPA promoter.

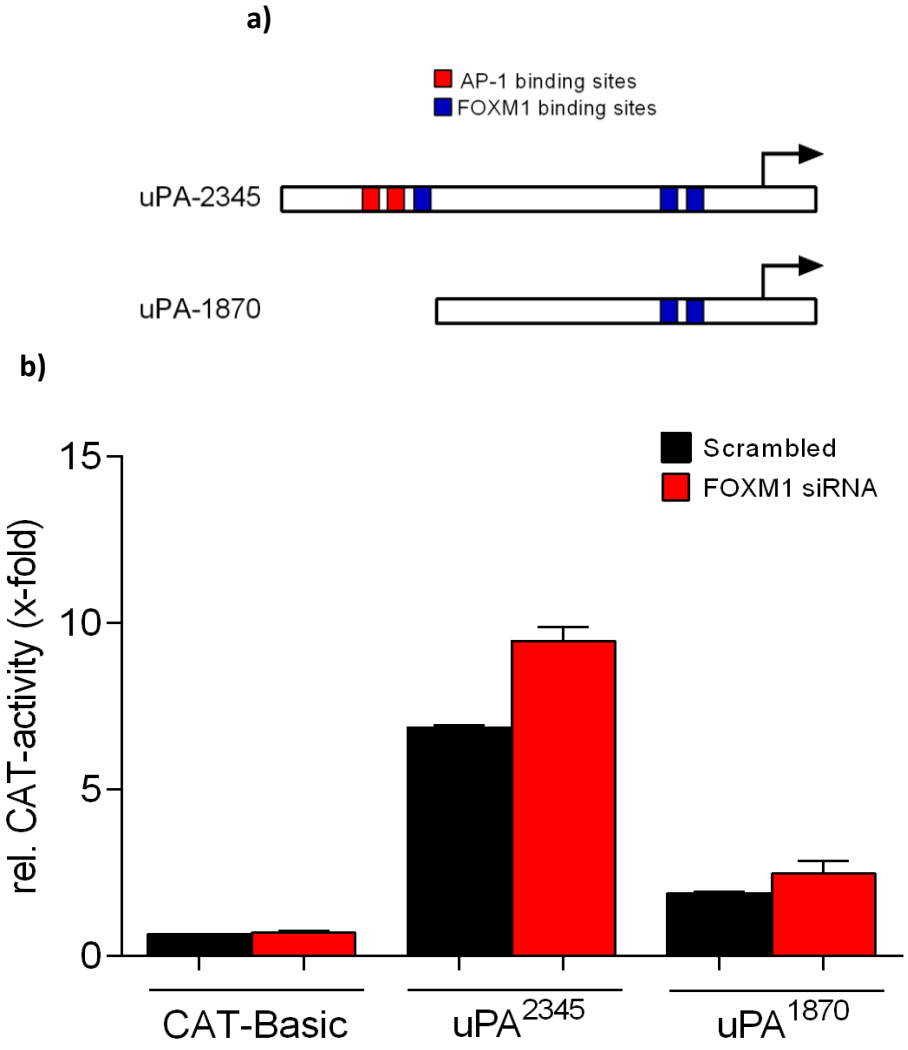


Figure 46: The AP-1 binding site is necessary for FOXM1 mediated activation of the uPA promoter. The wild-type uPA^{-2345} promoter possesses Forkhead and AP-1 recognition motifs. The uPA^{-1870} promoter construct lacks the AP-1 recognition motif (a). FaDu cells were transfected with the uPA^{-2345}-CAT, uPA-^{1870}-CAT reporter constructs alone or co-transfected with a FOXM1 cDNA expression plasmid. Protein lysates were isolated and CAT activity was measured using the CAT-ELISA kit (Roche, Germany). Transfection with the CAT-Basic vector served as a negative control. Statistics was done by Students T-test *p≤0.05. Bars show mean values ± SD from three independent experiments in triplicates (b).

The functional analyses using the CAT reporter constructs can not distinguish between direct and indirect action of FOXM1 and AP-1 on transcription of the uPA promoter. The chromatin immunoprecipitation (ChIP) method is the method of choice to study DNA binding of transcription factors. A ChIP Assay, using antibodies against FOXM1 and AP-1 members, as well as primers spanning the AP-1 recognition motif and the Forkhead-specific sequence in the uPA promoter, was performed to examine if FOXM1 or AP-1 directly interact with the uPA promoter (Figure **47a**).

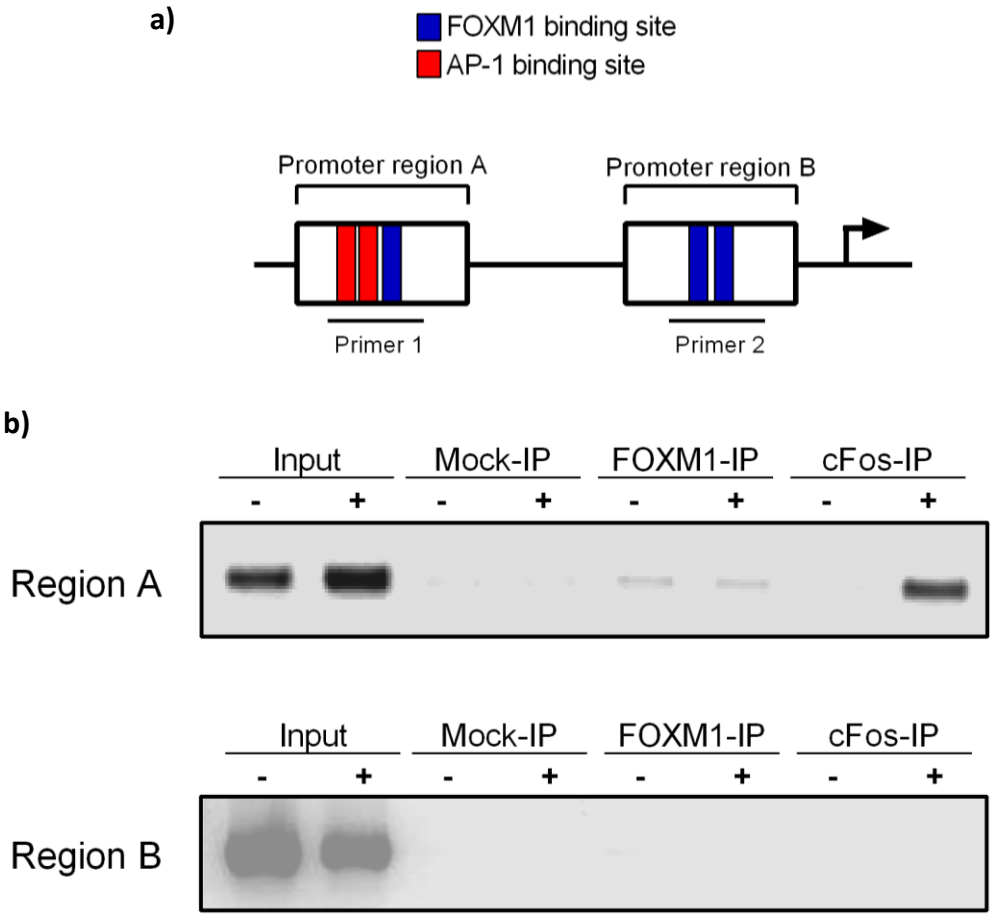


Figure 47: Induction of FOXM1 recruits c-Fos to the AP-1 binding site of the uPA promoter. Graph demonstrating positions of primers designed to measure FOXM1 and c-Fos binding in two regions of the uPA promoter by ChIP assay (a). ChIP assay was performed using A549 cells transfected in advance (24 hours) with a FOXM1 expression plasmidand antibodies against FOXM1 and c-Fos proteins (b). Semi-quantitative PCR was used to detect FOXM1and c-Fos binding (a) within the immunoprecipitated DNA fragments of the uPA promoter.

Using the primers spanning the Forkhead-specific sequence within the uPA promoter region B, it could be observed that FOXM1 does not bind the uPA promoter. Even as FOXM1 was overexpressed no FOXM1 binding was detected in that region of the uPA promoter. For c-Fos no binding to this region of the promoter could be observed. In contrast to region B, weak FOXM1 binding has been detected in region A, which could not be further increased by FOXM1 overexpression. In the control cells no binding of AP-1 at the promoter region A has been observed. However, upon overexpression of FOXM1 a very strong binding signal for AP-1 appears (Figure **47b**), indicating that FOXM1 overexpression induces the binding of AP-1 to the uPA promoter region A.

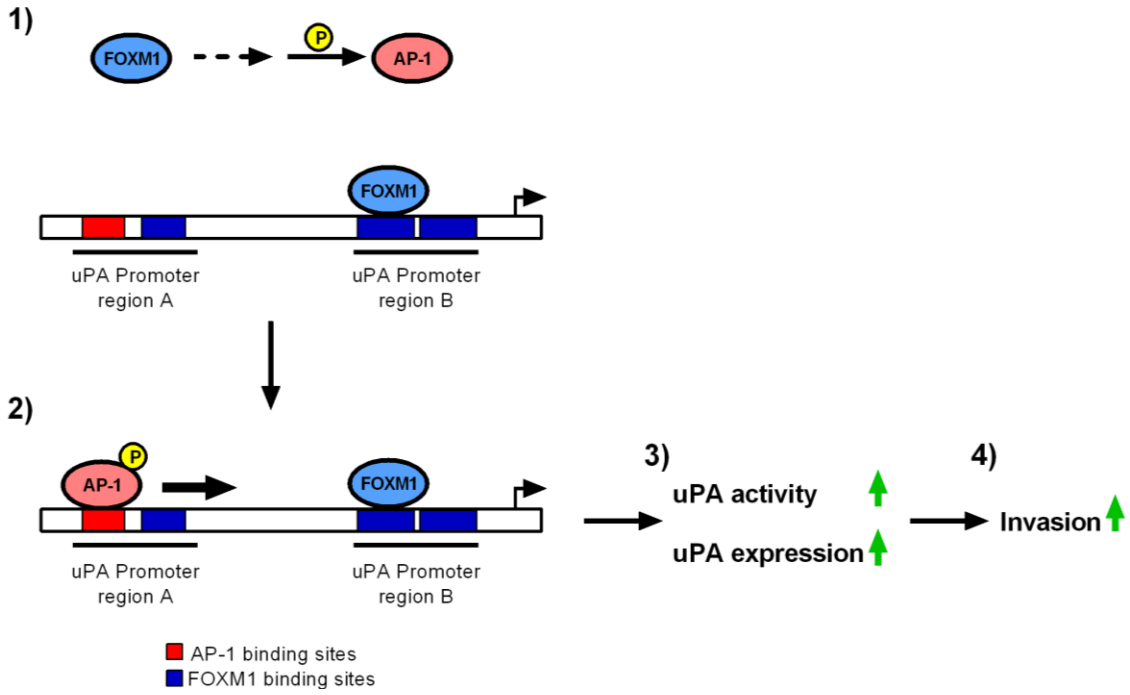


Figure 48: Mechanism of FOXM1/AP-1 dependent activation of the uPA promoter. FOXM1 activation leads to the phosphorylation of AP-1 members which recruits them to the AP-1 binding sites within the uPA promoter. As result, uPA expression and proteolytic activity is elevated, which this in turn enhances tumor cell invasion.

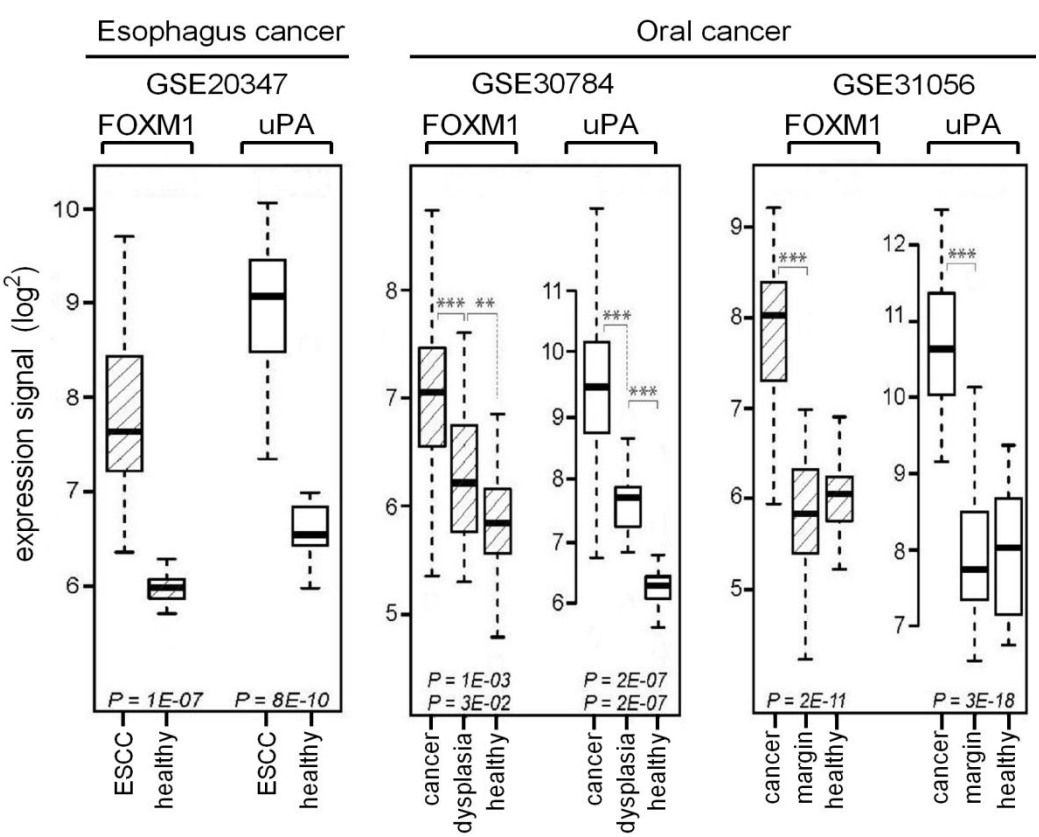
According to our gained data, it becomes evident that FOXM1 regulates *in vitro* invasion of HNSCC cells via an AP-1-dependent modulation of uPA expression and activity. This is achieved by initial FOXM1 mediated activation of the main AP-1 members c-Fos and c-Jun via phosphorylation. Upon phosphorylation, AP-1 is recruited to the AP-1 binding site of the uPA promoter, where it is involved in the increase of uPA activity and expression, which finally leads to enhanced invasion (Figure **48**).

3.5 Influence of the FOXM1-uPA axis on cancer and recurrent HNSCC

3.5.1 FOXM1 and uPA expression correlate in various cancer types

After we established the link between FOXM1-dependent regulation of uPA, we aimed to proof that this mechanism is not limited to the analyzed cell lines, but represents a general mechanism for tumor progression in head and neck cancer as well as in other epithelial cancer types. For this purpose, FOXM1 and uPA expression were examined in publically available gene expression data sets of different tumors. Regarding the head and neck tumor entities, FOXM1 and uPA were overexpressed in dysplastic lesions and, more dominant, in malignant tumors of the oral cavity (GSE30784; GSE31056) as compared to healthy tissue. There was no change in expression between healthy tissue and tumor margin. In esophageal squamous cell carcinoma (ESCC) FOXM1 and uPA were also overexpressed as compared to the healthy control (Figure **49a**).

a)



b)

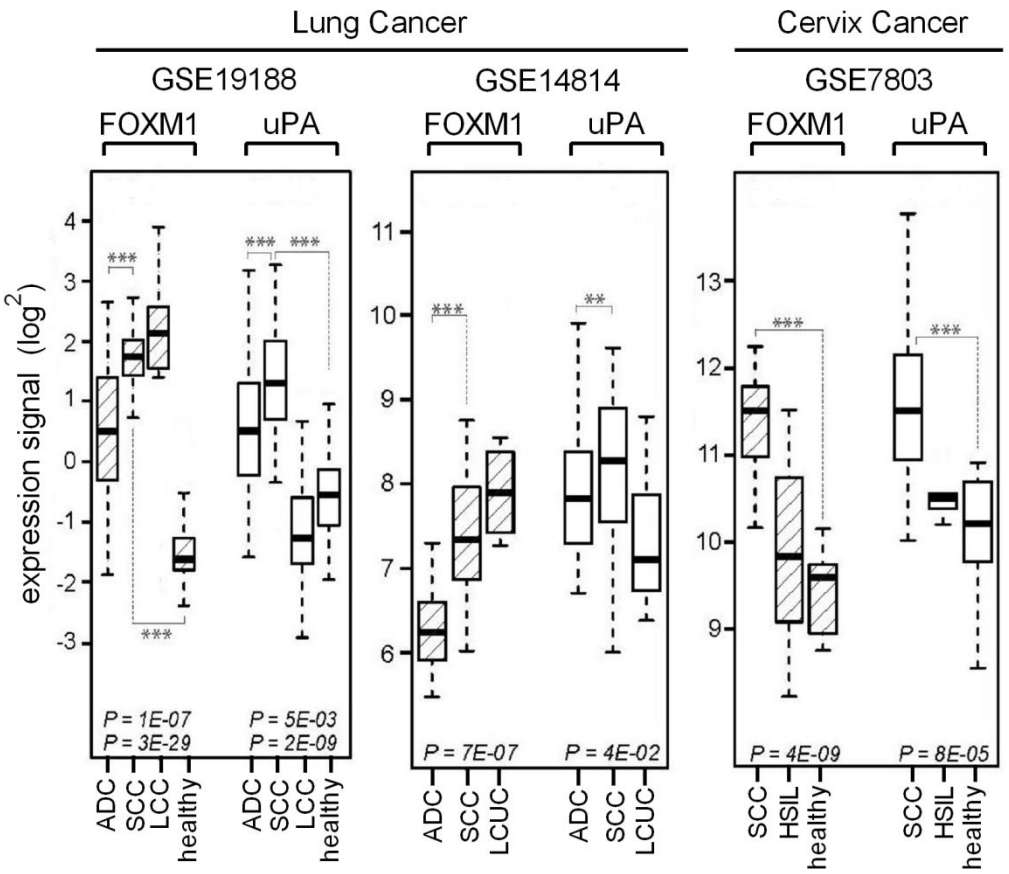


Figure 49: FOXM1 are co-expressed in different epithelial tumors and preinvasive lesions. Expression of FOXM1 and uPA was analyzed in publically available gene expression data sets of preinvasive lesion and malignant tumors of the lung, cervix (b), esophagus, and oral cavity (a). Results are displayed as Box plot diagrams.

In other cancer types, like cervix cancer, FOXM1 and uPA were both overexpressed in preinvasive, high grade cervical squamous intraepithelial lesions (HSIL) and more predominant in squamous cell carcinoma (SCC) of the cervix as compared to healthy tissue. In gene expression data sets from lung cancer FOXM1 and uPA overexpression positively correlated in squamous cell carcinoma (SCC) and adenocarcinoma (ADC). In lung cell undifferentiated carcinoma (LCUC) and large cell carcinoma (LCC), however, high expression levels of FOXM1 were accompanied by low relatively levels of uPA expression (Analyzed in collaboration with Benjamin Otto) (Figure **49b**).

3.5.1 Differential expression of FOXM1 and uPA in recurrent HNSCC tumors

In order to examine if FOXM1 and uPA are differentially expressed between primary and recurrent head and neck tumors, we adapted a previously established mouse model (Behren et al., 2010). eGFP labeled SCC7 cells were injected into nude mice (2 million per mice). After 3 weeks the developing tumor was resected and after another 2 weeks in 55% of mice developed recurrent tumors. The tumors samples were finally resected and together with the primary tumors further analyzed using immunohistochemistry and real-time qPCR (Figure **50**).

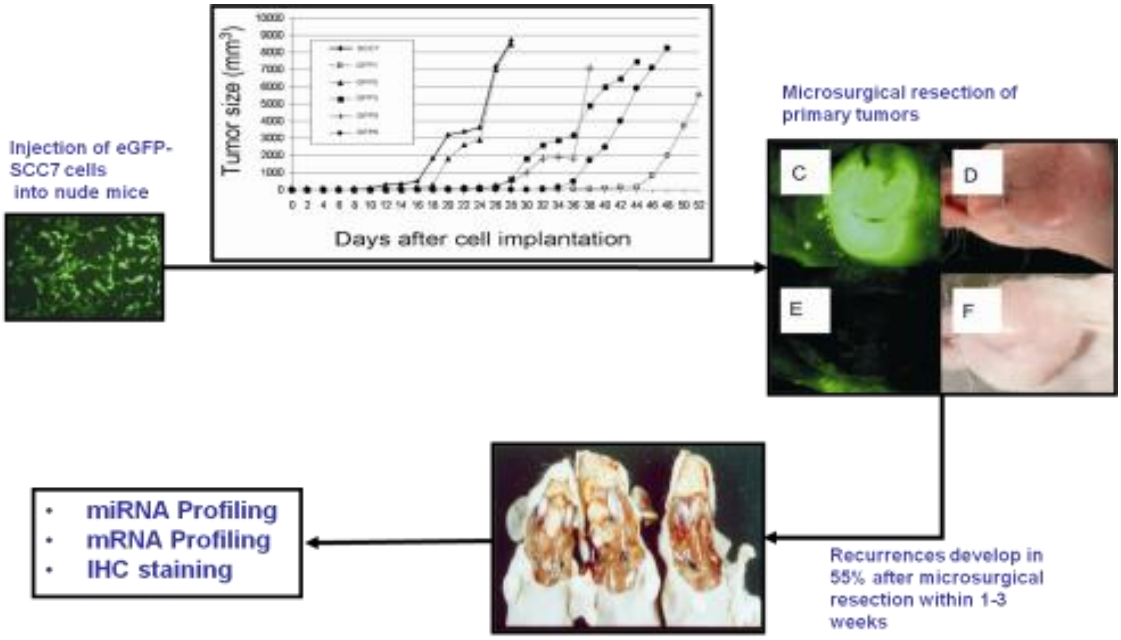


Figure 50: Experimental setup for the development of SCC7 induced mouse tumors. eGFP-labeled SCC7 cells were injected into nude mice where a tumor develops after 3-4 weeks. After resection of the primary tumor, 55% of the mice developed a recurrent tumor after 2 weeks. The recurrent tumor is also resected and together with the primary tumor analyzed for the expression of the target genes using immunohistochemical staining and mRNA profiling.

Staining of the p38 protein kinase did not show any significant difference between the primary and recurrent tumor samples (Figure **51a**). FOXM1 staining also did not reveal any difference in the expression level between primary and recurrent tumors (Figure **51b**). uPA, however, was significantly higher expressed in recurrent tumors, as compared to primary tumors (Figure **51c**). Those data were also confirmed by real-time qPCR, (data not shown).

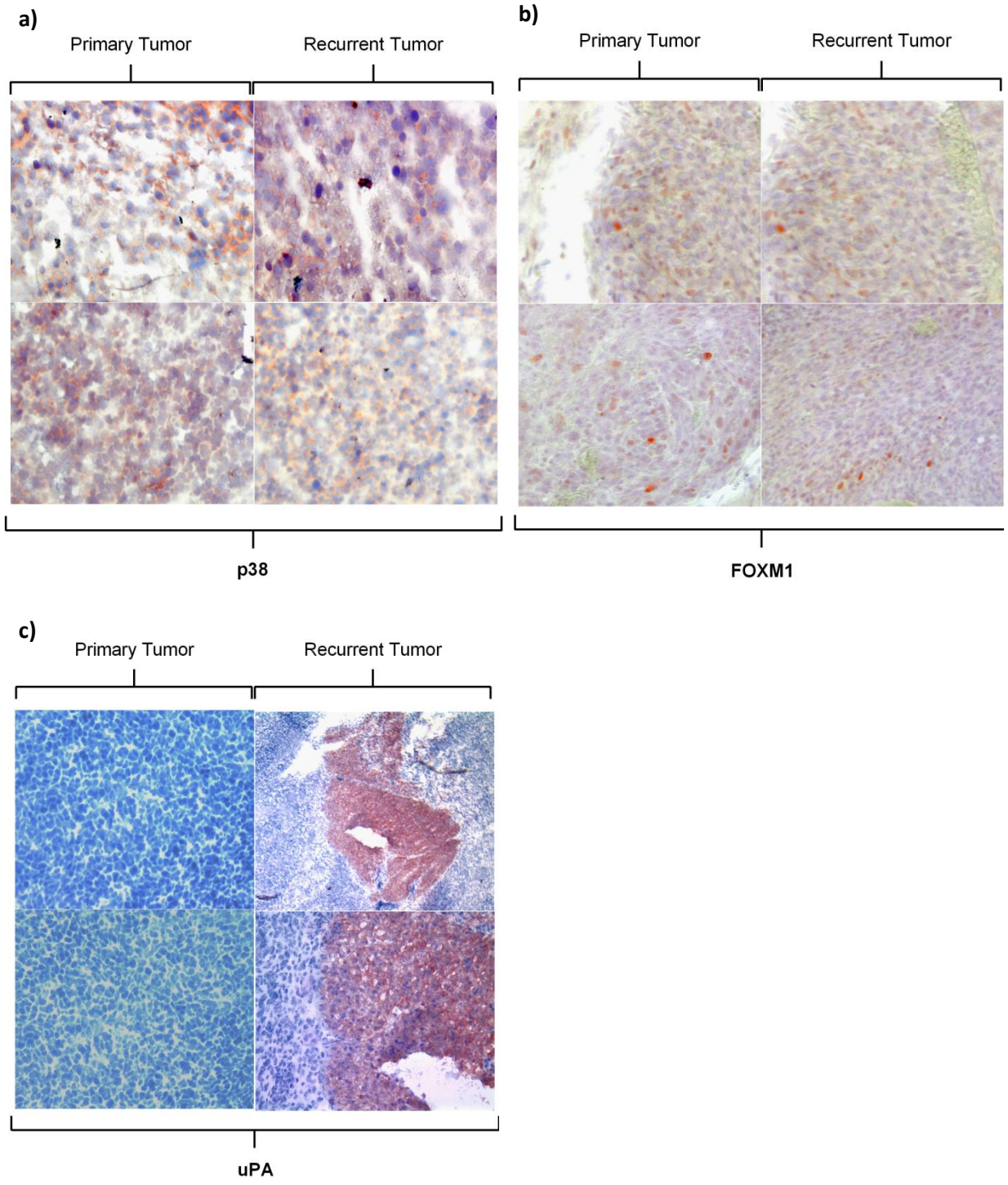


Figure 51: Differential expression of target genes between primary and recurrent SCC7 tumors. SCC7 induced primary and recurrent mouse tumors were isolated and examined for p38 (a), FOXM1 (b) and uPA (c) expression using immunohistochemistry.

uPA which has been shown to be regulated by FOXM1 and AP-1, is significantly upregulated in recurrent tumors as examined by immunohistochemical staining and mRNA profiling (data not shown). These data implicate a strong importance for FOXM1, AP-1, and uPA in the development of recurrent tumors.

4 Discussion

4.1 FOXM1 expression in HNSCC is regulated by SAPKs

Suited within the reactive stroma, tumor cells are surrounded by a hostile microenvironment and subjected to various stress conditions like metabolite deficiency, hypoxia and the constant necessity to escape the immunological protection barrier of the tumor host. Invasion, which is a hallmark of tumor progression, is linked to stress conditions, since the destruction of the ECM, the breakup of cell-cell contacts, and the migration process itself, pose a critical mechanical stress to tumor cells. Further, invasion and metastasis enable the tumor to escape from stress-induced damage in the primary tumor site (Kraning-Rush CM et al., 2012). Stress triggers the activation of SAPKs, like p38, leading to enhanced invasion, which in turn is the main cause for development of recurrent tumors. The exact mechanisms of how stress can induce invasion of tumor cells still remains unclear.

In our previous investigations we identified possible downstream targets of p38 by a computational-based analysis, where FOXM1 was one of them (Behren et al., 2009). After constitutively activating the Ha-Ras and MKK3 (p38) pathways in mouse fibroblasts we confirmed that FOXM1 protein and mRNA expression levels were increased effectively. However, constitutively active Ha-Ras elevated FOXM1 protein levels much stronger than MKK3 activation, where an elevation in FOXM1 protein level was barely detected. This could be related to the fact, that p38 activity in the MKK3- active cells was very weak, as western blots of MKK3-activated fibroblasts only showed faint elevation of phospho-p38 levels compared to wild-type NIH3T3 cells. Another explanation is that Ha-Ras operates upstream of MKK3 (Pelech SL et al., 1995; McDermott EP et al., 2002), and beside p38 also triggers other MAPK pathways, like ERK1/2, which is a very potent activator of FOXM1 (Lok GT et al., 2011). To confirm that p38 is indeed involved in the regulation of FOXM1 expression, we applied the p38 inhibitor SB203580 in Ha-Ras activated NIH3T3 mouse fibroblasts, which have elevated FOXM1 mRNA levels compared to NIH3T3 wild-type cells. Upon p38 inhibition, FOXM1 mRNA levels were significantly decreased in Ha-Ras activated mouse fibroblasts, though they still remained higher than in wild-type NIH3T3 cells. This indicates that p38 is also involved, at least partially, in Ha-Ras-mediated FOXM1 expression. Previous publications already demonstrated that ERK or p38,

both downstream of Ras, regulate the expression of FOXM1 (Behren et al., 2009; Chan DW et al., 2012).

We hypothesized that p38-dependent regulation of FOXM1 in fibroblast cells, which usually are the main components of the tumor-surrounding stromal tissue (Bhowmick NA et al., 2004), is a general mechanism which also occurs in epithelial tumor cells as well as in human HNSCC cells. In epithelial SCC7 cells, which were later used in our animal experiments, inhibition of p38 reduced FOXM1 protein levels markedly, while mRNA levels were decreased only marginally. The same observation could be made for human HNSCC cells lines. Upon p38 inhibition in the cell lines SCC-25 and FaDu, which express high and moderate levels of FOXM1, protein levels of FOXM1 were clearly reduced, whereas FOXM1 mRNA levels were reduced to a less degree. These results demonstrate that in epithelial tumors and human HNSCC FOXM1 is regulated via p38, preferentially on protein expression or stabilization and less through alterations of mRNA expression. Our results from human HNSCC and murine SCC7 cells reflect the known mechanisms by which the activity of FOXM1 is regulated through phosphorylation by various kinases and not necessarily through *de novo* synthesis (Fu Z et al., 2008). Further, in the course of tumor initiation of epidermal basal cell carcinoma, FOXM1 expression was upregulated upon UV light stress rather through protein stabilization and accumulation than through mRNA *de novo synthesis* (Teh MT et al., 2010). This mechanism of regulation makes it is possible for cancer cells to react to external stress stimuli faster and more flexible by protein modifications or phosphorylation, than by turning on the whole transcriptional machinery.

4.2 FOXM1 enhances invasion of HNSCC cells

The link between SAPKs and invasion has already been examined very well in the recent years by showing that stress-activated p38 and JNK MAP kinases enhance invasion and EMT of cancer cells (Denkert C et al., 2002; Shin I et al., 2005; Wang J et al., 2010). We confirmed the role of p38 in invasive processes of mouse fibroblasts by inhibiting p38 using the SB203580 inhibitor in MKK3 activated NIH3T3 cells, which decreased invasion almost to the levels of wild-type NIH3T3 cells, pointing out the crucial role of p38 in invasive processes in those cell types.

The role for FOXM1 in invasive processes of human HNSCC cells has not been examined yet. Though most head and neck tumors are squamous cell carcinomas, HNSCC turn out to be a very

heterogeneous malignancy, which makes a successful treatment very complicated (Bragado P et al., 2012). Different tumors from different HNSCC patients, as well as distinct cell populations within one tumor, vary in their genetic composition, expression of oncogenes, and in their phenotypes, like growth rate or invasion (Freier K et al., 2003; Song J et al., 2010; Prince ME et al., 2007). The cell lines SCC-25, FaDu, and CAL-27 used in our study, reflect this heterogeneity. They differ markedly with respect to their ability to grow and to invade. SCC-25 is highly invasive and slowly growing, while FaDu and CAL-27 invade less but grow relatively fast. Interestingly, the protein and mRNA expression of FOXM1, a well known cell cycle regulator, (Wierstra I et al., 2007), correlate with the invasiveness of HNSCC cells and not with their growth rate. Besides, p38 activity also correlates with FOXM1 expression and invasion of HNSCC cells. The highly invasive cell line SCC-25 expresses the highest amounts of FOXM1 and also displays the highest p38 activity. FaDu and CAL-27 expresses less FOXM1, invade less, and also have a lower activity of p38. This results support recent findings that FOXM1 is not only exclusively involved in proliferation but also in the regulation of other phenotypes, like invasion and metastasis (Raychaudhuri et al., 2011) and further supports our hypothesis that the p38-FOXM1 axis is important for invasion and aggressiveness of HNSCC cells. By this means, in HNSCC tumors, subjected to stress conditions, p38-mediated invasion and metastasis via FOXM1 may represent a proper survival mechanism for HNSCC cells, to escape harmful stress-conditions within the primary tumor site and to avoid stress-induced damage.

FOXM1 expression in immunohistochemical stainings of SCC7-induced mouse tumors was mostly limited to the tumors margins. Highly invasive cell populations are usually located at the border of tumors, where upon EMT, and loss of cell-cell contacts, they disseminate and invade the surrounding tissue or form metastasis (Zlobec I et al., 2009). A high expression of FOXM1 in those areas was another hint for the involvement of FOXM1 in invasion of epithelial tumor cells. Analysis of invasive and non-invasive cell populations of HNSCC cell lines revealed that FOXM1 expression was upregulated in the invasive cell populations of all analyzed HNSCC cells, compared to their non-invasive counterparts. These data strongly indicate that FOXM1 is involved in the regulation of invasive phenotypes of HNSCC cells. In a functional analysis FOXM1 expression was downregulated by Siomycin A. This treatment significantly inhibited invasion of HNSCC cells in a concentration depended manner, indicating that FOXM1 directly influences invasive properties of head and neck tumors. Siomycin A is a thiazole antibiotic, which previously has been shown to block FOXM1 transcriptional activity and expression (Bhat UG et al., 2009). Analysis of cleaved Caspase-3 in a western blot revealed that, in contrast to siRNA knockdown, Siomycin A induced apoptosis of HNSCC cells. Those results indicate that the observed decreased invasiveness of HNSCC cells, after FOXM1 knockdown via Siomycin A, could be due to elevated apoptosis and not necessarily due to altered invasion properties. Thus, we

conducted further FOXM1 knockdown experiments using specific siRNA, in order to confirm the results obtained with Siomycin A. Knockdown with siRNA decreased invasiveness in SCC-25 and FaDu cells significantly. In CAL-27 cells invasion was decreased as well, even though not significantly. This could be due to the fact that CAL-27 expresses less FOXM1 and have a low invasion rate making further depletion of FOXM1 less effective. In the conducted rescue experiment, where re-expression of FOXM1 reestablished invasion of FOXM1-depleted HNSCC cells, we demonstrated that our previously observed results were FOXM1-specific and not caused by off-target effects.

FOXM1 has been implicated in invasive processes of cervical (He SY et al., 2012), pancreatic (Huang C et al., 2012), ovarian (Lok TM et al., 2011), colorectal (Chu XY et al., 2012), and breast (Ahmad A et al., 2010) cancer. Invasion of tumor cells into the surrounding tissue is a crucial process for the early development of head and neck tumors and for the development of locoregional recurrences (Fagan JJ et al., 1998; Hatano H et al., 2008). Since in our study we show that FOXM1 regulates invasion of head and neck cancer cells, it implicates that FOXM1 is involved in early carcinogenesis, and the development of recurrent head and neck tumors by enhanced invasion. Our findings were confirmed by other studies, suggesting a contribution of FOXM1 to early head and neck cancer carcinogenesis induced by tobacco (Gemenetzidis E et al., 2009). The influence of FOXM1 in the development of recurrent tumors, however, still has not been examined.

4.3 FOXM1 mediates invasion of HNSCC cells via the regulation of uPA

Transcription factors are not able to directly modulate processes that are necessary for the destruction of the ECM, cell-cell-, and cell-ECM contacts. Instead, they trigger the activation of downstream genes which have the capability to degrade components of the ECM and by this means facilitate invasion and metastasis of tumor cells. The serine protease uPA, which is a known target of p38, has already been shown to induce invasion, especially in breast cancer (Montero L et al., 1999; Tang L et al., 2012).
FOXM1 has been shown to regulate genes, probably through binding to the conventional FOXM1 binding sites (Wierstra et al., 2006). Analysis of the uPA promoter sequence revealed

that it possesses several of those binding sites. In addition new ChIP-Seq data confirmed the presence of FOXM1 binding peaks within the uPA promoter, making it a preferential target for FOXM1 mediated invasion. Further, western blot analysis showed that the expression of uPA correlates with FOXM1 expression. uPA protein is expressed at high levels in the invasive cell line SCC-25, which also has high FOXM1 levels, while it is less expressed in the cell lines FaDu and CAL-27. Together with the ChIP-Seq data, the correlation between invasiveness, uPA-, and FOXM1 expression suggested that uPA might be a transcriptional target of FOXM1.

To examine if FOXM1 transactivates the uPA promoter in HNSCC, the cell lines FaDu and CAL-27 were transfected with a plasmid, containing the wild-type uPA promoter, which was fused to a CAT reporter (uPA^{-2345}-CAT). In FaDu cells the activity of the uPA promoter was almost 2-fold higher compared to the empty CAT-Basic vector control. uPA activity in CAL-27 cells was also elevated compared to the CAT-Basic transfected control, but less higher than in FaDu. Simultaneous overexpression of FOXM1 increased the activity of the uPA promoter in both HNSCC cell lines significantly compared to the uPA^{-2345}-CAT transfected samples. Upon FOXM1 transfection, the activity of the uPA promoter in FaDu cells was increased 5-fold, while in CAL-27 cells only 3-fold. This can be explained by the fact, that CAL-27 cells have a lower uPA expression compared to FaDu cells, which makes an increased activation after FOXM1 overexpression less effective. More interestingly, according to the Cancer Cell Line Encyclopedia (CCLE) database, in CAL-27 the AP-1 member c-Fos has been shown to be mutated at the position p.E137K.

Transactivation of a promoter region by transcription factors usually causes genes to be expressed. We tested if the observed transactivation of the uPA promoter by FOXM1 has any influence on uPA expression. FOXM1 levels in HNSCC were altered using siRNA-knockdown and overexpression via a FOXM1 expression plasmid. Inhibition of FOXM1 decreased uPA mRNA levels significantly, while overexpression of FOXM1 elevated uPA mRNA levels to a significant extent. Further, uPA protein levels were significantly affected after alteration of FOXM1 expression levels. These data demonstrate that FOXM1 regulates transcription of uPA in HNSCC cells. FOXM1 overexpression leads to a transactivation of the uPA promoter, which in turn enhances its mRNA and protein synthesis.

uPA induces invasion and EMT in cancer cells by converting plasminogen into its active form plasmin, which then degradates ECM components (Tryggvason K et al., 1987). For uPA to become active, tumor cells have to secrete the pro-uPA form into the extracellular space, where it binds the uPAR and is converted into its active form (Matsuoka H et al., 2006). In order to have a significant effect on invasion, the previously shown transcriptional regulation of uPA via FOXM1 should have an influence on the enzymatic activity of uPA in the cell culture

supernatant. Enzymatic based analysis makes it possible to measure the amount of active uPA in the supernatant of cultured cells. Upon inhibition of FOXM1 using siRNA, uPA activity in the supernatant of FaDu cells was decreased to a significant degree, proving that FOXM1 mediates regulation of uPA activity in HNSCC cells. Further, p38 inhibition, using the p38 inhibitor SB203580, had the same effect, confirming previous results from breast tumors, where it has been shown that p38 influences the enzymatic activity of uPA (Montero L et al., 1999).

To confirm that the FOXM1-uPA axis regulates invasion of HNSCC cells, FOXM1 expression was upregulated in FaDu cells, which lead to a significant increase of invasiveness. After cells were simultaneously treated with the FOXM1 expression plasmid plus the uPA inhibitor UK122, there was no significant increase of invasion. Those data clearly show that uPA is necessary for FOXM1-mediated enhancement of invasiveness in HNSCC cells. However, upon inhibition of uPA, FOXM1 overexpression still elevated invasion to some extent, though this increase was not significant. A reasonable answer for this is the fact that FOXM1 has been shown to mediate invasion also via other pathways than uPA. In the oral cavity of squamous cell carcinoma MMP-2 is involved in FOXM1-mediated invasiveness (Chen CH et al., 2009), in breast cancer cells VEGF plays an important role in invasive processes regulated by FOXM1 (Ahmad A et al., 2009).
Our findings demonstrate that uPA is a critical factor in FOXM1 mediated invasiveness of HNSCC cells. FOXM1 transactivates the uPA promoter, leading to an increase of its expression and consequently enzymatic activity, and finally elevates *in vitro* invasion of HNSCC cells. However, the exact mechanism of FOXM1-mediated regulation of uPA still needs to be examined.

4.4 FOXM1 regulates expression of uPA in a Ras-depended manner

Transfection of mouse fibroblast NIH3T3 cells with the wild-type uPA promoter construct driven CAT reporter (uPA^{-2345}-CAT), did not reveal any basal uPA promoter activity as compared to the control CAT-Basic vector. Overexpression of FOXM1 in the wild-type NIH3T3 cells elevated the activity of the uPA promoter, but not more than 2-fold. However, in NIH3T3 mouse fibroblasts with an active Ha-Ras background FOXM1 overexpression elevated uPA promoter activity to more than 10-fold compared to the negative control, indicating that active Ras is necessary for FOXM1 to activate the uPA promoter efficiently and that it plays an important role in FOXM1-

mediated tumorgenesis. Generally, K-Ras mutations occur in only less than 5% of HNSCC patients (Langer CJ 2012), whereas Ha-Ras mutations were found in about 22% of HNSCC patients, predominantly in patients from India and Southeast Asia (Anderson AA et al., 1994; Kiaris H et al., 1995). While mutations rarely occur, overexpression of members of the Ras protein family is a relatively common event in HNSCC with 65% positive incidence for Ha-Ras, 45% for K-Ras, and 32% for N-ras (McDonald JS et al., 1994). In total, amplification of wild-type Ha-Ras or K-Ras, together with mutations of Ras family members, occur in over 80% of HNSCC tumors (Bornstein et al., 2009). This deregulation of the Ras pathway plays an important role in the initiation and progression of HNSCC (Caulin C et al., 2004; Hoa M et al., 2002). These findings were further confirmed by results obtained together with our collaboration partners from Cincinnati. Tumors from transgenic mice which overexpress K-Ras, FOXM1, or K-Ras plus FOXM1 together, were analyzed for the expression of AP-1 members, uPA and for tumor growth. While with overexpression of FOXM1 or K-Ras alone there was no increase in uPA expression or tumor growth, mice with FOXM1 plus K-Ras overexpression displayed significantly elevated mRNA levels of uPA and increased tumor growth. Expression levels of AP-1 members remained unchanged in all three cohorts (Misetic et al., under submission). Recent publications from other groups support our findings. Bornstein et al. showed that in Smad4 knockout mice an additional activation of Ras (*K-ras*^{G12D}Smad4$^{+/-}$) is needed to develop HNSCC rapidly, while neither heterozygous deletion of Smad4 nor K-Ras mutations alone were able to induce HNSCC formation (Bornstein S et al., 2009). In another study activation of either K-Ras or H-Ras only in combination with TGFβRII deletion lead to the formation of HNSCC tumors, which displayed increased invasiveness, inflammation, and angiogenesis (Lu SL et al., 2006). In summary, these findings demonstrate that overexpression of FOXM1 or Ras alone is not sufficient for a strong induction of uPA expression and therefore tumor invasion. Instead a “second hit” is necessary to induce a malignant and invasive phenotype more efficiently.

4.5 FOXM1 regulates uPA via AP-1

The molecular mechanism of the FOXM1-mediated regulation of uPA expression remains to be clarified. Based on the occurrence of conventional Forkhead-specific recognition motifs in the promoter region of the uPA gene we assumed that FOXM1 binds to this sequence. However, our data from this study indicates that FOXM1 binds to another region, where also a well-known AP-1 binding site is located (Verde P et al., 1988). Analyzing the public FOXM1 ChIP-Seq data from

five human tumor cell lines, strong enrichment of the AP-1 recognition motif has been obtained for this region. Additional bioinformatical data revealed that AP-1 recognition motifs are gerally more frequent near the center of FOXM1 binding peaks. In consideration of those new findings FOXM1 might either activate directly the uPA promoter or via AP-1 members.

To examine if FOXM1 does influence AP-1 activity at all, we transfected HNSCC cells with a plasmid containing AP-1 responsive elements fused to a CAT reporter (5xTRE-CAT). FOXM1 levels were altered using siRNA knockdown or overexpression via an expression plasmid. FOXM1 depletion decreased overall AP-1 activity significantly, while FOXM1 overexpression lead to a significant increase of AP-1 activity. Phorbol 12-myristate 13-acetate (PMA) is a well known potent AP-1 activator (Angel and Karin, 1991), which mediates a wide range of cancer-specific phenotypes via enhanced AP-1 activity, like transformation of epidermal cells (Dong Z et al., 1994; Bruder JT et al., 1992), or cellular invasion (Dong Z et al., 1997). PMA has also been shown to involve the Ras or ERK in the activation of AP-1 (Katagiri et al., 1994; Frost JA et al., 1994). We hypothesized that FOXM1 might also be involved in PMA-mediated activation of AP-1. PMA stimulation of HNSCC cells elevated AP-1 significantly compared to control. Simultaneous inhibition of FOXM1 via siRNA inhibited PMA-induced AP-1 activity significantly, but only to a weak (0.4-fold) extent. We show that FOXM1 partially is involved in PMA-mediated activation of AP-1. The fact that FOXM1 knockdown only weakly decreased PMA-stimulated AP-1 activity, can be explained by the fact that PMA activates several pathways (Huang TS et al., 1995), which can activate AP-1 independently of FOXM1.

The activity of AP-1 can be regulated through increased expression, phosphorylation of their subunits, or by other post-transcriptional modifications (Karin M et al., 1995; Ozolins et al., 1999; Biswal S et al., 2002; Fujioka S et al., 2004). After it has been demonstrated that FOXM1 enhances AP-1 activity, we raised the question if FOXM1 modulates AP-1 activity by increased transcription of its main protein members, or by mediating phosphorylation of its subunits. Observations from transgenic mice, obtained in cooperation with our partners from Cincinnati, revealed that neither K-Ras or FOXM1 overexpression, nor simultaneous overexpression of both genes increased the expression of the main AP-1 member's c-Fos, c-Jun, or ATF-2 (Misetic et a., under submission). Therefore, we performed western blots in A549 cells where FOXM1 expression was altered and checked the expression and phosphorylation status of the main AP-1 members. After inhibition or upregulation of FOXM1, there was no change in expression levels of c-Fos or c-Jun, except a slight decrease of c-Jun expression after FOXM1 overexpression. However, after FOXM1 overexpression an increase in phosphorylation of c-Fos and c-Jun was observed. Those data indicate that FOXM1 leads to an activation of AP-1 via phosphorylation of its subunits and not via elevated expression levels. Our findings mirror other publications by

which activation of AP-1 via MAPKs is regulated through increased phosphorylation (Karin M et al., 1995). Further, it has been demonstrated that the AP-1 subunit c-Fos is phosphorylated by members of the p38 MAPK family (Tanos T et al., 2005), which in our work have been shown to regulate FOXM1, integrating it into this regulatory axis. Nevertheless, in further studies it still has to be examined which kinase is involved in the FOXM1 mediated phosphorylation of AP-1.

After it has been demonstrated that FOXM1 regulates AP-1 activity and that the uPA promoter possesses several AP-1 binding sites, we obtained deletion mutants of the uPA wild-type promoter. The deletion mutant uPA^{-1870}-CAT only possessed 1870 basepairs of the original 2345 basepair long wild-type uPA promoter, and lacked the AP-1 binding sites. CAT-Assays showed that after deletion of this promoter region, uPA promoter activity dropped down significantly, and FOXM1 overexpression was not able to increase uPA promoter activity further. This demonstrated that the region 1870-2345 of the uPA region is crucial for its activation via FOXM1. Since several AP-1 binding sites are suited within this region, it becomes evident that AP-1 is necessary for FOXM1 mediated activation of the uPA promoter.

To further prove our findings, we performed ChIP-Assays using antibodies against FOXM1, c-Fos and primers spanning the region where the AP-1 binding sites are located (Region A). In addition we used primers that also span a region further downstream (Region B) that harbors the putative Forkhead-specific recognition motifs, which were discovered by less precise random-oligonucleotide sequencing (Yao KM et al., 1997). Analyzing Region B of the uPA promoter, no binding of either FOXM1 or c-Fos was observed. Since there are no AP-1 recognition motifs in this region, it is not surprising that no c-Fos binding was observed. The lack of FOXM1 binding in this region, though it contains the conventional Forkhead-specific recognition motif , could be due to the fact that the FOXM1 ‘winged helix’ DNA-binding domain lacks typical features required for DNA interaction and, as result, binds only weakly to TAAACA sequence specific for Forkhead family members, as it has recently been discovered (Littler et al., 2010). It is also very likely that there are no FOXM1 binding sites in this region at all, since bioinformatic analysis of the FOXM1 Chip-Seq data revealed that FOXM1 binding sites are located within the Region A and not B. Indeed, in A549 cells weak FOXM1 binding signal has been observed in the Region A. The binding intensity could not be further increased by overexpression of FOXM1, showing that FOXM1 weakly binds to this region and it is not involved directly in the activation of the uPA promoter. For c-Fos weak binding within this region in the A549 cells was observed as well. However, after FOXM1 overexpression the binding signal increased drastically, indicating that FOXM1 activation induces the binding of c-Fos to the uPA promoter.
To confirm that AP-1 is involved in FOXM1 mediated invasion, we performed invasion assays where FOXM1 and c-Fos levels were modulated. Overexpression of FOXM1 increased

invasiveness of HNSCC cells as expected. Upon c-Fos inhibition FOXM1 was not anymore able to increase invasion of HNSCC cells, indicating that AP-1 is involved in FOXM1 mediated invasion.
Taken together, we show that uPA possesses both AP-1 and FOXM1 binding sites within the promoter Region A. Overexpression of FOXM1 leads to phosphorylation of the main AP-1 members, by a yet unknown mechanism. Upon phosphorylation, the AP-1 members are recruited to the AP-1 binding site of the uPA promoter, which is in direct proximity to the FOXM1 binding site, and initiate the transactivation of the uPA promoter.

These observations suggest that FOXM1 does not bind as a single transcription factor and thus activates transcription of target genes. Due to its weak binding to the conventional TAAACA sequence (Littler et al., 2010), it is conceivable that transcriptional activity of FOXM1 relies on the interaction with other DNA-binding proteins, which recruit FOXM1 to its target genes. In that case, FOXM1 specificity is mainly determined by its interaction partners, for example by NfkB1 that interacts with FOXM1 upon doxorubicin treatment to confer drug resistance (Park YY et al., 2012) and or by the MMB transcriptional activator complex that together with FOXM1 regulates cell cycle related genes (Chen X et al., 2013). This principal has also been discovered for other genes. For TGFβ-mediated invasion the interactions between Smad2/3 and Fra1 is required to induce the binding of Smad proteins to the promoter of PAI-1 and MMP-10 (Sundqvist A et al., 2012).
It would also explain why FOXM1 is often described as a “so-called” master regulator, playing a dual role enabling the regulation of proliferation and invasion and the transition between them. By interacting with different transcription factors and thereby binding different promoters, FOXM1 triggers different cellular actions, depending on the required phenotype. Thereby, FOXM1 contributes to a balance of invasively growing tumors, where individually migrating cells and groups of collectively moving cells can switch between ‘go’ and ‘growth’ states (Raychaudhuri P et a., 2011; Wierstra I et al., 2007).

4.6 The relevance of the FOXM1-uPA for cancer and tumor recurrence

In our study, by the use of *in vitro* experiments and mouse models, we demonstrated that FOXM1-mediated invasion via uPA is a general mechanism, which could be observed in several cell systems. In order to examine the clinical significance of our findings, we analyzed several bioinformatical data sets from patients with different tumors and compared FOXM1 and uPA expression. Indeed, FOXM1 and uPA were concordantly upregulated in preinvasive lesions and malignant tumors of the oral cavity, esophagus, cervix, and lung. This confirms our findings, that FOXM1 dependent regulation of uPA is a general mechanism, underlying malignant progression in diverse tumor entities. Positive correlation between high FOXM1 and uPA levels in a broad range of tumors and preinvasive lesions substantitates FOXM1 and uPA as a prognostic factor for poor outcome, recurrent tumor development, and as a potential target for adjuvant therapy (Koo CY et al., 2012; Halasi M et al., 2013).

Using our mouse model of oral cancer, based on transplantation of SCC7 cells, we wanted to examine if there is a differentiated expression of SAPKs, FOXM1, and uPA between SCC7-induced primary tumors and recurrent tumors. Immunohistochemical staining did not reveal any differences in expression of FOXM1 and p38 in recurrent tumors compared to the primary tumors. uPA expression, however, was elevated in recurrent tumors. These data were all confirmed by RT-PCR analysis (performed by Dr. Peter Hofner; data not shown). We explain these observations by the fact that unlike uPA, MAPKs and FOXM1 are mainly regulated through phosphorylation than by change in their expressional level (Chen YJ et al., 2009; Shetty SK et al., 2011), though for FOXM1 regulation via alteration of expression levels has also been shown to play a role (Behren et al., 2010; Millour J et al., 2011). Since antibodies for detection of the phosphorylated form of FOXM1 in paraffin sections are not available, it was not possible to measure phospho-FOXM1 levels in primary and recurrent mouse tumors. However, in immunohistostainings (performed by Babitha George; data not shown), an increase in phospho-ERK and phospho-c-Jun levels in recurrent tumors has been observed. Since we have shown that FOXM1 regulates invasion of HNSCC tumors via elevated uPA expression and uPA mRNA and protein levels were upregulated in recurrent tumors, as well as phosphorylated members of the MAPK and AP-1 family, it is evident that the MAPK-FOXM1-AP1-uPA axis is involved in the development of recurrent head and neck tumors. Further experiments will be performed to abalyze the exact mechanism of how FOXM1 is involved in the development of recurrent tumors.

4.7 Conclusions

The initial aim of this thesis was to analyze if p38 and FOXM1 regulate invasion of HNSCC tumors and to decipher the exact mechanism. We have been able to successfully provide compelling evidence for a crucial role of FOXM1 in transcriptional regulation of uPA, an important regulator of tumor invasion. Using chemical inhibitors, we identified FOXM1 as a downstream target of p38. We also showed that FOXM1 regulates invasion via enhanced expression, and proteolytic activity of uPA and that AP-1 is involved in this process. We therefore suggest a new mechanism by which stress-activated p38, through increasing FOXM1 protein levels, directs FOXM1-dependent activation of the AP-1 complex through phosphorylation. This activation leads to the expression of AP-1-regulated genes like uPA, which facilitate the invasive phenotype of HNSCC cells (Figure **52**).

We confirmed our findings in several epithelial cancer cell lines by the use of *in vitro* and *in vivo* models and bioinformatics. Analyzing data sets from patients with different tumors entities, we observed a concordant upregulation of both FOXM1 and uPA in all preinvasive and malignant tumor samples, suggesting a general role for the FOXM1-uPA axis in tumor development. In recurrent tumors we observed an upregulation of uPA, indicating that the FOXM1-mediated invasion through upregulation of uPA expression plays a role in the development of recurrent HNSCC tumors. *In vitro* experiments, mouse models, bioinformatical analysis, clinical patient data all confirm our findings. Nevertheless, there are still unknown factors within the cascade that need to be further investigated. More importantly, the role of FOXM1 in the development of recurrent tumors has to be examined in more detail and to be proven by additional experiments. Once it the exact mechanisms underlying the MAPK-FOXM1-uPA cascade in recurrent tumor development have been completely revealed, biological agents, targeting these specific pathways, can be tested in order to decrease the incidence of recurrent tumor development and thus to improve the survival of head and neck cancer patients.

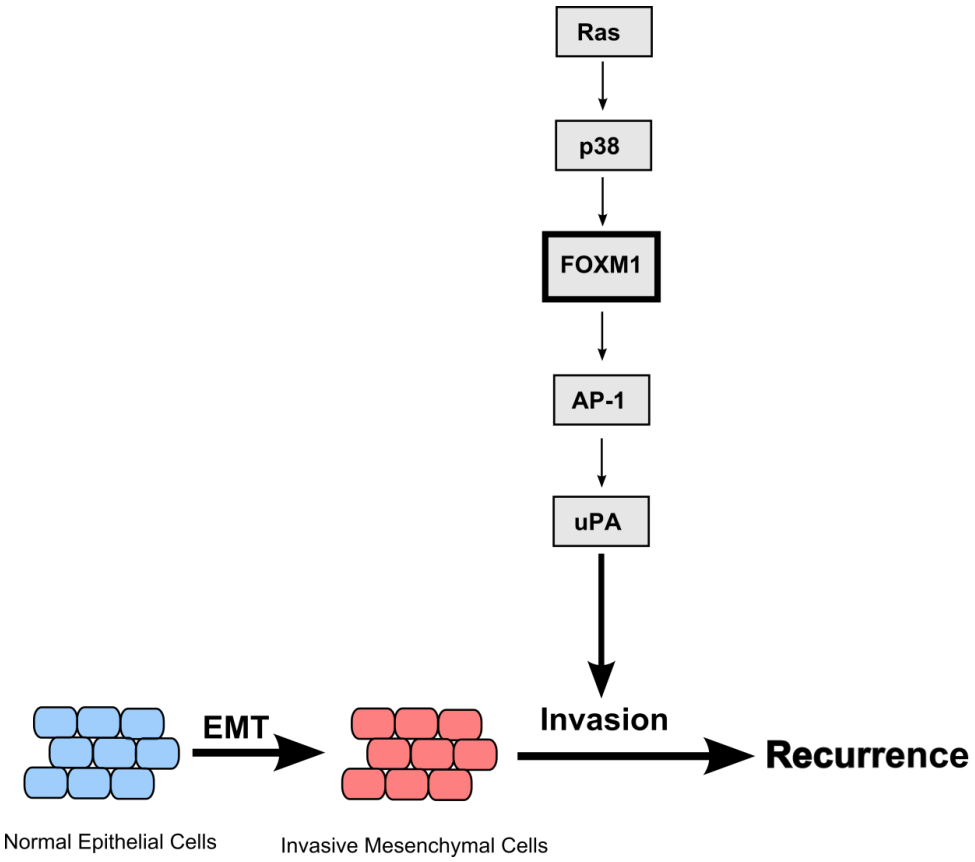


Figure 52: Summary of the findings gained within this work. The scheme illustrates a new mechanism, discovered in this work, of how the p38-FOXM1 axis regulates invasion of HNSCC cells. Under the control of Ras-MAPK(p38) signaling FOXM1 regulates its downstream target uPA via an AP-1-dependent mechanism. This mechanism potentiates invasiveness of HNSCC cells, contributing to the development of recurrent tumors. Recent data indicates that FOXM1 might also be involved in the process of EMT. Further studies have do be conducted to proof this hypothesis.

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AFFIDAVIT

I hereby declare that I have drawn up this thesis autonomously and without illicit assistance.

No supplemental materials or references other than the ones specified have been used.

Heidelberg, February 2013

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Vinko Misetic