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ROLE AND FUNCTION OF c-JUN PROTEIN COMPLEX IN CANCER CELL BEHAVIOR

by

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To Herman, Nuutti-Noël and Sampo,
my three best biological achievements,
and Sirpa.

"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

Sir Winston Churchill (1874-1965) following the victory at El Alamein. London, November 10th 1942.

Antoine MIALON
Role and function of c-Jun protein complex in cancer cell behavior

ABSTRACT

Transcription factors play a crucial role in the regulation of cell behavior by modulating gene expression profiles. Previous studies have described a dual role for the AP-1 family transcription factor c-Jun in the regulation of cellular fate. In various cell types weak and transient activations of c-Jun N-terminal kinase (JNK) and c-Jun appear to contribute to proliferation and survival, whereas strong and prolonged activation of JNK and c-Jun result in apoptosis. These opposite roles played by c-Jun are cell type specific and the molecular mechanisms defining these antonymous c-Jun-mediated responses remain incompletely understood.

c-Jun activity in transformed cells is regulated by signalling cascades downstream of oncoproteins such as Ras and Raf. In addition, the pro-proliferative role and the survival promoting function for c-Jun has been described in various cancer models. Furthermore, c-Jun was described to be overexpressed in different cancer types. However, the molecular mechanisms by which c-Jun exerts these oncogenic functions are not all clearly established. Therefore it is of primary interest to further identify molecular mechanisms and functions for c-Jun in cancer.

Regulation of gene expression is tightly dependent on accurate protein-protein interactions. Therefore, co-factors for c-Jun may define the functions for c-Jun in cancer. Identification of protein-protein interactions promoting cancer may provide novel possibilities for cancer treatment. In this study, we show that DNA topoisomerase I (TopoI) is a transcriptional co-factor for c-Jun. Moreover, c-Jun and TopoI together promote expression of epidermal growth factor receptor (EGFR) in cancer cells. We also show that the clinically used TopoI inhibitor topotecan reduces EGFR expression. Importantly, the effect of TopoI on EGFR transcription was shown to depend on c-Jun as $Jun^{-/-}$ cells or cells treated with JNK inhibitor SP600125 are resistant to topotecan treatment both in regulation of EGFR expression and cell proliferation.

Moreover, c-Jun regulates the nucleolar localization and the function of the ribonucleic acid (RNA) helicase DDX21, a previously identified member of c-Jun protein complex. In addition, c-Jun stimulates rRNA processing by supporting DDX21 rRNA binding. Finally, this study characterizes a DDX21 dependent expression of cyclin dependent kinase (Cdk) 6, a correlation of DDX21 expression with prostate cancer progression and a substrate binding dependency of DDX21 nucleolar localization in prostate cancer cells.

Taken together, the results of this study validate the c-Jun-TopoI interaction and precise the c-Jun-DDX21 interaction. Moreover, these results show the importance for protein-protein interaction in the regulation of their cellular functions in cancer cell behavior. Finally, the results presented here disclose new exciting therapeutic opportunities for cancer treatment.

TIIVISTELMÄ

Transkriptiotekijät ovat tärkeässä osassa solun toiminnan säätelyssä, muunnellen solun geenien ilmentymisprofiileja. Aiemmissa tutkimuksissa on kuvattu AP-1 transkriptiotekijä c-Junin kaksinaisrooli solun kohtalon säätelyssä. Normaaleissa soluissa solustressi aktivoi JNK (eng. c-Jun N-terminal kinase) viestiketjun, joka puolestaan johtaa c-Junin aktivoitumiseen ja ohjelmoidun solukuoleman käynnistymiseen. Transformoituneissa soluissa JNK-välitteinen c-Jun aktiivisuus sen sijaan stimuloi solujen proliferaatiota ja selviytymistä.

Useat onkogeeniset signaalinvälitystiet lisäävät c-Jun:in aktiivisuutta syöpäsoluissa, ja c-Jun proteiinin lisääntynyttä ilmentymistä on osoitettu useassa ihmisen syöpätyypissä. Vaikka c-Jun:in onkin osoitettu useissa malleissa lisäävän syöpäsolujen proliferaatiota ja selviytymistä, molekyylitason mekanismit jotka osallistuvat c-Jun välitteiseen solujen kasvun säätelyyn ovat vielä epäselviä. Tämän vuoksi olisi tärkeää pyrkiä karakterisomaan c-Jun:in toimintamekanismeja syövässä.

Aikaisemmat työt ovat osoittaneet että transkriptiotekijöiden kanssa interaktoivat proteiinit voivat muuntaa niiden aktiivisuutta Siten on oletettavaa että myös c-Jun:in ko-faktorit voivat selittää miksi solut reagoivat eri tavalla c-Jun:in aktiivisuutta lisääviin ärsykkeisiin. Tässä tutkimuksessa osoitamme, että topoisomeraasi I (TopoI) on c-Jun:in ko-faktori EGFR:n (eng. epidermal growth factor receptor) ilmentymisen säätelyssä syöpäsoluissa. Lisäksi c-Jun säätelee ribonukleiinihappo (RNA) helikaasi DDX21:n, aiemmin identifioidun c-Jun kompleksin proteiinin, tumajyväslokalisaatiota ja toimintaa. Tutkimuksen yhtenä tärkeimpänä havaintona osoitetaan että c-Jun edistää ribosomaalisen RNA:n (rRNA) prosessointia vaikuttamalla DDX21:n tumajyväslokalisaatioon. Tämä tulos paljastaa täysin uuden geenin luennasta riippumattoman mekanismin jolla c-Jun säätelee syöpäsolujen toimintaa. Lopuksi, tämä tutkimus karakterisoi DDX21 riippuvaisen CDK6:n (eng. cyclin dependent kinase 6) ilmentymisen, DDX21:n ilmentymisen ja eturauhassyövän etenemisen välisen korrelaation ja DDX21:n substraattiriippuvaisen tumajyväslokalisaation eturauhassyöpäsoluissa.

Yhteenvetona tämän tutkimuksen tulokset tarjoavat uutta tietoa mekanismeista jotka osallistuvat c-Jun välitteiseen proliferaation säätelyyn. Tutkimuksen tulokset antavat myös uutta tärkeää tietoa c-Junin kanssa vuorovaikutuksessa olevien proteiinien roolista syöpäsolun viestinvälityksessä. Mikä tärkeintä, tässä esitetyt tulokset tuovat esiin uusia terapeuttisia mahdollisuuksia syövän hoitoon.

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ABBREVIATIONS

AA	Amino acid
ADPR	Adenosine diphosphate ribosylation
Amp	Ampicillin
AP-1	Activator protein 1
ATF	Activating transcription factor
ATP	Adenosine 5'-triphosphate
Bcl2	B cell lymphomas 2
BRCA1	Breast cancer protein 1
BRCA2	Breast cancer 2
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
bZIP	Basic leucine-zipper domain
C/EBPβ	CCAAT/enhancer binding protein beta
cAMP	Cyclic adenosine monophosphate
CBP	CRE binding protein
CD95-L	CD95-ligand
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin dependent kinase inhibitor 2A
CEF	Chicken embryonal fibroblasts
CNS	Central nervous system
COBRA1	Cofactor of the breast cancer susceptibility gene 1
CRE	cAMP response element
CREB/ATF	CRE binding protein
DBD	DNA-binding domain
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DRE	Differentiation response element
DRF	Differentiation response factor
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid

EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
FBJ	Finkel, Biskis, Jinkins
FBR	Finkel, Biskis, Reilly
Fbw7	F-box and WD repeat domain-containing 7
FCS	Fetal calf serum
Fra-1	Fos related antigen-1
Fra-2	Fos related antigen-2
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase 3
GST	Glutathione S-transferase
hAR	Human androgen receptor
HB-EGF	Heparin-binding epidermal growth factor
HDAC3	Histone deacetylase 3
HEK	Human embryonal kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG	High mobility group
HR	Hormone refractory
hTERT	Human telomerase reverse transcriptase gene
huCOP1	Human constitutive photomorphogenesis protein 1
IL-4	Interleukin 4
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
LB	Luria-Bertani
LPS	Lipopolysaccharide
MAF	Musculoaponeurotic fibrosarcoma
MAPKKK	Mitogen-activated protein kinase kinase kinase
MDM2	Murine double minute 2
MEF	Mouse embryonal fibroblasts
MEKK1	Mitogen activated protein kinase kinase kinase 1
MEN1	Menin

MIF	Migration inhibitory factor
MLK	Mixed lineage kinase
mRNA	Messenger RNA
NLS	Nuclear localization sequence
NOR	Nucleolar-organizing region
NPC	Nasopharyngeal carcinomas
NQO	4-nitroquinolineoxide
NSCLC	Non-small cell lung carcinoma
NSF	Normal skin fibroblasts
OTF	Octamer transcription factor
PCD	Programmed cell death
PI	Protease inhibitors
PMSF	Phenylmethylsulphonylfluoride
Pol II	RNA polymerase II
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Room temperature
S	svedberg
SAPK	Stress activated protein kinase
SCC	Squamous cell carcinomas
SCLC	Small-cell lung cancer
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
SV40	Simian virus 40
TAD	Trans-activating domain
TAFs	Tightly associated factors
TAP	Tandem affinity purification
TBP	TATA-binding protein

TFII	Transcription factor for RNA polymerase II
TFIID	Transcription factor for RNA polymerase II D
TFIIF	Transcription factor for RNA polymerase II F
TFIIH	Transcription factor for RNA polymerase II H
TGFα	Transforming growth factor alpha
TIC	Transcription initiation complex
Topo3α	Topoisomerase 3α
Topo3β	Topoisomerase 3β
TopoI	Topoisomerase I
TopoIcc	TopoI cleavable complex
TopoIMT	Mitochondrial topoisomerase I
TPA	12-*O*-tetradecanoyl-phorbol 13-acetate
TRE	TPA response element
tRNA	Transfer RNA
UV	Ultraviolet

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-III.

I. Antoine Mialon, Matti Sankinen, Henrik Söderström, Teemu T. Junttila, Tim Holmström, Riku Koivusalo, Anastassios C. Papageorgiou, Randall S. Johnson, Sakari Hietanen, Klaus Elenius, and Jukka Westermarck. 2005. DNA Topoisomerase I Is a Cofactor for c-Jun in the Regulation of Epidermal Growth Factor Receptor Expression and Cancer Cell Proliferation. Mol Cell Biol. 25(12):5040-5051.

II. Tim H. Holmström, Antoine Mialon, Marko Kallio, Yvonne Nymalm, Leni Mannermaa, Tina Holm, Henrik Johansson, Elizabeth Black, David Gillespie, Tiina A. Salminen, Ülo Langel, Benigno C. Valdez, and Jukka Westermarck. 2008. c-Jun supports ribosomal RNA processing and nucleolar localization of a RNA helicase DDX21. J. Biol. Chem. 283(11):7046-7053.

III. Antoine Mialon, Kati Porkka, Tim Holmström, Tapio Visakorpi, and Jukka Westermarck. 2008. The nucleolar RNA helicase DDX21 correlates with prostate cancer progression and regulates Cdk6 expression. Manuscript.

INTRODUCTION

"Growth for the sake of growth is the ideology of the cancer cell." This quote from the American writer Edward Abbey (1927-1989) might summarize appropriately the nature of cancer. However, regardless of the efforts and discoveries during the last decades to find better cancer treatments, this disease is still affecting worldwide 10.9 million new patients each year and 6.7 million persons die from the disease (http://info.cancerresearchuk.org). The variety of cancer types and patient profiles complicates the treatment possibilities. It is therefore of primary importance to precisely identify the molecular properties of each cancer type in order to develop new cancer treatments.

Cancer genetics from the late 1980´s has described the importance of oncogenic transcription factors in the regulation of genes involved in cancer progression. More detailed analyses have shown that the composition of protein complexes formed on the promoter regions of overexpressed genes modifies gene activity. The AP-1 transcription factors c-Jun was described to positively regulate cancer growth survival and proliferation. In addition c-Jun is overexpressed in various cancer types. However the molecular mechanisms by which c-Jun exerts positive role in cancer behavior remain unclear.

In this study we analyzed the role for DNA topoisomerase I (TopoI)-c-Jun interaction and DDX21-c-Jun interaction in the regulation of cancer cell behavior. Most important results of this study revealed that TopoI-c-Jun interaction positively regulates epidermal growth factor receptor (EGFR) expression and cancer cell proliferation. In addition we show that inhibition of TopoI by topotecan treatment reduces the expression of EGFR. Importantly, c-Jun$^{-/-}$ cells or cells treated with JNK inhibitor SP600125 were found to be resistant to topotecan treatment.

Nucleolar RNA helicase DDX21 has been shown previously to function as a transcriptional co-factor for c-Jun. However, whether c-Jun in turn regulates DDX21 functions have not been studied yet. Here, we demonstrate that c-Jun regulates the nucleolar localization of DDX21. We also demonstrate that c-Jun stimulates rRNA processing by supporting DDX21 rRNA binding. Importantly, these results characterize a novel non-transcriptional role for c-Jun. Finally, we analyze the clinical roles for DDX21 in prostate cancer and we show that DDX21 nucleolar recruitment correlates with prostate cancer progression and stimulates cyclin dependent kinase (CDK)-6 expression.

Taken together, these results identify novel roles for c-Jun, TopoI and DDX21 in the positive regulation of cancer cell behavior. Moreover, based on the results of this work, it is clear that the oncogenic functions of c-Jun are modulated by its interaction with Topo I and DDX21. Therefore, targeting of c-Jun interactions with TopoI and DDX21 are discussed as a novel approach for cancer treatment.

REVIEW OF THE LITERATURE

1 Regulation of gene expression

A cell has to constantly perform its attributed duties, and in order to fulfill these requirements, proteins are synthesized on demand. The information coding for the correct protein sequence is stored in the deoxyribonucleic acid (DNA) in the nucleus of the cell. Therefore, the mechanism converting DNA to proteins is a key component of the proper functionality of a cell. These mechanisms are divided into two main stages: transcription from DNA to ribonucleic acid (RNA), and translation from RNA to protein. Transcription and translation are subjected to regulation in order to process the correct information at the required time.

DNA consists of two complementary polynucleotides chains held together by hydrogen bonds. The three-dimensional structure of DNA forms a double helix condensed in chromosomes which are composed of nucleosomes. Nucleosomes are composed of a short length of DNA wrapped around a core of histone proteins. Nucleosomes are the basic units of the chromosome structure and represent a higher degree of DNA compaction. Initiation of transcription requires a release of this DNA compaction and modulation of histone post-translational modifications to activate the transcription machinery. Thereafter, one strand of DNA is used as template for RNA transcription. The strand of RNA produced is complementary to the template DNA strand, as in DNA replication. Nonetheless, the single stranded RNA transcript does not form hydrogen bonds with the template and, once the RNA synthesis is completed, the DNA double helix can re-form. Several categories of RNA transcripts are produced. The messenger RNAs (mRNAs) carry the information for protein synthesis and are used as templates for translation into proteins.

Transcription is performed by a group of enzymes called RNA polymerases. Three types of RNA polymerases (I, II and III) exist, each with distinct functions. RNA polymerase I is used to transcribe ribosomal RNA (rRNA) genes (5.8S, 18S and 28S). RNA polymerase II (Pol II) transcribes all protein-coding genes, small nucleolar RNAs (snoRNAs) genes, and part of the small nuclear RNAs (snRNAs) genes. Finally, RNA polymerase III transcribes transfer RNAs (tRNAs) genes, 5S rRNA gene and other snRNAs. However, not all of DNA is transcribed into RNA. DNA is divided into two principal categories, non-coding and coding sequences (Fig.1). The majority of the genome is composed of non-coding sequences whereas the coding sequence represents just a small percent of all the DNA content of a cell. Despite this fact, the non-coding sequences, by housing various types of DNA elements, play important roles in the regulation of gene expression, evolution and development (Shapiro and von Sternberg, 2005; Taft et al., 2007). In

addition, non-coding sequences have been found to be DNA spacers allowing protein transcription complexes to bind the promoter region of the gene to transcribe.

Coding and non-coding sequences alternate all along the genome. Thus, RNA polymerases need a distinct signal in the DNA to transcribe a gene accurately. Unlike prokaryotes, eukaryotic Pol II requires additional proteins, called general transcription factors or transcription factor for RNA polymerase II (TFII), in order to initiate transcription. These transcription factors are protein complexes binding to specific DNA sequences in the promoter region upstream of the transcription start site of the gene. The assembly of the transcription initiation complex (TIC) occurs in a highly organized fashion (Fig.2). Initially, the transcription factor for RNA polymerase II D (TFIID) binds through its TATA-binding protein (TBP) to the TATA box. Generally located 25 nucleotides upstream of the transcription start site, this region is rich in thymine and adenosine nucleotides. Other boxes, such as BRE and INR are found upstream of the transcription start site of genes whereas DPE are downstream from this point. The binding of various transcription factors to these sequences is one of the numerous levels of the regulation of gene expression.

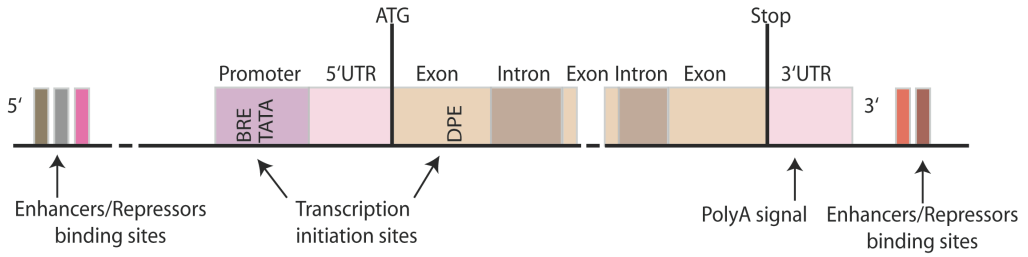

Figure 1. General gene structure.

The binding of TFII changes DNA conformation and serves as a docking site for the assembly of the transcription initiation complex around Pol II. The complex is then guided to the transcription start point with the aid of the transcription factor for RNA polymerase II H (TFIIH) opening the double stranded DNA through its DNA helicase function. The interaction between these proteins allows RNA synthesis to occur once all key effectors are correctly positioned. The final event before the launch of RNA transcript production is the phosphorylation of the Pol II tail by TFIIH which also exhibits a kinase function (Dvir et al., 2001). Pol II is thereafter liberated from the transcription initiation complex and can start transcribing the gene. The regulation of such a complex mechanism occurs at several levels and depends on various factors transiently associated with this protein complex.

The composition of the TIC varies not only between cell types, but also within the tissue it is expressed. Some co-regulators, named tightly associated factors (TAFs), are expressed or repressed in certain cellular contexts. Thereby modulation of gene expression is highly dependent on the nature of the complex members.

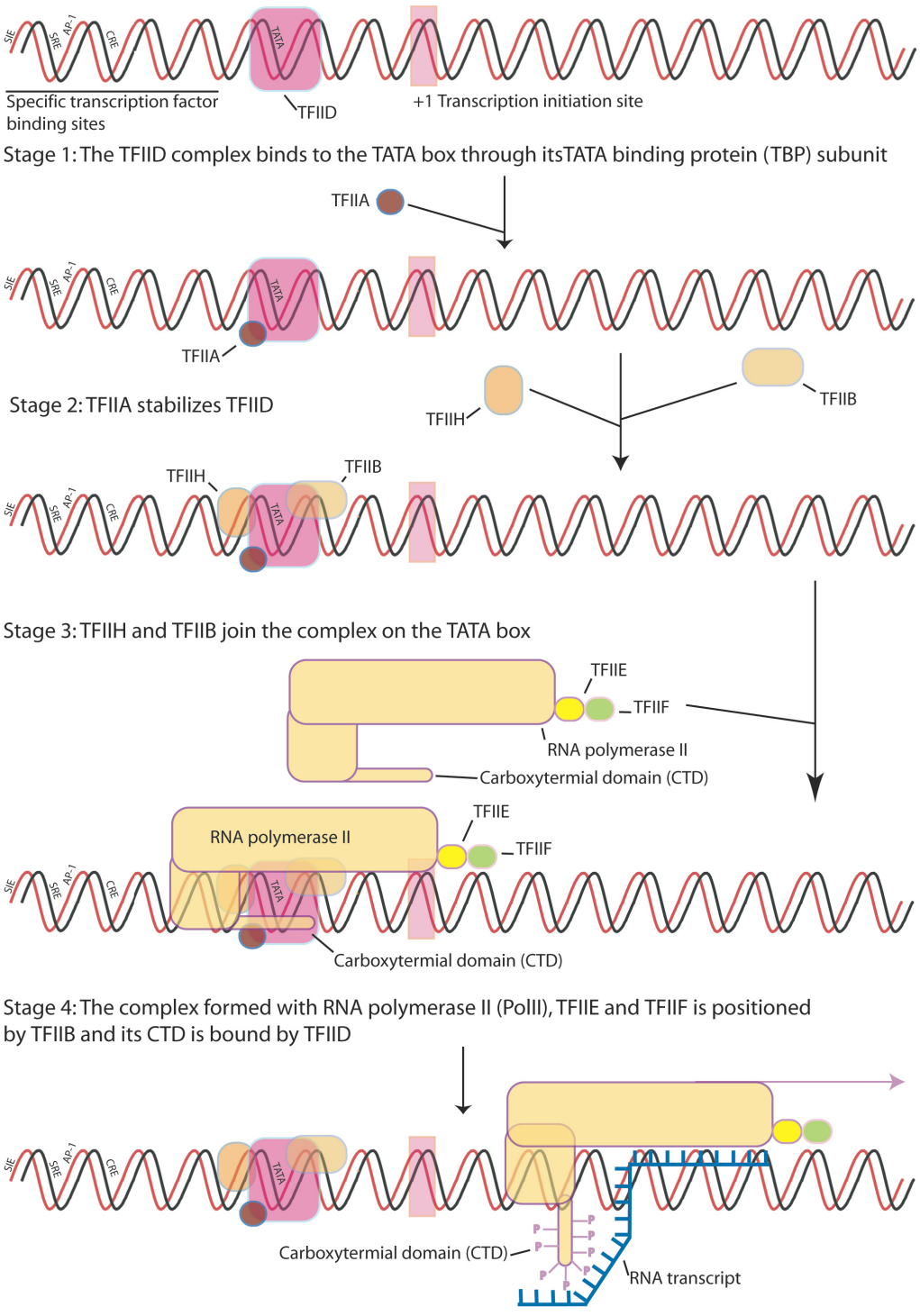

Figure 2. Initiation of transcription.

The formation of the TIC is dependent on the DNA binding of a second type of transcription factors upstream of the TIC assembly site (Fig.1 and 2). Upstream transcription factors are composed of two basic domains: the DNA-binding domain (DBD), and the trans-activating domain (TAD). The DBD is the protein domain where the physical interaction between the protein and DNA occurs. The DNA sequences that bind transcription factors are named response elements (Babu et al., 2004). The TAD contains binding sites for other proteins such as transcriptional co-regulators which tune the activity of upstream transcription factors. The interaction of upstream transcription factors and co-regulator proteins enhances or represses the transcription activity of the TIC.

Upon stimulation of the cellular membrane a signalling cascade is triggered leading to the final activation of the upstream transcription factor by post-translational modifications such as phosphorylation. Other modes of regulation of upstream transcription factor activities are cellular localization, autoregulation of expression, DNA compaction of the promoter site, and interaction of transcriptional co-regulators (Herdegen and Leah, 1998).

Another mode of gene expression regulation by upstream transcription factors consists of modification of the chromatin structure. DNA compaction, and epigenetic modifications such as DNA methylation, and histone post-translational modifications represent important levels of regulation of gene expression and potent mechanism preventing arbitrary transcription of undesired genes (Berger, 2007). DNA is highly compacted into nucleosomes and general transcription factors are not able to bind DNA with this level of compaction. The presence or absence of upstream transcription factor proteins affects the level of DNA compaction and the exposure of the promoter region to the TIC, thereby changing the expression level of the target gene.

Upstream transcription factor proteins might not be able to bind DNA sequences strongly enough. However, together with other weak DNA binders belonging to the same transcriptional complex, they can actively bind DNA sequences and enhance transcription. This presents a strong level of gene expression regulation. Indeed, when regulatory proteins bind DNA as dimers, the nature of the dimer directly influences the expression of the target gene.

Once the TIC is formed by the initial binding of the transcription factor and the subsequent binding of co-activator or co-repressor proteins, further regulation of the transcription occurs (Cramer, 2004). An enhancer sequence, where regulatory protein might bind may be far upstream from the transcription start point. This requires that a mediator protein binds the TIC in order to allow the gene transcription to occur. This distant regulatory element acts on the TIC by binding it through a loop formed by the DNA sequence between its binding site and the TIC (Alberts, 2002).

2 AP-1

Among the first established mammalian transcriptions factors, activator protein 1 (AP-1) was originally described as a transcription factor binding to 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA)-inducible enhancer elements of the methallothionein I gene and simian virus 40 (SV40) (Angel et al., 1987; Lee et al., 1987a; Lee et al., 1987b). AP-1 was also described to be activated by various extracellular stimuli (Angel et al., 1987). AP-1 is constituted of different homodimer and heterodimer complexes of the JUN, FOS, activating transcription factor (ATF), and musculoaponeurotic fibrosarcoma (MAF) protein subfamilies (Shaulian and Karin, 2002) Jun proteins also form heterodimers with MyoD, NFat or c-rel transcription factors (Angel and Karin, 1991). The regulation of dimerization was described to be under the control of the mitogen activated protein kinase kinase kinase 1 (MEKK1) (Cuevas et al., 2005). AP-1 proteins share a basic leucine-zipper domain (bZIP) through which they dimerize and a basic DNA binding domain (Bohmann et al., 1987; Landschulz et al., 1988; Maki et al., 1987).

2.1 AP-1 proteins

2.1.1 Fos protein family

The Fos protein family consists of c-Fos, FosB, Fos related antigen-1 (Fra-1) and Fos related antigen-2 (Fra-2) (Matsui et al., 1990; Zerial et al., 1989). c-Fos was identified as the counterpart of viral v-fos isolated from Finkel, Biskis, Jinkins (FBJ) and Finkel, Biskis, Reilly (FBR) murine osteosarcoma viruses (Curran et al., 1982; Curran and Teich, 1982). FosB was later identified as a growth factor induced gene which interacts with Jun proteins to enhance their binding activity (Zerial et al., 1989). Fra-1 and Fra-2 were identified in monocytes and in corneal epithelial cells after TPA activation (Adhikary et al., 2004; Matsui et al., 1990).

All Fos proteins share a bZIP domain and a basic DNA binding domain. However, Fra-1 does not possess a transactivation domain (Matsuo et al., 2000). All Fos proteins have the capacity to form heterodimers with Jun proteins, but they are not able to homodimerize (Zenz et al., 2008). c-Fos has been extensively studied and it is the only family member which possesses an ERK-binding domain called the DEF domain (Murphy et al., 2002). The half-life of Fos protein is short and c-Fos is degraded through the destabilizer domains in the C- and N-terminus by ubiquitin-independent and ubiquitin dependent mechanisms, respectively (Bossis et al., 2003).

Fos proteins show similar and variable expression patterns. In most cell types c-Fos is expressed at very low levels but, as a immediate-early gene, it is rapidly induced upon mitogenic stimulation (Greenberg and Ziff, 1984). The Fra-2 expression pattern differs the most from the other Fos proteins and was found to be expressed in differentiating epithelia (Carrasco and Bravo, 1995). However, all Fos proteins were described to be

commonly expressed in the central nervous system (CNS) and during bone and cartilage development (Carrasco and Bravo, 1995; Dony and Gruss, 1987; Gruda et al., 1996; Karreth et al., 2004; Wagner and Eferl, 2005).

Fos proteins all play a role in oncogenesis. Nevertheless, each protein plays a different role depending on the cancer type. The earliest discovery of the role for Fos proteins in carcinogenesis was observed in osteosarcomas where FBJ murine osteogenic sarcoma virus encoding *v-fos* supports osteosarcoma formation (Grigoriadis et al., 1993). Thereafter, observations from human osteosarcomas showed c-Fos overexpression (Franchi et al., 1998). In breast cancer, a correlation between c-Fos and FosB downregulation, Fra-1 and Fra-2 upregulation and phosphorylation, and mammary carcinoma progression was established (Milde-Langosch et al., 2004). In endometrical carcinomas c-Fos overexpression corresponds to tumor progression and de-differentiation. FosB and Fra-1 expression could not be linked to any prognosis whereas Fra-2 overexpression was found more often in non-endometrioid than in endometrioid tumors (Bamberger et al., 2001). However, recent reports suggest a possible tumor suppressor role for c-Fos in gastric cancer and in hepatocellular tumorigenesis (Jin et al., 2007a; Mikula et al., 2003). Fos proteins have antagonistic roles in cervical cancer. In non-malignant cells, c-Fos is downregulated and Fra-1 suppresses tumourigenicity by forming an AP-1 complex with c-Jun and thereafter reduces the DNA binding capacity of AP-1. In contrast, in aggressive tumors, Fra-1 expression is downregulated and c-Fos level is upregulated (Soto et al., 1999). Skin tumors follow the same scheme, supporting a model in which c-Fos is necessary for the first steps in carcinogenesis and Fra-1 contribute to transformation and invasion (Milde-Langosch, 2005; Serewko et al., 2002).

Fra-1 activation seems to be an early event in the genesis of cancer thyroid although c-Fos and Fra-2 expression does not change (Liu et al., 1999). In ovarian cancer, the opposite observations have been made compared to cervical cancer. c-Fos and Fra-1 play similar positive roles in cell motility and adhesion (Hapke et al., 2003). In mesotheliomas, only Fra-1 expression is induced in an activated extracellular signal-regulated kinase (ERK) dependent manner. This induction is necessary for cell transformation but not sufficient to transform cells alone (Ramos-Nino et al., 2002). Similar to the two previous examples, in colorectal cancer Fra-1 expression is increased; its phosphorylation occurs through the ERK pathway and it is required for the motility and invasiveness characteristics of colon carcinomas (Vial et al., 2003). In esophageal cancer Fra-1 and c-Fos are overexpressed only in well-differentiated tumors compared to poorly differentiated tumors. These results suggest that c-Fos and Fra-1 overexpressions are early events in esophageal cancer (Hu et al., 2001; Wu et al., 2004). In addition, in lung cancer, c-Fos and Fra-1 expressions increase and c-Fos is the strongest predictor of short survival in non-small cell lung carcinoma (NSCLC) (Levin et al., 1995; Milde-Langosch, 2005).

The role of Fos proteins has been studied extensively in several mice models (Zenz et al., 2008). Transgenic, conditional or knockout mice for all different Fos protein members demonstrated as important role for Fos proteins in bone and skin cancer, and brain development. In more details, Fos overexpressing mice develop chondrogenic tumors and mice lacking Fos are deficient for osteoclasts exhibiting osteopetrotic phenotype (Wagner and Eferl, 2005). Mice overexpressing FosB and Fra-1 in osteoblasts develop osteoclerosis (Jochum et al., 2000; Sabatakos et al., 2000). In addition, Fra-2 overexpressing mice result in ocular malformations (McHenry et al., 1998). More importantly, knockout mice for Fra-1 and Fra-2 are lethal indicating a major role for these two proteins in mouse development. Mice conditionally knockout in chondrocyte display cartilage defects, retarded growth and kyphosis-like phenotype (Karreth et al., 2004).

In summary, the former idea that Fra-1 and Fra-2 might negatively regulate oncogenesis was supported by the absence of transactivation domain in their C-terminal end. However, recent evidences describe a role for c-Fos in the early events of carcinogenesis and positive roles for Fra-1 and Fra-2 in cell transformation and invasion.

2.1.2 Jun proteins

The Jun protein family is composed of three members (c-Jun, JunB and JunD) which exhibit distinct functions and, in some cases, play antagonistic roles.

c-Jun

c-Jun is the oldest member of this protein family and being the main focus of this study, it will be extensively described in the next section.

JunB

Although derived from a common ancestor gene, sharing 45% amino acid (AA) sequence homology, *junb* and *c-jun* differ both structurally and functionally (Kockel et al., 2001; Ryder et al., 1989). Even though JunB possesses a JNK docking domain between amino acids 35 and 61 no JNK phosphorylation sites on JunB have been described to date. This indicates that JunB activation is independent of JNK activation. However, the cell cycle specific kinase $p34^{cdc2}$–cyclin B kinase was found to phosphorylate JunB on non-conserved sites on c-Jun and JunD (Bakiri et al., 2000). In contrast with c-Jun, where phosphorylation trans-activates the protein, phosphorylation of JunB triggers its degradation (Fuchs et al., 1997). In T-cells, sumoylation regulates the activity of JunB and the subsequent cellular activation (Garaude et al., 2008). Further differences have been noticed between JunB and c-Jun. JunB does not form homodimers as spontaneously as c-Jun and JunB homodimers bind the AP-1 region in a weaker manner (Deng and Karin, 1993). Such differences between these transcription factor characteristics induce different mode of gene regulation and signalling pathway activation (Hsu et al.,

1993). For example, in HT-1080 fibrosarcoma cells, where JunB mediates collagenase expression in response to tumor promoter okadaic acid (OA) treatment, c-Jun is not induced (Westermarck et al., 1994). Furthermore, in F9 embryonal carcinoma cells TPA treatment leads to JunB increase but fails to activate c-Jun (Selvaraj and Prywes, 2004). Human T-cell leukemia virus type I protein drives the relocalization of JunB to the nucleolar bodies and induces the subsequent inhibition of JunB function *in vivo* (Hivin et al., 2007). When exogenous c-Jun is expressed in these cells, the endogenous *c-jun* gene is induced whereas *junb* or *c-fos* are not. Finally, when co-transfected with c-Jun, JunB inhibits c-Jun-mediated *cyclin D1* gene regulation (Bakiri et al., 2000). Altogether, these observations describe differences between c-Jun and JunB in gene regulation. Moreover, tissue expression in adult mice differs between c-Jun and JunB suggesting possible tissue specific AP-1 target gene regulation (Ryder et al., 1989). Other models demonstrate that JunB regulates interleukin 4 (IL-4) in a c-Jun independent manner. JunB, by regulating IL-4 expression, influences T-helper cell differentiation (Garaude et al., 2008; Li et al., 1999). Similarly, JunB was described as an essential and specific protein in myeloid cell differentiation, Th2 cells differentiation and during placentation (Passegue et al., 2001; Schorpp-Kistner et al., 1999; Voice et al., 2004). JunB also plays an opposite role to c-Jun in cell proliferation and oncogenesis. JunB induces cyclin-dependent kinase (CDK) inhibitor which reduces cell proliferation and triggers premature senescence (Konishi et al., 2008; Passegue and Wagner, 2000).

Despite these factors, in the absence of c-Jun, JunB can replace c-Jun and prevent p53 expression (Passegue and Wagner, 2000). Moreover, JunB can rescue *c-Jun*$^{-/-}$ mice embryo phenotypes in a dose dependent manner. JunB can also partially replace c-Jun in the regulation of *c-jun* expression (Passegue et al., 2002).

JunD

JunD is the third JUN protein family member discovered. Amino acid sequence alignments describe a 53% homology between JunD and c-Jun. However, two domains within the sequence remain conserved (DNA binding domain and bZIP domain) whereas inter-domain regions vary considerably (Ryder et al., 1989). Furthermore, JunD binds DNA sequences (TGACTCA) similar to c-Jun. Moreover, JunD, like c-Jun, participates in the trans-activation of TRE containing promoters such as c-myb or human telomerase reverse transcriptase gene (hTERT) (Kuhlmann et al., 2007; Nicolaides et al., 1992). It has two protein forms: long and short, differing in their N-terminal amino acids content. JunD-S does not include the 43 N-terminal amino acids. The N-terminal domain of JunD is the binding site for the tumor suppressor Menin (MEN1). Upon the binding of MEN1 on JunD, the transcriptional activity of JunD is repressed (Agarwal et al., 1999). Moreover, in the presence of MEN1, ERK phosphorylation of JunD and Elk-1 is inhibited. JNK phosphorylation of JunD and c-Jun are reduced upon MEN1 expression and *c-fos* promoter activity is impeded (Gallo et al., 2002). In addition, the interaction

between JunD and MEN1 regulates c-Jun-mediated transcription (Ikeo et al., 2004). Despite the presence of JNK-mediated phosphorylation sites on serines 90 and 100 on JunD-L and serines 47 and 57 on JunD-S, JNK phosphorylation of JunD is less efficient than c-Jun phosphorylation. This is explained by the heterodimerization of JunD with JNK docking site carrying partners such as c-Jun (Kallunki et al., 1996).

Ubiquitination of JunD is not sufficient to drive its degradation and the protein exhibits a high stability (Musti et al., 1996). However, the control of JunD expression and activity are regulated at transcriptional, translational and post-translational levels (Hernandez et al., 2008).

JunD also exhibits specific biological activities which are often opposite and antagonistic to c-Jun activities. In fibroblasts, JunD protects cells from p53 induced senescence and apoptosis (Weitzman et al., 2000). Moreover, cell growth is not affected by JunD expression (Castellazzi et al., 1991). However, in lymphocytes, JunD regulates proliferation (Meixner et al., 2004). Furthermore, following polyamine depletion, JunD expression, in contrast to c-Jun and JunB, increases resulting in unbalanced AP-1 complex composition. This modification of AP-1 composition affects intestinal cell growth (Patel and Wang, 1999). For example, JunD antagonizes Ras mediated transformation and JunB is much less efficient than c-Jun in transforming rat fibroblasts (Pfarr et al., 1994; Schutte et al., 1989b). Finally, using *junD* deficient cell model, tumor angiogenesis was demonstrated to be under the control of JunD expression (Gerald et al., 2004).

In summary, redundant and distinct functions are divided among the three members of the Jun protein family. This allows the action specificity of these members but, on the other hand enables them to compensate each other's function in essential cellular mechanisms.

3 Biology of c-Jun

3.1 c-Jun structure

The proto-oncogene *c-jun* was initially discovered as a human counterpart of the avian sarcoma virus 17 (the name Jun comes from the Japanese translation of the number 17 "ju-nana") (Maki et al., 1987). It is a 3,1kb intronless gene, located on chromosome 1 (1p32-p31). Its promoter region exhibits the AP-1 autoregulation site and a differentiation response element (DRE) to which differentiation response factor (DRF) complex binds (Angel et al., 1988; Kitabayashi et al., 1992). In addition, using the promoter trapping method, c-Jun promoter binding pre-initiation complex was purified and RNA polymerase II, TBP, TFIIF subunit RAP74, and transcription factor SP1 were isolated (Jiang et al., 2006). The protein encoded is 331 amino acids long and is composed of four main domains: the δ-domain, the transactivation domain, the DNA binding domain

and the bZIP domain. The δ-domain (amino acids 31 to 60) has been described as the docking site for JNK and thereby the site of regulation of c-Jun phosphorylation and ubiquitination (Adler et al., 1994; Treier et al., 1994). The transactivation domain contains two main phosphorylation sites: serine 63 and 73 (Ser-63 and Ser-73) (Derijard et al., 1994). Two other JNK phosphorylation sites on threonines 91 and 93 were described to be specifically up regulated by stress and pro-inflammatory signals. Moreover, a recent report demonstrated that a preceding MAPK phosphorylation of threonine 95 is required in order to phosphorylate Thr-91 and Thr-93 (Vinciguerra et al., 2008). Two other phosphorylation sites are present on c-Jun on threonine 239, mediated by glycogen synthase kinase 3 (GSK3), and on serine 243 (Wei et al., 2005). The DNA binding domain is closely linked to the bZIP domain, each of them possessing precise and distinct functions. The DNA binding domain is the basic region which physically binds the TRE or the cyclic adenosine monophosphate (cAMP) response element (CRE) DNA sequence (Bohmann et al., 1987). The bZIP domain is the signature sequence of AP-1 proteins. It has five heptad repeats of leucine residues where dimerization between members of this protein family occurs (Landschulz et al., 1988; Rasmussen et al., 1991). In addition to these four domains, c-Jun possesses a nuclear localization sequence (NLS) driving the continuous translocation of c-Jun to the nucleus (Chida and Vogt, 1992).

3.2 Regulation of c-Jun activity

3.2.1 c-Jun activity

c-Jun is the downstream effector of a signaling cascade initiated upon binding of various ligands to membrane receptors. These stimuli include, growth factors (Quantin and Breathnach, 1988), cytokines (Brenner et al., 1989), infections, or mitogens. Each stimulus triggers distinct signaling pathways. Pro-inflammatory cytokines, genotoxic stress and mitogens such as lipopolysaccharide (LPS) launch the JNK/stress activated protein kinase (JNK/SAPK) pathway (Chang and Karin, 2001). Mitogen-activated protein kinase kinase kinases (MAPKKK) consisting of MAPKKK1, -2, -3 and -4, and mixed lineage kinases (MLKs), Tpl2, ASKs, TAOs and TAK1 phosphorylate MAPKK4 and MAPKK7 (Kyriakis and Avruch, 2001). These two MAPKKs further activate JNKs which translocate to the nucleus, thereby phosphorylating and activating c-Jun. In order to specify the signalling cascade, the consecutive kinases are brought together by scaffolding proteins specific for each kinase cascade (Yasuda et al., 1999). However, the phosphorylation of c-Jun by JNK might not be required for activation of c-Jun transcriptional activity but is necessary to displace a transcriptional repressor complex composed of the histone deacetylase 3 (HDAC3) bound to c-Jun (Weiss et al., 2003).

On the other hand, serum and growth factor activate ERK which translocates to the nucleus to phosphorylate ternary complex factors (TCFs). These TCFs bind to the *fos* promoter inducing Fos protein synthesis which heterodimerises with c-Jun (Hill et al.,

1994). However, in certain cellular contexts, phosphorylation of Ser-63 and Ser-73 on c-Jun occurs upon ERK signalling pathway activation including Ras, Raf, MEK and the subsequent ERK1 and ERK2 activations (Leppa et al., 1998).

Cellular stress response requires rapid activation of immediate-early proteins. This is autonomous of *de novo* proteins synthesis and includes DNA binding proteins such as transcription factors. c-Jun, as an immediate-early gene, is rapidly activated upon various stimuli. Two types of regulation of c-Jun activation have been described: transcriptional and post-translational. At the transcriptional level, by binding to a TPA response element (TRE) of its promoter region, c-Jun auto-regulates its expression (Angel et al., 1988). Similarly, in F9 cells the DRF complex, composed of the AP-1 protein ATF-2 and p300, binds to the DRE of c-Jun promoter in order to drive c-Jun expression (Kawasaki et al., 1998). Nevertheless, post-translational regulation and particularly phosphorylation and dephosphorylation are a major means of regulation of c-Jun activation (Boyle et al., 1991; Pulverer et al., 1991). In resting fibroblasts, c-Jun dephosphorylation occurs on serine and threonine residues (Thr-239, Ser-243, Ser-249) upstream of the DNA binding domain. It follows the activation of protein kinase C and is concomitant with an increase of AP-1 binding activity. Phosphorylation of c-Jun protein on Thr-239 by GSK-3 leads to reduction of c-Jun homodimers binding to TRE sites. Other post-translational modifications such as sumoylation, ubiquitination and acetylation regulate c-Jun function. c-Jun is modified by SUMO-1 on lysine 229 (Lys-229) and negatively regulates c-Jun transcriptional activity (Muller et al., 2000). The stability of c-Jun protein is regulated by ubiquitination of c-Jun in the δ-domain by the ubiquitin ligase F-box and WD repeat domain-containing 7 (Fbw7) and is enhanced by GSK3 phosphorylation of c-Jun (Treier et al., 1994; Wei et al., 2005). However, phosphorylation of c-Jun by JNK protects c-Jun from ubiquitination and thereby contributes to the longer half-life of the protein (Musti et al., 1997). This highlights the ambivalent role of JNK in c-Jun stability in response to environmental stress. Finally, the lysine residue 271 (Lys-271) of c-Jun is acetylated *in vivo* and is essential for collagenase promoter repression by adenovirus E1A protein (Vries et al., 2001).

3.2.2 c-Jun dimerization

c-Jun, as a transcription factor, is a DNA binding protein (Bohmann et al., 1987). In order to bind DNA, c-Jun needs to form homo- or heterodimers. The nature of these dimers and the sequences flanking the binding sites determine the affinity of c-Jun containing dimers for the DNA binding sequences (Smeal et al., 1989). Jun-Jun homodimers as well as Jun-Fos and Jun-Fra heterodimers bind with high affinity to a phorbol ester- and growth factor-inducible element. This element is a seven base pair consensus sequence (TGAGTCA). On the other hand, Jun-ATF heterodimers bind with highest affinity to a cyclic AMP-responsive element (CRE) sequence (TGACNTCA) (van Dam and

Castellazzi, 2001). Furthermore, the components of AP-1 dimers modulate AP-1 activity since heterodimers formed with c-Fos and c-Jun increase c-Jun transcriptional activity whereas heterodimers formed with c-Jun and JunB decrease it (Chiu et al., 1989). In addition, initiation of AP-1-mediated transcription is modulated by the interaction of the AP-1 dimers with co-activators. AP-1 dimers formed with c-Fos and c-Jun display higher affinity for BAF60a, a member of the SWI.SNF chromatin remodeling complex, than dimers formed with JunD and Fra-2 and thereby tunes the subsequent transcriptional response (Ito et al., 2001).

3.3 c-Jun; its cellular roles and functions

AP-1 dimers are involved in various cellular events including development, cellular proliferation, differentiation, transformation and death. The plethoric events controlled by activation of AP-1 transcription factors depends on the constitution of the AP-1 dimers, their abundance, the cell type and its environment as well as the stimulus which triggered the AP-1 activity.

3.3.1 The role and expression of c-Jun in development and differentiation

c-Jun is widely expressed during embryogenesis and as early as during neurulation (Bennett et al., 1997). The role of c-Jun in embryogenesis has been extensively studied in mouse embryos. *c-Jun*$^{-/-}$ mouse embryos die at days 12-14 of gestation (Hilberg et al., 1993). Further investigations of these mutant embryos described impaired hepatogenesis, altered liver erythropoiesis, oedema and non-proliferative fibroblasts (Johnson et al., 1993). Nevertheless, analysis of fetal liver mRNAs of AP-1 target genes do not show expressional differences (Eferl et al., 1999). Moreover, *c-Jun*$^{-/-}$ mouse embryos show heart outflow tract malformations (incomplete separation of the aorta and the pulmonary artery) (Eferl et al., 1999). Such heart malformations are similar to those observed in mice with neural crest cell defects (Kirby and Waldo, 1995). Further investigations of the neural crest of *c-Jun*$^{-/-}$ embryos do not show any neural crest cell distribution defect. This suggests a specific defect of neural crest cell function in the heart and not a general neural crest defect (Eferl et al., 1999).

High expression of c-Jun in the developing brain was observed and c-Jun phosphorylation has been noted under neuronal differentiation (Kockel et al., 1997; Mellstrom et al., 1991). Nuclear phosphorylated c-Jun is highly expressed in neuroblasts migrating towards the forebrain and hindbrain (Raivich and Behrens, 2006). Additionally, c-Jun homodimers and c-Jun-MafB heterodimers regulate the transcription of *Hoxb3* genes in the fifth rhombomere of the hindbrain. Later, high levels of c-Jun expression are detected in sensory, motor and sympathetic neurons (Mechta-Grigoriou et al., 2003). Postnatally, in the central nervous system, the highest levels of c-Jun were detected at day 15 and thereafter decreased to low levels at the adult stage. High c-Jun expression levels are

found in the visual, olfactory and telencephalon areas (Wilkinson et al., 1989). More detailed analysis demonstrated that apoptotic-looking postnatal sympathetic neurons express c-Jun suggesting that c-Jun is involved in cell death (Messina et al., 1996). Phosphorylation of c-Jun is also required for the initiation of an early and reversible event of embryonic programmed cell death (PCD) (Sun et al., 2005). Moreover, c-Jun neuronal conditional excision does not show any apparent effect on brain morphology or facial motorneurons (Behrens et al., 2002). Thus, c-Jun deletion might be compensated by other Jun proteins (Raivich et al., 2004). Finally, c-Jun expression was detected in mouse embryos between day 14.5 and 17.5 in cartilage, skeletal muscle, gut, kidney and the adrenal gland (Wilkinson et al., 1989). Importantly, these functions for c-Jun have to be considered in the specific cellular context in which they were studied since c-Jun necessity is tissue specific (Eferl et al., 1999; Yamamoto et al., 2007).

Differentiation is the cellular process by which a cell specializes and acquires more specific functions. The role for c-Jun in this process has been investigated in various cell models. In neural cells, phosphorylated c-Jun induces differentiation and neurite outgrow. Constitutively phosphorylated c-Jun mutants (c-Jun^{Asp}) injected even at low concentration increases the formation of long neurites whereas alanine c-Jun mutants (c-Jun^{Ala}), where phosphorylation is prevented, reduce PC-12 cells differentiation. Phosphorylation by ERK preferably to more classical JNK pathway is demonstrated in PC-12 although the role of JNK3 phosphorylation is not discussed (Leppa et al., 1998). The role for c-Jun in PC-12 differentiation was recently emphasized since it was shown to be an indispensable phenomenon in the differentiation of rat pheochromocytoma-derived cell line (Vaque et al., 2008). Additionally, c-Jun's positive role in differentiation has been described in other models such as F9 mouse embryonal carcinomas or in leukemic cell line U-937 (Szabo et al., 1994; Yamaguchi-Iwai et al., 1990). In mouse epidermis, keratinocytes conditionally inhibited for c-Jun displayed reduced EGFR expression in the eyelids and resulted in a similar open eyes at birth phenotype observed in EGFR null mice. In spite of normal skin development, an increase of keratinocyte differentiation was noticed (Zenz et al., 2003).

3.3.2 The role of c-Jun in cellular proliferation

Involvement of c-Jun in embryogenesis and organ development suggests a potential role for AP-1 complexes and particularly c-Jun in cellular proliferation control. Moreover, AP-1 transcription factors regulate the expression and function of cell-cycle regulators: cyclin D1 (Bakiri et al., 2000), cyclin A (Katabami et al., 2005), cyclin E (Hennigan and Stambrook, 2001), p53 (Schreiber et al., 1999), p21^{Cip1} (Shaulian et al., 2000), p16^{Ink4a} (Passegue and Wagner, 2000) and p19^{ARF} (Weitzman et al., 2000). The control of expression of pro- and anti-proliferative genes indicates the possibility of various routes to govern cellular proliferation. Moreover, organ specific c-Jun depletion shows variable results

concerning the expression levels of AP-1 target genes. Fibroblasts lacking c-Jun exhibit high expression levels of p53 and its target gene $p21^{Cip1}$ thereby inhibiting expression of cyclin D1 (Schreiber et al., 1999). Moreover, hepatocyte regeneration is controlled by c-Jun by repressing p53/p21 and p38α pathways (Stepniak et al., 2006). Comparably, in keratinocytes lacking c-Jun, p53 and $p21^{Cip1}$ protein levels are not affected. This indicates that various molecular mechanisms direct cell proliferation in these different cell types. For example, keratinocytes lacking c-Jun exert a cell proliferation defect through the EGFR and its ligand, heparin-binding epidermal growth factor (HB-EGF) (Zenz et al., 2003).

Experiments done with MEFs and in acute myeloid leukemia (erythroleukemia) treated with antisense RNA specific for *c-fos* or *c-jun* showed reduction of cellular proliferation (Holt et al., 1986; Smith and Prochownik, 1992). Antibody microinjections against c-Fos and c-Jun also demonstrated the role of AP-1 complexes in cell cycle re-entry of serum starved cells (Shaulian and Karin, 2001). Notably, specific antibodies against any Jun protein inhibit cell cycle entry whereas all forms of Fos protein should be inactivated by an antibody to reach similar effects. This suggests distinct and essential involvement of each Jun protein and compensatory roles for Fos protein (Kovary and Bravo, 1991). Further analysis of the roles of c-Jun in cell cycle progression suggest a requirement of c-Jun in G1 to S phase progression and a role for c-Jun induction in ultraviolet (UV) light irradiated cells to re-enter the cell cycle from the G2/M interphase (Schreiber et al., 1999; Shaulian et al., 2000). Nonetheless, c-Jun expression levels do not vary during cell cycle progression but its phosphorylation status increases when cells proceed from the G2 to M phases (Bakiri et al., 2000). Moreover, knock-in mouse fibroblasts expressing c-Jun JNK phosphorylation sites alanine mutant ($c\text{-}Jun^{Ala63/73}$ mice) show proliferation defects indicating a potential role for c-Jun activation in cellular proliferation (Behrens et al., 1999). *c-jun*$^{-/-}$ fibroblasts reveal deficient growth capacities. These cells can be passed only a few times before exhibiting senescent phenotype (Johnson et al., 1993). Rescue experiments by re-introducing c-Jun in these cells resulted in increased proliferation. Similarly, immortalized *c-jun*$^{-/-}$ fibroblasts display proliferation defects and deficient cell cycle re-entry after UV-irradiation. These phenotypes can be rescued by c-Jun re-introduction (Schreiber et al., 1999; Shaulian et al., 2000).

3.3.3 The role of c-Jun in cell death

Early evidence indicating a potential role for AP-1 transcription factors in apoptosis were found when AP-1 proteins were induced upon withdrawal of growth factors from neuronal and lymphoid cell cultures (Colotta et al., 1992; Ham et al., 1995). However, recent report described that inactivation of c-Jun by SP600125 treatment in sympathetic neurons did not affect the viability of these neuronal cells (Lindwall and Kanje, 2005). It is therefore important to consider the cellular model chosen to describe functional role

for c-Jun. Nonetheless, c-Jun is strongly induced when cells are exposed to genotoxic stress such as UV light or alkylating agents (Devary et al., 1991; Karin, 1998). These suggestions were confirmed by overexpression experiments describing the direct induction of apoptosis by c-Jun in a number of cell lines (Bossy-Wetzel et al., 1997; Podar et al., 2007). Reverse experiments where c-Jun is depleted by antisense RNA or where c-Jun is blocked by specific antibody reveal a survival increase in growth factor deprived lymphoids or neurons (Colotta et al., 1992; Estus et al., 1994). Equivalent observations are made in neurons subject to expression of c-Jun$^{Ala63/73}$ mutant (Le-Niculescu et al., 1999). Furthermore, c-Jun$^{Ala63/73}$ mice are resistant to kainic acid induced cell death (Behrens et al., 1999). Moreover, the specific neuronal form of JNK, JNK3, once depleted, reduces the activity of AP-1 and kainic acid apoptosis (Yang et al., 1997). In motorneurons, inhibition of c-Jun signaling reduced PCD in chicken embryos (Sun et al., 2005). Similar results were obtained in c-Jun depleted fibroblasts and *jnk1*$^{-/-}$ *jnk2*$^{-/-}$ MEFs upon UV light induced apoptosis (Shaulian et al., 2000). Moreover, alkylating agents induced apoptosis through CD95-ligand (CD95-L) are reduced in c-Jun$^{-/-}$ indicating a role for CD95 signalling in c-Jun induced apoptosis (Kolbus et al., 2000).

3.3.4 Non-transcriptional roles of c-Jun

c-Jun functions are described in the context of transcription, dimer formation or DNA binding. However, many independent studies evoked various non-transcriptional roles for c-Jun. Human androgen receptor (hAR) increase of gene expression by c-Jun is mediated by c-Jun domains different from the DNA binding domain and the transactivation domain (Bubulya et al., 1996). The bZIP domain and part of the N-terminus of c-Jun are required but not sufficient to enhance hAR transactivation (Wise et al., 1998). The composition of the c-Jun protein complex determines the stimulation of hAR transactivation. Further investigations demonstrated that c-Jun transactivation is distinct from its co-activation function. Moreover, these functions play opposite roles in prostate cancer cells, c-Jun co-activation function exhibiting a proliferative function whereas c-Jun transactivation is antiproliferative (Chen et al., 2006).

The cellular context in which c-Jun exhibits its functions is determinant for the cellular response. In non-differentiated neural cells, microinjection of c-Jun antagonizes the apoptotic effects of MEKK signaling (Leppa et al., 2001). A similar apoptosis protective function was described for JunD and JunB but not for Fos family members. Moreover, this anti-apoptotic function of c-Jun does not depend on its phosphorylation status by JNK. This was demonstrated by microinjections of c-Jun mutants to mimic constitutive phosphorylation status or the constitutive non-phosphorylated form of the protein (Leppa et al., 2001). In addition, the binding of JNK on c-Jun is not required for c-Jun to protect undifferentiated PC-12 cells from MEKK induced apoptosis since deletion of the JNK docking site on c-Jun does affect the anti-apoptotic effect of c-Jun. Further

analyses of the non-transcriptional roles for c-Jun indicated that c-Jun can protect undifferentiated PC-12 cells from MEKK induced apoptosis in dimerization and DNA binding independent manners (Leppa et al., 2001). In c-Jun$^{-/-}$ liver cells, the general gene expression is not significantly different from wt cells (Eferl et al., 1999). Therefore, the apoptotic phenotype noticed in hepatocytes lacking c-Jun is most likely not due to the transcriptional role for c-Jun. Moreover, the activation of c-Jun is essential for its transcriptional activity and, transgenic mice expressing c-Jun Ser-63 and Ser-73 mutated for alanine did not differ from wt in embryogenesis, liver formation, and developmental regulation of cell differentiation indicating that the JNK phosphorylation is not essential for these mechanisms (Eferl et al., 1999). In adipocytes, overexpression of c-Jun leads to negative regulation of adipogenesis. This is reminiscent of what was observed in undifferentiated human liposarcomas and in the mouse LM3D cell line, suggesting that c-Jun overexpression might block adipogenesis in human tumors through an interaction with CCAAT/enhancer binding protein beta (C/EBPβ).

4 c-Jun, the oncoprotein

4.1 Cancer

Depending on the environmental stresses and the somatic signals consequently triggered, cells react and behave in order to maintain their homeostatic balance. Cells are constantly subject to response towards cell survival or apoptosis. When damages caused by these environmental stresses are too severe, apoptosis is engaged and the mutated cells die. However, a mutation might give one cell a selective advantage with, for example, the capacity to divide in an uncontrolled manner or to resist apoptosis signals. Therefore, this mutated cell gives rise to mutant clones. Further mutations can occur in these cells and natural selection advantages might drive these cells towards tumor formation.

Cancer is the general name for a group of diseases characterized by an abnormal cellular proliferation within any normal tissue. Although abnormal, oncogenesis is not a random process. Cancer cells originate from a single clone and result from a multistep process driving normal cells toward transformed stages where cells become immortalized and hyper-proliferative. Moreover recent observations done in various cancer samples indicate that genetic mutations occur in a tissue specific context and as a function of the previous mutations which occurred in the sample (Yeang et al., 2008). To become neoplastic and escape the natural defense of the organism, cells have to acquire six main capabilities: evading apoptosis, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, self-sufficiency in growth signals, and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

A tumor might evolve towards a more malignant stage where these transformed cells can migrate from their tissue of origin and invade different tissues to form metastases. Metastasis is the principal cause of death following tumor formation. Therefore metastasis is a key factor in carcinogenesis. To form a metastasis, tumor must acquire several characteristics: separation from primary tumor, invasion and migration through connective tissue, penetration into and translocation in blood vessels, interaction with endothelial cells, migration through blood vessels and invasion into a different tissue.

Different cancer types can be categorized by various qualities. Based on their tissue of origin, neoplasms fall in four main classes: carcinomas (epithelial tissue), sarcomas (mesenchymal tissue), lymphomas (hematopoietic tissue) and germ cell tumor (totipotent cells). These are all genetic pathologies originating from quantitative and qualitative modifications of the host genome. Most of these modifications affect somatic cells and are, therefore, not hereditary.

There are several ways to classify genes involved in cancer development but two main groups of genes play key roles in tumorigenesis: oncogenes and tumor suppressor genes. Over 100 oncogenes have so far been discovered. Their hyperactivity positively regulates cellular transformation and cell proliferation. Several proto-oncogenes are involved in signaling pathways regulating cell response to mitogenic signals, growth factors such as transforming growth factor alpha (TGFα), EGFR, anti-apoptotic molecules such as B cell lymphomas 2 (Bcl2) and transcription factors (MYC, FOS, JUN). The second gene group includes tumor suppressor genes such as retinoblastoma protein (Rb), breast cancer 1 and 2 (BRCA1 and BRCA2) proteins, p53, protein phosphatase 2A (PP2A) or cyclin dependent kinase inhibitor 2A (CDKN2A). They act in an opposite manner to oncogenes by negatively regulating cellular proliferation and becoming inactivated upon transformation (Alberts, 2002).

4.2 c-Jun in cellular transformation

4.2.1 Role for c-Jun in cancer

Originally c-Jun, was identified as the mammalian homolog of the retroviral oncoprotein v-Jun. (Angel et al., 1988; Bohmann et al., 1987; Maki et al., 1987). Moreover, various transforming factors trigger AP-1 activity (Angel and Karin, 1991). Tumor promoter TPA (phorbol ester) and serum were among the earliest stimuli demonstrated to activate AP-1 members (Angel et al., 1987). Carcinogens such as 4-nitroquinolineoxide (NQO) or UV light induce DNA damage ultimately driving cells towards transformation and activation of AP-1 regulated genes (Stein et al., 1989). Finally, oncogenes and, in particular, Ha-*ras* enhance AP-1 activity (Binetruy et al., 1991).

c-Jun overexpression studies have provided evidence that c-Jun overexpression is enough to transform immortalized rat fibroblasts but is not sufficient to transform rat primary embryo fibroblast (REF) (Schutte et al., 1989a). In human nasopharyngeal carcinomas (NPC), c-Jun was shown to stimulate cell growth (Jin et al., 2007b). Furthermore, c-Jun overexpression showed an important role in the development of skin and liver tumors (Eferl et al., 2003; Young et al., 1999). Similarly, c-Jun amplification was described to block adipocytic differentiation in aggressive sarcomas (Mariani et al., 2007). Several phenotypes indicate a clear role for c-Jun in oncogenesis. For example, c-Jun was reported to positively regulate the growth and angiogenesis of solid squamous cell carcinomas (SCC) (Zhang et al., 2006). Moreover, Jun expressing cells exhibit higher mobility, invasiveness, and loss of polarity in mammary epithelial cells (Bos et al., 1999; Fialka et al., 1996). In several cancer cell types, elevated levels of c-Jun expression were described (Langer et al., 2006; Mariani et al., 2007; Mathas et al., 2002; Podar et al., 2007; Rangatia et al., 2003; Vleugel et al., 2006). However, the human oncogenic nature for c-Jun is considered in the discussion section.

The nature of the AP-1 dimer modulates its role in tumorigenesis. Depending on the dimer composition and the cellular context, AP-1 triggers various genetic programs leading to distinct cellular fates. Whereas some AP-1 members activate cancer promoting genes, others stimulate apoptotic pathway (Ameyar-Zazoua et al., 2005). Using recombinant constructs, Jun homodimers display oncogenic capacities. c-Jun homodimers, together with activated Ras, can transform mammalian cells (Vandel et al., 1996). Moreover, these homodimers are more oncogenic than v-Jun in avian cells (Jurdic et al., 1995). c-Jun-c-Fos induces anchorage-independent growth whereas c-Jun-Atf2 heterodimers give rise to growth factor independence (van Dam and Castellazzi, 2001). However, sumoylation down-regulates c-Jun-c-Fos heterodimer activity (Bossis et al., 2005). Additionally, c-Jun together with Fra-1, which is induced in response to *Ha-ras* expression, form an AP-1 heterodimer required for transformation of immortalized mouse fibroblasts by Ha-Ras (Johnson et al., 1996; Mechta et al., 1997; Schutte et al., 1989b). On the other hand, c-Jun-Fra-2 heterodimers suppress the growth of immortalized fibroblasts (Bakiri et al., 2002).

The DNA binding capacity of the AP-1 dimer affects the oncogenicity of c-Jun, whilst the target gene of the AP-1 transcription depends on AP-1 composition (van Dam et al., 1998). Mutational analysis of the Jun DNA binding domain showed full inhibition of transformation (Basso et al., 2000).

4.2.2 Signaling pathways relevant for c-Jun activity in cancer

Ha-Ras activates ERK and JNK inducing elevated c-Fos protein and c-Jun phosphorylation levels (Binetruy et al., 1991). Mutations in the transactivation domain of c-Jun blocked transformation induced by oncoproteins such as Ras and Raf and

reversed the transformed phenotype (Rapp et al., 1994). Moreover, the most important difference between c-Jun and v-Jun is the presence of the delta domain (amino acids 34-60) in c-Jun. This domain is the docking site for JNK and, therefore, is a key regulating sequence for c-Jun phosphorylation on serines 63 and 73 (Dai et al., 1995). Furthermore, c-Jun phosphorylation on serines 63 and 73 is required for transcriptional activation and transformation of mammalian cells (Smeal et al., 1991). Finally, v-Jun is a less potent oncogenic factor than c-Jun upon Ha-Ras stimulation (Bos et al., 1990). This correlates with the transforming potentials of v-Jun and c-Jun in REF indicating that c-Jun should be activated by phosphorylation in order to be oncogenic (Alani et al., 1991). However, c-Jun activation by JNK can be stimulated by other factors such as migration inhibitory factor (MIF) (Coleman et al., 2008). Nevertheless, it is important to consider the cellular context in which the transformation occurs since c-Jun phosphorylation is not required for transforming chicken embryonal fibroblasts (CEF) (Ui et al., 1998) and c-Jun was found to be a non-determining factor in colitis-associated cancer in mice (Hasselblatt et al., 2008).

5 c-Jun protein complex

5.1 c-Jun interactome

The necessity of proper spatial and temporal gene expression entails an extremely precise regulation mechanism. The early hypothesis regarding how transcription occurs was oversimplified. The chromatin structures and the flanking regions of coding sequences appeared to be essential key regulators of gene expression (Dynan, 1989). Later, once biochemical and genetic assays were developed, it was found that the regulation of such an accurate mechanism requires series of events carefully organized and rigorously controlled by transcriptional activators and repressors. The notion of proteins co-working and influencing each other's functions shed light on this yet unapproached field of science (Goodrich et al., 1996).

Therefore, taking into consideration the cellular context, the requirement of rapid and precise response a cell has to give following any stimuli or stress, and the amount of events to be managed in short period of time, the analysis of the protein complex copurifying with a transcription factor such as c-Jun provides a large amount of information regarding its cellular primary and secondary roles. The accurate roles played by these complex members may also be defined by protein complex analysis approach.

A protein complex consists of the assembly of individual proteins and possibly nucleotides to form a protein-apparatus dedicated to precise functions. Generally, proteins are linked to each other in two different ways: covalently and non-covalently. A covalent bond between two proteins demands a higher amount of energy to be created but also presents

more solid interaction compared to non-covalent link. However, non-covalent links are major binding mechanisms found in protein complexes.

Moreover, various stimuli trigger the activation or the repression of distinct transcription factors and co-regulator complexes resulting in a different affinity for the promoter and variation in the expression level of the target gene (Tsai et al., 2000). Therefore, the nature of the proteins interacting with c-Jun is of primary importance to determine the molecular functions of c-Jun protein complexes. Proteins interacting with c-Jun were classified into four categories: structurally related basic region - leucine zipper proteins; unrelated DNA binding proteins; transcriptional co-activators that do not bind DNA directly; and structural components of the nucleus (Chinenov and Kerppola, 2001). Interaction between c-Jun protein and human constitutive photomorphogenesis protein 1 (huCOP1) or with the cofactor of the breast cancer susceptibility gene (COBRA1) modulates its transcriptional activity (Bianchi et al., 2003; Zhong et al., 2004). Further modulation of c-Jun-mediated transcription results from the interaction of the adenovirus E1A protein with c-Jun and is dependent on the acetylation on lysine 271 by p300 acetyltransferase (Vries et al., 2001). Interaction with E3 ligase Fwb7 upon phosphorylation by GSK3 and previous phosphorylation on serine 243 drives c-Jun to polyubiquitination and proteasomal degradation (Nateri et al., 2004). In addition, the interferon-gamma (IFN-γ) promoter is recognized by CREB protein, ATF-2, and c-Jun to stimulate its expression. However, neither cyclic AMP response element, ATF-1, nor c-Fos bind the IFN-γ promoter region (Samten et al., 2008). Finally, c-Jun-c-Fos heterodimer was described to promote breast cancer cell growth whereas c-Jun homodimers did not (Lu et al., 2005). Altogether, these interactions between c-Jun, co-activators and dimerization partners, the resulting modulation of the roles for c-Jun, and the cellular consequences are cell type specific, cancer stage dependent, and also conditional on a plethora of cellular factors such as the activation of signalling pathway and the expression of oncogenes or tumor suppressors (Eferl and Wagner, 2003).

Recent progress in bioinformatics allowed the elaboration of *in silico* protein complexes based on literature cross analysis. Figure 3 illustrates the protein network interacting with c-Jun which can be drawn using this bioinformatic method. Moreover, using the tandem affinity purification (TAP) method, c-Jun protein complex was purified (Westermarck et al., 2002) (Table 1). Interestingly, based on TAP method results and c-Jun interactome analysis, a large number of co-purified and c-Jun interacting proteins with c-Jun were nucleolar proteins, whereas c-Jun was described to be localized in the nucleus (Sassone-Corsi et al., 1988). Moreover, several of these nucleolar proteins such as splicing factor HCC1, topoisomerase I (TopoI) and the RNA helicase DDX21 were previously described to be implicated in transcriptional regulation (Jung et al., 2002).

5.2 Topoisomerase I and DDX21, two nucleolar proteins involved in the regulation of transcription.

A large number of the proteins co-purified with c-Jun are nucleolar proteins (Table1). Moreover, among these nucleolar proteins nucleophosmin, DDX5, DDX17, DDX21, TopoI, and Nucleolin are involved in the regulation of transcription (Fuller-Pace and Ali, 2008; Garg et al., 1987; Liu et al., 2007; Roger et al., 2002; Westermarck et al., 2002). To clearly define the roles of these nucleolar proteins in relation to c-Jun roles in cancer cell behavior, we further investigated the relation between TopoI and c-Jun, and DDX21 and c-Jun.

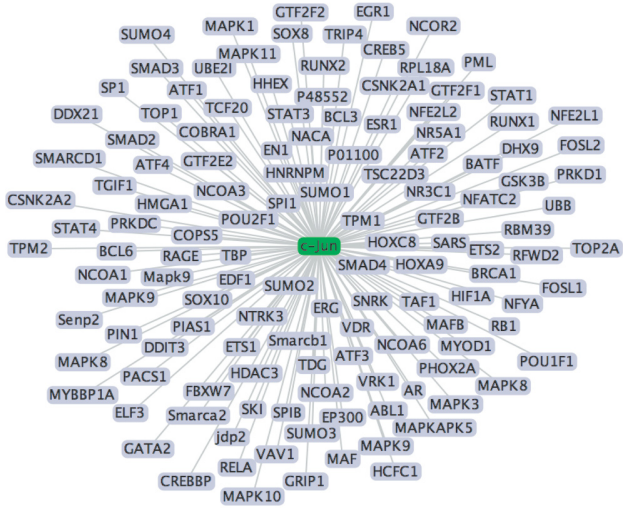

Figure 3. c-Jun interactome. List of c-Jun interacting proteins based on the PINA interactome website (http://csbi.ltdk.helsinki.fi/pina/home.do).

The nucleolus is a sub-compartment of the nucleus described as a control center for cellular survival and proliferation (Carmo-Fonseca et al., 2000). However, the original and the most described function of the nucleolus corresponds to ribosome biosynthesis (Fig.4) (Warner, 1990). Structurally, the nucleolus is composed of three components the functions of which are closely related to ribosomal biosynthesis. The fibrillar centres are the most central components of nucleoli where rRNA gene copies are arranged in tandem arrays at chromosomal loci named nucleolar-organizing regions (NORs) (Pinol-Roma, 1999). NORs are associated in complexes with many of the over 350 ribosomal and non-ribosomal nucleolar proteins to assist the nascent rRNA transcripts (Andersen et al., 2002). These rRNA transcripts migrate under the guidance of snoRNAs towards a more external region of the nucleolus, the dense fibrillar component, where early post-transcriptional modifications (methylation and pseudouridylation) occur (Verheggen et al., 1998). Further external movements lead the processed rRNA to the third and most

external nucleolar component, the granular component. Here, late rRNA processing events take place and pre-ribosomal particles are ready for export to the cytoplasm where their association in functional ribosomes allows translation to occur (Grummt, 1999).

Table 1. c-Jun TAP co-purified proteins.

Protein name	Cellular localization	Principal function	Confirmed interaction with c-Jun
40S Ribosomal Protein S3	Cytoplasm, Nucleus	Structural constituent of ribosome	No
60S Acidic Ribosomal Protein P0	Nucleolus	Protein biosynthesis	No
Fibrillarin	Nucleolus	Ribosome biosynthesis	No
Nucleophosmin	Nucleolus	Ribosomal protein assembly and transport	No
Actin	Ubiquitous	Cytoskeleton component	No
60S Ribosomal Protein L3	Cytoplasm	Structural constituent of ribosome	No
DDX5	Nucleolus	RNA helicase	No
DDX17	Nucleolus	ATPase activity in presence of RNA	No
Heterogeneous Nucleolar Ribonucleoprotein M	Nucleolus	Pre-mRNA processing	Yes
DDX21	Nucleolus	RNA helicase, RNA foldase	Yes
TopoI	Nucleolus	DNA supercoiling regulation	Yes
Nucleolin	Nucleolus	Control of transcription of ribosomal RNA	No
DDX9/RHA	Nucleus	RNA helicase	Yes

In more detail, the rDNA is transcribed as a 45S precursor rRNA of 13000 nucleotides. After post-transcriptional modifications, this rRNA precursor is cleaved in three rRNA particles: 18S, 5.8S and 28S rRNA. Further association with externally produced 5SrRNA and ribosomal proteins leads to the formation of the two ribosomal particles; 18S is incorporated into the small 40S ribosomal subunit, whereas 5S, 5.8S, and 28S rRNA are incorporated into the large 60S ribosomal subunit (Olson and Dundr, 2005). In addition to the primary function of the nucleolus consisting of ribosome biogenesis, recent reports have indicated functions for the nucleolus in mitosis, cell-cycle regulation, stress sensing and the abundance of the tumor suppressor p53 (Boisvert et al., 2007; Rubbi and Milner, 2003; Zhang and Xiong, 2001).

5.2.1 Topoisomerase I

The DNA Topoisomerase family includes a group of DNA enzymes originally isolated from the bacteria *Escherichia coli* (Wang, 1971). These enzymes, because of their primary function, are involved in most of the cellular mechanisms where DNA is utilized: replication, transcription, recombination, and chromatin modeling (Wang, 1998). The level of compaction of DNA in a cell is such that the access to the information requires a separation mechanism of the double stranded DNA. Topoisomerases perform this function by relaxing positive and/or negative supercoiling tension introduced in the DNA molecules (Wu et al., 1988). Topoisomerases cut DNA strand(s) in two distinct

fashions. These different modes of action give the bases for the classification of the members of this protein family. The type I DNA topoisomerase subfamily cut one strand of the DNA and pass the non-segmented strand in the break in the opposing strand. Type II DNA topoisomerase cut the two strands of DNA and pass a duplex region through the gap generated in DNA. Further divisions (IA, IB, IIA, IIB) of each subfamily are based on structural and functional features.

The human TopoI is a nucleolar protein expressed ubiquitously among most tissues (Alsner et al., 1992). Three others topoisomerase I genes encode for the mitochondrial version of the protein (TopoIMT), topoisomerase 3α (Topo3α), and topoisomerase 3β (Topo3β) (Champoux, 2001; Zhang et al., 2001). TopoI possesses homologues in all eukaryotes and diverges from other DNA topoisomerases both structurally and at the sequence level. Regardless of these differences, TopoI is an essential protein in eukaryotic embryogenesis since knockout mice die between the 4- and 16- cell stages. Moreover, TopoI functions are conserved in eukaryotes and it is able to relax both positive and negative DNA supercoils in Mg^{2+} and adenosine 5'-triphosphate (ATP) independent manners (Sherratt and Wigley, 1998; Wang, 1971). The crystal structure was obtained for most of the protein except the N-terminal part. It forms an empty cylinder shape in the center of the tertiary structure of the protein where DNA can interact with the protein and form the cleavage complex (Stewart et al., 1998).

The TopoI gene is located on the chromosome region 20q12-q13.2 (Juan et al., 1988). The promoter region of TopoI does not contain TATA or CCAAT boxes. However, other binding sequences are found for general transcription factors such as Sp1, octamer transcription factor (OTF), or stress induced response transcription factors like CRE binding protein (CREB/ATF), NF-κB and Myc (Heiland et al., 1993). It is composed of 21 exons covering 85 kilobases of genomic DNA (Kunze et al., 1991). TopoI is a 91-kDa protein divided into four distinct domains. The N-terminal domain is 212 amino acids long and includes the functional activity site. It is a hydrophobic region sensitive to proteases and contains four NLS (Stewart et al., 1996). This is the region of the protein where most of the known interactors (nucleolin, p53, SV40 T-antigen) and transcription factor bind TopoI (Albor et al., 1998; Bharti et al., 1996; Kretzschmar et al., 1993; Simmons et al., 1996) . In addition, the N-terminal end of TopoI hosts the binding domain for the splicing factor SF2/ASF (Alsner et al., 1992). The largest part of the protein is composed of the core domain between amino acid 213 and 635 (Redinbo et al., 1998). The core domain is followed by a 76 amino acids long linker domain with no known functional domain. The 52 amino acids globular C-terminal region of the protein includes tyrosine residue 723 which is the active site of the protein. Tyr723 forms a covalent bond with the 3'-end of the DNA in order to perform the supercoil relaxing function of TopoI.

The DNA substrate for TopoI is a 5 nucleotide sequence 5'-(A/T)(G/C)(A/T)T-3'. TopoI binds at the -1 position of this sequence and further nicks the DNA (Tanizawa et al., 1993). The DNA cleavage is obtained by transesterification between the enzyme tyrosyl and the DNA phosphate groups (Wang, 1971). A consequent DNA-protein 3'-phosphotyrosyl bond takes place until the relaxation reaction is completed. The helical duplex downstream of the cleavage site thereafter rotates to relieve extra torsions within the DNA molecule. Once the torsions are removed, the DNA is religated by a reverse transesterification reaction (Stewart et al., 1998).

In addition to its DNA relaxing function, TopoI has been found to play a role as a modulator of transcription, as a kinase, in recombination, in DNA damage response and in mitosis. The formation of a protein complex between TopoI and the general transcription factor TFIID facilitates the interaction between TFIID and TFIIA (Shykind et al., 1997). Thereby the protein complex exclusively increases the basal transcription of genes with a TATA box in their promoter region. This occurs in a TopoI DNA-binding independent manner. However, when TopoI binds THIID or TBP, the transcription is repressed (Mondal and Parvin, 2003). In addition, upon DNA damage TopoI is recruited by the TFIID protein complex to facilitate the repair procedure. Paradoxically, TopoI interaction depends on the XPD helicase, a member of the THIID protein complex (Vichi et al., 1997). The kinase activity of TopoI on the splicing factor SF2/ASF is another mechanism by which it affects gene expression (Labourier et al., 1998). TopoI, in its N-terminal domain, binds the splicing factor SF2/ASF and catalyses its phosphorylation by binding ATP in the globular domain (Rossi et al., 1998). Additionally, the ligation-religation roles played by TopoI on DNA molecules as well as the structural and functional similarities shared with recombinases implicate TopoI in illegitimate recombination (Zhu and Schiestl, 1996). Moreover, TopoI is associated with the integration of the viral DNA of SV40 and viruses without the integrase gene (Bullock et al., 1984). Finally, during mitosis, TopoI was shown to participate in the 13S condensing protein complex which introduces positive supercoils in DNA and thereby compacts chromatin fibers (Kimura and Hirano, 1997).

The critical roles played by TopoI requires close regulation. TopoI regulation occurs at two levels: post-translational modifications of the protein and its interaction with other cellular proteins. TopoI is a substrate for casein kinase II and protein kinase C and is therefore a phosphoprotein (Samuels et al., 1989; Staron et al., 1996). The phosphorylation of TopoI increases its ability to relax supercoils. Furthermore, during mitosis TopoI is hyper-phosphorylated suggesting that it is a substrate for mitotic kinases (D'Arpa and Liu, 1995). TopoI is also a subject to poly adenosine diphosphate ribosylation (ADPR) posttranslational modification (Ferro et al., 1983). This modification occurs upon DNA damage decreasing the catalytic activity of TopoI and participating in the temporary replication shutdown mechanism required for DNA repair (Kasid et al., 1989). Another level of control of TopoI activity occurs through interaction with other proteins. The

nature of the protein complex associated with TopoI determines the modulation of its role. Several chromatin-associated proteins such as high mobility group 1, 2 and 17 (HMG) and histone H1 stimulates TopoI activity (Javaherian and Liu, 1983). As a nucleolar protein, TopoI was found to physically interact with nucleolar proteins nucleolin and RNA polymerase I (Bharti et al., 1996; Rose et al., 1988). The tumor suppressor p53 also interacts with TopoI (Gobert et al., 1996). However, this interaction depends on the nature of p53. In wild-type p53 cells, TopoI interaction with p53 is succinct and occurs only after genotoxic stress. On the other hand, in p53-mutated cells the interaction between these two proteins is constitutive (Gobert et al., 1999). Moreover, the balance between repairing DNA following genotoxic aggression and illegitimate recombination favorable to cancer formation indicates a dual role for TopoI in cancer (Bronstein et al., 1996).

Development of cancer relevant protein inhibitors with specific activity on cancer cells is a constant challenge for researchers. Camptothecin is an anti-cancer molecule extracted from the bark of *Camptotheca acuminata* (Wall and Wani, 1995). Further research demonstrated its specific activity as a TopoI inhibitor. Camptothecin treatment inhibits TopoI by binding the TopoI cleavable complexes (TopoIccs). It thereby prevents the religation of the nicked DNA strands and induces the accumulation of TopoIccs (Hsiang et al., 1985). This accumulation is reversible upon camptothecin removal. Moreover, camptothecin penetrates cells rapidly and has a low affinity for TopoIccs. This allows very precise control of the inhibition. However, this low affinity and fast recovery after camptothecin exposure requires long treatment in order to convert TopoIccs inhibition in DNA damage. This DNA damage is obtained because TopoI inhibitors slow down the TopoI religation step. The TopoIccs cripple with replication and transcription complexes and create covalent irreversible DNA-protein complexes at the 5'end of the nicked DNA template (Del Bino et al., 1991; Horwitz and Horwitz, 1973). However, this exposure to TopoI inhibitor induces side effects which limit its dosage and therefore limits its anti-tumor efficacy. Other non-camptothecins TopoI inhibitors such as indolocarbazole, phenanthridine and indenoisoquinoline derivatives are under development (Meng et al., 2003; Pommier, 2006).

Among the camptothecins derivatives, topotecan (Hycamtin) is a specific TopoI inhibitor exhibiting wide-range antitumor activity (Houghton et al., 1996; Sato et al., 2008; Takimoto and Arbuck, 1997). It is clinically approved (FDA approved on June 14th, 2006) for the treatment of ovarian carcinoma, small-cell lung cancer (SCLC) and advanced cervical cancer (Armstrong et al., 2005). Moreover, the side effects of the drug such as myelosuppression and its toxicity are limited, predictable, and reversible. X-ray analysis of TopoI bound to DNA and in presence of topotecan clarifies the mechanism of action of TopoI inhibition. Topotecan binds to the cleaved DNA mimicking a DNA base pair and thereby preventing DNA religation by an uncompetitive inhibition (Staker et al., 2002).

5.2.2 DDX21

Among the various protein families implicated in ribosome biogenesis, the DExD/H protein family is characterized by highly conserved motifs including the eponymous amino acid sequence (Gorbalenya et al., 1989; Linder, 2006; Linder et al., 1989; Tanner and Linder, 2001). Originally, based on sequence homology to DNA helicases, DExD/H proteins were classified as the ATP-dependent RNA helicase family. All members of this family exhibit a helicase domain consisting of two sub-structures forming a rift where ATP can bind (Caruthers and McKay, 2002). Nevertheless, in spite of common sequence motifs and common functional roles played by members of this family, more specific roles have been assigned to each member (Kim et al., 1992; Lee and Hurwitz, 1992). Based on less conserved sequences flanking the helicases core (Tanner, 2003), DExD/H proteins were described to be involved in various cellular functions such as transcription (Rajendran et al., 2003; Westermarck et al., 2002), pre-mRNA splicing (Jankowsky et al., 2001), ribosome biogenesis (Charollais et al., 2003; Valdez et al., 1997b), and RNA decay (Py et al., 1996).

Within this protein family, DDX21, also known as RHII/Guα, was originally isolated from a patient (named Gu) suffering of a watermelon stomach disease (Valdez et al., 1996). The DDX21 gene is located on chromosome 10 (10q21) and spans over 29kb of genomic DNA. Upstream of a promoter without a functional TATAA box, a c-Myc/USF site and a CCAAT site were found (Zhu et al., 2001). The cDNA sequence consists of 15 exons and encodes a 783 amino acid protein expressed in all tissues (Ou et al., 1999; Valdez et al., 1996; Valdez et al., 2002a). DDX21 is localized in the nucleolus and upon cytotoxic drug treatments such as actinomycin D, toyocamycin or mycophenolic acid, DDX21 translocalizes to the nucleoplasm (Perlaky et al., 1997). DDX21 has two paralogues, α and β arising from gene duplication and differentially regulated. The β form has 3 pseudogenes and is subjected to alternative splicing whereas the α form shows neither pseudogene nor alternative spliced variants (Valdez et al., 2002b).

The human DDX21 sequence is 82,1% identical to the mouse protein and 100% similar to the 9 conserved motifs throughout RNA helicases (Zhu et al., 2001). The mouse protein is encoded by a cDNA compiling 14 exons. Nevertheless, the mouse protein possesses three 37 amino acid repeats rich in basic residues at the N-terminus of the protein. Moreover, the mouse promoter region is very divergent from the human sequence. Upstream of this promoter region a silencer region composed of three 38 nucleotides repeats was detected. Finally, downstream of the stop codon, at nucleotide positions 240 and 2100, 2 polyadenylation sites were found (Valdez and Wang, 2000).

DDX21 possesses two main functional domains, a helicase domain and a foldase domain located in separate regions of the protein (Valdez et al., 1997b). The helicase domain, in the first three-quarters of the N-terminal parts of the protein, unwinds double stranded

RNA stretches in an ATP-dependent manner. This activity occurs in a 5' to 3' exclusive direction (Flores-Rozas and Hurwitz, 1993; Valdez et al., 1996). Moreover, the ATPase function is necessary for the production of 18S and 28S rRNAs (Yang et al., 2005b). The anticancer drug adriamycin also drives nucleoplasmic redistribution of DDX21 and has been shown to inhibit the RNA helicase activity in a dose dependent and reversible manner (Valdez et al., 1998). The mode of action of adriamycin is to bind RNA substrates and thereby prevent interaction between DDX21 and RNA (Zhu et al., 1999).The folding domain is located between the amino acids 731-783. The folding activity introduces intramolecular secondary structures into single stranded RNA molecules. To perform this activity, DDX21 needs to bind to RNA. The binding occurs in two distinct sites required for folding activity. The first site includes the three last FRGQR repeats between amino acids 731 and 758. In more detail, mutation analysis of these repeats showed that the second and the fourth repeat are essential for the folding activity but not for the binding activity. The second RNA binding site is located at the very C-terminal end of the protein and three amino acids have been shown to be critical for binding (K774, R775 and K779) (Valdez, 2000). When deleted, and in the presence of intact FRGQR repeats, RNA folding activity is abolished but RNA binding is maintained.

These two distinct activities do not require similar conditions to be triggered. ATP and Mg^{2+} have shown opposite effects on RNA unwinding and RNA folding activities of DDX21 (Valdez et al., 1997b). Firstly, RNA folding activity is stimulated at low ATP concentration (0.5 mM) and inhibited at high ATP concentration (5 mM). RNA helicase activity is specifically dependent on ATP whereas RNA folding activity is present even in the absence of ATP and can also be stimulated by GTP. Second, Mg^{2+} at low concentration is required for RNA unwinding whereas absence of Mg^{2+} does not affect RNA folding. Moreover, in *Xenopus laevis* oocytes, DDX21 depletion by antisense RNA results in aberrant ribosomal biogenesis. In particular, DDX21 is involved in the processing of 20S to 18S rRNA and stabilizes 28S rRNA (Yang et al., 2003). Using short interfering RNA (siRNA), in mammalian cells, DDX21 has been shown to be involved in 18S and 28S rRNA production (Henning et al., 2003). In addition, Hela cells transfected with deletion constructs of wt DDX21 show cell growth impediment indicating that DDX21 is required for cell growth (Ou et al., 1999).

Using a GST pull-down assay, DDX21 was co-purified from a pre-ribosomal ribonucleoprotein together with human parvulin protein (Fujiyama et al., 2002). Using TAP and mass spectrometry, DDX21 has been found to be a co-factor for c-Jun transcription (Westermarck et al., 2002). DDX21 was also shown to interact with ribosomal protein L4 in an ATPase dependent manner. Moreover, this interaction is necessary for correct rRNA processing (Yang et al., 2005b). Finally, using the yeast two-hybrid method, DDX21 was found to interact with the protein inhibitor of activated STAT1 which upon interaction with DDX21 drives DDX21 proteolytic cleavage (Valdez et al., 1997a).

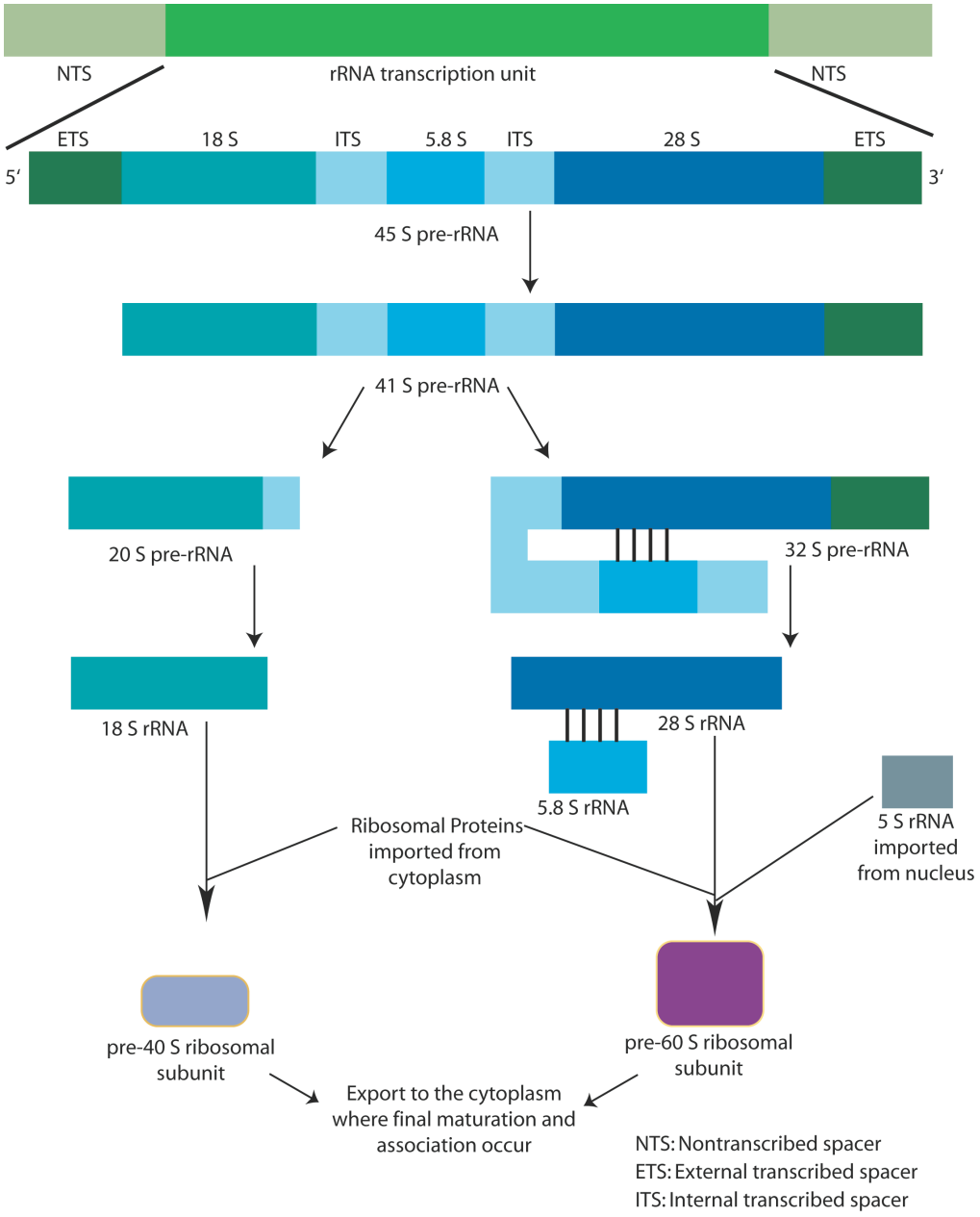

Figure 4. Ribosome biogenesis.

Several other human RNA helicases involved in ribosome biogenesis have been described to be overexpressed in various cancer types (Abdelhaleem, 2004). Similarly, DDX21 is overexpressed in prostate cancer cells, germ cells, colorectal, acute leukemia, myeloma, ovarian and lung cancer (Andersson et al., 2007; Ki et al., 2007; Lapointe et al., 2004; Sperger et al., 2003; Talbot et al., 2005; Welsh et al., 2001; Zhan et al., 2002). However, no evidence has been presented to explain either DDX21 over-expression or DDX21 specific roles in cancer cell behavior.

AIMS OF THE STUDY

The specific objectives of this study were

Aim I: To understand the role for Topoisomerase I in c-Jun-mediated regulation of cancer cell behavior

Aim II: To understand the role for DDX21 in c-Jun-mediated regulation of cancer cell behavior

Aim III: To determine mechanisms by which DDX21 regulates cancer cell behavior

MATERIALS AND METHODS

Cell cultures

PC-3, human embryonal kidney cells (HEK) 293, HT-1080, Hela, wt and c-Jun$^{-/-}$ mouse embryonal fibroblast (MEF) cells were obtained from ATCC. HEK 293, HT-1080, Hela and MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 6 nmol/l glutamine, nonessential amino acids, 100 U/ml penicillin, 100 μg streptomycin, and 10% fetal calf serum (FCS) (Invitrogen). PC-3 cells were cultured in HAM F-12 medium (Lonza) supplemented with 6 nmol/l glutamine, nonessential amino acids, 100 U/ml penicillin, 100 μg streptomycin, and 10% FCS (Invitrogen).

Transient transfections and siRNA treatment

Subconfluent cells were transiently transfected with plasmids using FuGene 6 Transfection reagent (Roche) according to the manufacturer's protocol. For siRNA duplex oligonucleotide treatments, transfections were performed using Oligofectamine (Invitrogen) following the manufacturer's protocol. Cells were incubated with siRNA for the indicated length(s) of time. SiRNA oligonucleotide sequences are listed in Table 2.

Table 2. SiRNA oligonucleotide sequences.

SiRNA	Sequence targeted
c-Jun (used in II)	5′-GATCCTGAAACAGAGCATG-3′
c-Jun (used in II)	5′-AACGCAGCAGTTGCAAACA-3′
DDX21 (used in II)	5′-GGGAAATCACCCTGAAAGG-3′
DDX21 (used in II)	5′-AAGAGCAGCTGGGCGAGGAGA-3′
DDX21 (used in II)	5′-GTGTGGCATACAAGAAAGA-3′
DDX21 (used in III)	5′-CGGGAATTAAGTTCAAACGAA-3′
Cdk6.5	5′-AAGACTCAAGGTGGTCAGTAA-3′
Cdk6.6	5′-TCTGAAGTGTTTGACATTTAA-3′
Cdk6.2	5′-CACGTAGAGTGCAAATTAGAA-3′
Cdk6.3	5′-CAGATGAGTCTCAGTAATTCA-3′

Co-immunoprecipitations

Protein G-Sepharose (Pharmacia) beads were agitated on a tumbler for 2 hours at 4°C with c-Jun goat antibody and preimmune goat sera, with c-Jun rabbit antibody and GST antibody, with green fluorescent protein (GFP) antibody, or with HA antibody in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid with potassium hydroxide (HEPES-KOH) pH7.5, 400 mM NaCl, 0.25 mM ethylene glycol tetraacetic acid (EGTA), 1.5 mM

$MgCl_2$, 0.25% NP-40, protease inhibitors (PI) (Roche), 10 mM β-glycerolphosphate, 20 mM NaF and 0.5 mM Dithiothreitol (DTT). Before co-immunoprecipitation, HT-1080 nuclear extracts (preparation described in (Andrews and Faller, 1991)) were sonicated and pre-cleared for 2 hours at 4°C with goat or rabbit pre-immune serum immobilized to protein G-Sepharose beads, and supernatants from the pre-cleared samples were incubated with immobilized c-Jun, GFP, or HA antiserum or control goat or rabbit pre-immune serum overnight at 4°C. Thereafter, the sedimented beads were washed four times with 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.3% NP-40 AND 0.5 mM DTT, and bound protein were analyzed by western blotting.

Western blotting

For western blot analysis, cells were lysed in 150mM Tris HCl pH6.8, 1.2% Sodium dodecyl sulfate (SDS), 30% glycerol, 15% β-mercaptoethanol, and 0.02mg/ml bromophenol blue. Cell lysates were boiled and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to Immobilon-P membrane (Millopore). Primary and secondary antibodies used for protein detection are listed in Table 3. Secondary antibodies were detected by enhanced chemiluminescence (ECL) (Pierce).

Luciferase reporter assay

Cells were plated in a 96-well plate and transfected at 50% confluency with the following reporter plasmids: pf2Luc, together with CMVGalDBD, CMVJunGal (Stratagene), pEGFP, or Topo-GFP (Rallabhandi et al., 2002) expression constructs. 24 hours after transfection, firefly luciferase activity was measured by using the Dual-Glo luciferase assay system (Promega).

Immunofluorescence

Immunostaining of cells grown on coverslips was performed by simultaneous fixation and permeabilization in 20 mM PIPES pH 6.8, 4% formaldehyde, 0.2% Triton X-100, 10 mM EGTA, 1 mM $MgCl_2$, for 10 min at room temperature (RT). DNA was stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen). Primary antibodies used are listed in Table 3. Primary antibodies were detected with Cy2- or Cy3-conjugated secondary antibodies (Jackson Immunoresearch). Wide-field fluorescence microscope images were collected using a Leica DMRE microscope equipped with a high resolution Hamamatsu Photonics ORCA C4742-95 CCD camera. Fluorescence intensities were determined using the ImageJ64 imaging software (http://rsbweb.nih.gov/ij/index.html).

Table 3. Antibodies. WB: Western Blot, IP: Imunoprecipitation, IHC: Immunohistochemistry.

Antibody	Source	Supplier	Application
Actin	mouse	Sigma Aldrich	WB
B23	goat	Santa Cruz Biotechnology	IHC
Cdk1	mouse	Santa Cruz Biotechnology	WB
CDK4	rabbit	Santa Cruz Biotechnology	WB
Cdk6	rabbit	Santa Cruz Biotechnology	WB
c-Jun	mouse	Cell Signalling	WB, IHC
c-Jun (H-79)	rabbit	Santa Cruz Biotechnology	WB
Cyclin D1	mouse	Santa Cruz Biotechnology	WB
Cyclin E	rabbit	Santa Cruz Biotechnology	WB
DDX21	human	(Valdez et al., 1996)	WB, IHC
EGFR	rabbit	Santa Cruz Biotechnology	WB
ERK1,2	mouse	Cell Signaling Technology	WB
GST	mouse	Santa Cruz Biotechnology	WB, IP
HA	mouse	Santa Cruz Biotechnology	IHC, WB
His	rabbit	Santa Cruz Biotechnology	WB, IP
JNK2	rabbit	Santa Cruz Biotechnology	WB
Ki-67	mouse	Novocastra™ Laboratories Ltd.	IHC
MEK1,2	rabbit	Cell Signaling Technology	WB
p15	rabbit	Cell Signaling Technology	WB
p21	rabbit	Santa Cruz Biotechnology	WB
p53	mouse	Santa Cruz Biotechnology	WB
p-c-Jun	mouse	Santa Cruz Biotechnology	WB
PCNA	mouse	Santa Cruz Biotechnology	WB
p-EGFR	goat	Santa Cruz Biotechnology	WB
p-JNK	mouse	Santa Cruz Biotechnology	WB
TBP	rabbit	Santa Cruz Biotechnology	WB
Topol	rabbit	Santa Cruz Biotechnology	WB

Recombinant proteins synthesis

For glutathione S-transferase (GST) protein induction, BL21 bacterial strain competent cells (Sigma Aldrich) were heat-shock transformed with pGEX-4T-2 plasmids (HE Healthcare) and grown overnight on a Luria-Bertani broth (LB) agar plate at +37°C. Transformed colonies were selected by ampicillin (Amp) resistance. One colony was inoculated into a 30ml LB+Amp culture and grown in a shaking incubator overnight at +37°C. 10ml of the overnight culture were pelleted at RT (2000 rpm/5min) and resuspended in 10ml of fresh LB+Amp. This was used to inoculate a 500ml LB+Amp culture and grown in a shaking incubator at +37°C until an optic density of 0.8 was reached. Thereafter the culture was incubated at +22°C for 5 hours complemented with 0.1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). For protein purification, bacteria were pelleted at +4°C (4000rpm/10min) and resuspended in 13.5ml of cold phosphate buffered saline (PBS) complemented with PI (0.5 mM Phenylmethylsulphonylfluoride (PMSF), 0.5 mM DTT, and 1x Complete (Roche)) and 1mg/ml lysozyme. The bacteria

were incubated for 10 minutes on ice prior to sonication. 1.5ml of cold 10% Triton X-100 was added to the bacterial lysate and then pelleted at +4°C (10000rpm/20min). The supernatant was incubated on a rotating wheel for 1 hour at +4°C with 1ml of Glutathione Sepharose beads previously washed twice in 10ml of cold PBS. The beads were pelleted at +4°C (1000rpm/2min) and washed 3 times in 10ml cold PBS, and once in 10ml of cold 50mM Tris pH8.0. For protein elution, beads were incubated at RT for 3 minutes with 0.5ml of 10mM fresh Glutathione in 50mM Tris pH8.0 elution buffer. The beads were pelleted at +4°C (1000rpm/1min) and protein elution was repeated 2 more times until three fractions were obtained. The residual beads were pelleted at +4°C (14000rpm/1min), and the supernatant was dialyzed at +4°C for 2 hours against a dialysis buffer (20mM Tris pH8.0, 50mM NaCl, 0.1mM EDTA, 0.5mM DTT, 5%glycerol).

Purification of polyhistidine-tagged proteins was performed using the Protino® Ni-IDA 1000 packed columns kit (Macherey-Nagel) under denaturing conditions following the manufacturer's instructions.

in vitro RNA-binding assay

A fluorescent RNA substrate (Proligo) for RNA binding assay was designed from pBluescript II KS plasmid sequence. The FITC fluorescent label was coupled on the 5'end of the RNA sequence: 5'-FITC-CGCUCUAGAACUAGUGGAUCCCCCGGGC UGCAGGAAUUCGAUAUCAAGCU-3'. RNA binding assays were performed using glutathione-Sepharose 4B beads (Pharmacia). 25 µl of beads were washed three times in 1 ml of EBC buffer (140mM NaCl, 0.5% NP-40, 50mM Tris pH8.0) and blocked for 5 minutes with 5% bovine serum albumin (BSA) in an EBC buffer at room temperature. The beads were then washed in 1 ml of EBC complemented with PI. 1µg of GST proteins and the beads were thereafter incubated with rotation in 300 µl of the EBC buffer with PI for 1 hour at +4°C. After spinning (1000 rpm, 2 minutes at room temperature), the beads were added to 1µg of His-tagged proteins and 23 pmoles of fluorescent RNA in 500 µl His buffer with PI and rotated for one hour at room temperature. The beads were then washed three times in His buffer (20mM Tris pH7.4, 0.2mM ethylenediaminetetraacetic acid (EDTA), 0.1M NaCl) complemented with 0.1% NP-40 and PI. The fluorescence was then measured using the fluorescein (485/535 nm, 1s) protocol on the VICTOR2™ 1420 Multilabel Counter (Wallac).

Plasmid constructs

GFP vectors: DDX21 constructs were PCR amplified from pGEX4T-2 DDX21 vectors and cloned in the pEGFP-C1 in the XhoI and BamHI sites.

DsRed vectors: DDX21 constructs were PCR amplified from pEGFP-C1 DDX21 vectors and cloned in the pDsRed Express-1 vector using the XhoI and BamHI sites.

Mutant vectors: Mutagenesis was introduced using the QuikChange® II Site-Directed Mutagenesis Kit protocol (Stratagene). Mutagenesis primer sequences are listed in Table 4.

Table 4. Mutagenesis primers used to produce Ds-red-DDX21 KRK to ARK and ARA mutants.

Mutation	Sequences
DMK774AF (KRK->ARK)	5′-CAAAACAAAGGCCAGGCGCGGAGTTTCAG-3′
DMK774AR (KRK->ARK)	5′-CTGAAACTCCGCGCCTGGCCTTTGTTTTG-3′
DMK779AF (KRK->ARA)	5′-CGGAGTTTCAGTGCATTTGGTC-3′
DMK779AR (KRK->ARA)	5′-GACCAAATGCACTGAAACTCCG-3′

His pull-down and peptide interaction inhibition assay.

His pull-down interaction studies were performed using Ni-NTA agarose beads (QIAGEN). 25 µl of beads was washed three times in 1 ml of EBC buffer (140 mM NaCl, 0,5% Nonidet P-40 (NP-40), 50mM Tris (pH 8.0)) and blocked for 5 minutes with 5% BSA in an EBC buffer at room temperature. The beads were then washed in 1 ml of EBC complemented with PI. 1µg His-tagged c-Jun proteins (complemented with 20x molar ratio of peptides when required) and the beads were thereafter incubated with rotation in 300 µl of EBC buffer with PI for 1 hour at +4°C. After spinning, (1000 rpm, 2 minutes at room temperature), the beads were added to 1µg of GST proteins in 500 µl of His buffer (20 mM Tris pH 7.4, 0.2 mM EDTA, 0.1 M NaCl with PI and rotated for one hour at room temperature. The beads were then washed three times in His buffer complemented with 0.1% NP-40 and PI. Samples were analyzed by western blotting using GST, His or c-Jun antibodies (Santa Cruz Biotechnology).

Cell proliferation and cell viability assay

For the cell proliferation assay, the cells were plated on a 96-well plate and transfected with siRNA for 48 hours. Thereafter, cell proliferation was measured using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT assay) (Promega) following the manufacturer's protocol. For the cell survival assay, the cells were plated on a 96-well plate and transfected with siRNA for 48 hours. Thereafter, the cells were exposed to various ultraviolet (UV) doses, and cell survival was measured after 24 hours by MTT assay.

Cell migration assay

For the cell migration assay, the cells were transfected with DDX21 or Cdk6 siRNAs for 48 hours. Thereafter, the cells were scratched with a 1 ml pipette tip, and cell migration

was measured for 48 hours. Wide-field microscope images were collected every 12 hours using an Olympus CK2 microscope equipped with a digital Canon Powershot A510 camera. Cell migration was measured using the ImageJ64 imaging software.

Fluorescence recovery after photobleaching

For fluorescence recovery after photobleaching (FRAP) studies, the cells were transfected with the EGFP-DDX21 plasmid (Valdez et al., 1998) or the EGFP-DDX21 c-Jun binding mutant plasmid and grown on glass-bottom dishes (MatTek). The size and location of the photobleach and photoswitching area were adjusted with the Zeiss LSM 510 version 3.2 software. Fluorescence intensities were measured using photomultiplier tubes connected to the microscope and analyzed using the Zeiss LSM 510 version 3.2 software through EGFP (excitation, 405 nm; emission, 480 nm) and FITC channels (excitation, 490 nm; emission, 528 nm). Digital images were superimposed and assembled using Adobe Photoshop software.

RNase treatment

For RNase treatment, fixed cells were incubated in RNase A (Sigma–Aldrich) at 40 µg/ml in PBS for 2 hours at 37ºC. RNAse inhibitor, Ribolock RNase inhibitor (Fermentase), was incubated with RNAse following the manufacturer's protocol.

RESULTS

1 TopoI is an interacting partner for c-Jun

1.1 Identification of c-Jun-TopoI interaction

To discover novel mechanisms regulated by c-Jun in human cells, we purified proteins interacting with the amino-terminal transactivation domain of c-Jun *in vivo*. In this regard, the c-Jun amino-terminal sequence (from amino acids 1 to 223) was linked to the TAP domain (c-Jun$^{1-223}$TAP) (I, Fig.1A). Thereafter, HEK293 were transfected with c-Jun$^{1-223}$TAP for 24 hours. Thereafter, nuclear proteins from transfected and non-transfected cells were purified following the tandem affinity purification method (Puig et al., 2001). Purified proteins were separated by SDS-PAGE and gel was silver stained (I, Fig.1B). c-Jun interacting proteins were subsequently identified by mass spectrometric peptide sequencing. In comparison with mock transfected cells, eluates from c-Jun$^{1-223}$TAP transfected cells displayed c-Jun interacting proteins with known gene regulation functions (I, Fig. 1B and Table 1).

To verify whether c-Jun and TopoI operate in similar cellular compartment, a colocalization study of these proteins was performed in human HT-1080 fibrosarcomas cells transfected with TopoGFP and HA–c-Jun constructs. TopoI localized in the nucleolus and the majority of c-Jun in the nucleoplasm as previously reported (I, Fig.1C) (Chida et al., 1999; Rallabhandi et al., 2002). However, c-Jun was clearly concentrated in perinucleolar domains, where it colocalizes with TopoI (I. Fig.1C, white arrows).

To biochemically verify the interaction between TopoI and c-Jun, nuclear extracts of HT-1080 fibrosarcoma cells were subjected to coimmunoprecipitation analyses. Endogenous TopoI coimmunoprecipitated with endogenous c-Jun (I, Fig.2A). TopoI was previously described to interact with TBP and TFIID, and c-Jun has been demonstrated to bind to TBP *in vitro* (Franklin et al., 1995; Kretzschmar et al., 1993; Merino et al., 1993). Importantly, endogenous TBP was detected in c-Jun immunoprecipitate from HT-1080 cells (I, Fig.2A). However, cyclin D1, as a negative control, was not immunoprecipating with c-Jun from HT-1080 cells (I, Fig.2A). To confirm endogenous c-Jun-TopoI interaction, immunoprecipitation with a different c-Jun antibody raised in rabbit was performed. Coimmunoprecipitation between c-Jun and TopoI was noticed, indicating that the observed interaction was not due to unspecific binding of TopoI to the c-Jun antibody (I, Fig.2B). However, c-Jun did not coimmunoprecipitate with the GST antibody. Finally, a reciprocal experiment was performed with TopoI fused to green fluorescent protein (TopoGFP) in HT-1080 cells. Immunoprecipitation with GFP antibody demonstrated c-Jun-TopoI interaction from HT-1080 nuclear extracts (I, Fig.2C). These results confirm endogenous interaction between c-Jun and TopoI.

1.2 Regulation of c-Jun-TopoI interaction

To determine the possible role for c-Jun phosphorylation in the regulation of TopoI interaction, c-Jun-TopoI interaction analyses were performed in contexts where c-Jun phosphorylation was prevented. HT-1080 cells were first treated with a chemical JNK inhibitor SP600125 for two hours, and subsequently coimmunoprecipitation assays were performed. SP600125 clearly reduced c-Jun's phosphorylation status. However, SP600125 did not affect the expression levels of TopoI and c-Jun (I, Fig.2D, NP). TopoI interaction with c-Jun was reduced in SP600125 treated cells, indicating that c-Jun-TopoI interaction correlates with the phosphorylation of c-Jun.

An independent experiment was performed in which HT-1080 cells were transiently transfected with HA-tagged c-Jun constructs with N-terminal JNK phosphorylation sites mutated to alanines. Subsequent coimmunoprecipitation analyses were performed by using the HA antibody. Whereas the WT c-Jun construct co-immunoprecipitated with TopoI, no interaction between the alanine mutated c-Jun construct and TopoI was observed. Together, these results show that the interaction between c-Jun and TopoI is positively regulated by JNK-mediated c-Jun amino-terminal phosphorylation.

1.3 EGFR is a transcriptional target for c-Jun and TopoI

To determine the functional role for TopoI in c-Jun mediated transcription, a luciferase reporter assay was performed. The luciferase reporter activity driven by JunGal was found to be increased upon transfection of TopoI (I, Fig.2F). These results demonstrate that TopoI enhances c-Jun transcriptional activity.

EGFR has been demonstrated to be a c-Jun target gene in MCF-7 cells and keratinocytes (Johnson et al., 2000; Zenz et al., 2003). To verify whether EGFR was also a c-Jun target gene in HT-1080 cells, a c-Jun dominant negative deletion construct containing the DNA binding and dimerization domains (JunbZIP) was transiently transfected into HT-1080 cells. EGFR is highly expressed in HT-1080 cells, and EGFR protein expression decreased upon overexpression of dominant negative c-Jun (I, Fig.3A). To confirm these observations, HT-1080 cells were transfected with either c-Jun or scrambled short hairpin RNA (shRNA) constructs. EGFR expression was decreased upon c-Jun shRNA transfection when compared to scrambled transfected cells (I, Fig.3B). Together, these results indicate that c-Jun positively regulates EGFR expression in HT-1080 cells.

To verify the role for TopoI in the c-Jun mediated regulation of EGFR expression, the cells were treated with topotecan. A clear concentration-dependent decrease of expression of EGFR is observed when cells are exposed to topotecan (I, Fig.3C). Moreover, the p-EGFR protein level decreases proportionally with EGFR protein level upon topotecan treatment (I, Fig.3D). Taken together, these results demonstrate that TopoI supports the expression of activated EGFR in HT-1080.

To verify the specificity of the inhibition of EGFR expression upon topotecan treatment, HT-1080 cell lysates were treated with topotecan. Interestingly, even when a clear EGFR expression decrease was noticed upon topotecan treatment, neither MEK1,2 nor ERK1,2 expression levels were affected by TopoI inhibition (I, Fig.3E). To further study the specificity of inhibition of EGFR expression by topotecan treatment, expression levels of established c-Jun target genes were analyzed from cell lysates used in I, Fig.3D. We found that neither c-Jun nor CDK4 expression was affected by topotecan treatment (I, Fig.3F). Altogether, these results demonstrate that c-Jun and TopoI positively regulate EGFR expression in HT-1080 cells. Moreover, inhibition of EGFR expression by topotecan is not due to a general transcription impediment.

To analyze the dependency on c-Jun of the role played by TopoI in the regulation of expression of EGFR, EGFR expression was studied in c-Jun-deficient cells (MEF c-Jun$^{-/-}$). The basal level of EGFR expression in c-Jun$^{-/-}$ MEFs is reduced as compared to wt MEFs (I, Fig.4A). More interestingly, whereas in wt MEFs experiencing topotecan treatment, EGFR expression is reduced, no further decrease of EGFR expression was noticed in c-Jun$^{-/-}$ MEFs. These results indicate that c-Jun-deficient cells are protected from the effects of topotecan treatment (I, Fig.4A). These results were recapitulated at the mRNA level (I, Fig.4B). In addition, mRNA expression of *EGFR* was measured in SP600125-treated HT-1080 cells. Upon inhibition of JNK phosphorylation of c-Jun, *EGFR* expression decreased (I, Fig.4C). A similar reduction of *EGFR* expression was observed in topotecan-treated cells (I, Fig.4C). However, in cells where JNK phosphorylation of c-Jun was inhibited, no further reduction of *EGFR* expression upon topotecan treatment was observed (I, Fig.4C). These results confirmed that cells lacking c-Jun are not sensitive to topotecan treatment and that TopoI and c-Jun co-operate in the positive regulation of *EGFR* expression.

To investigate the specificity of the inhibition of EGFR expression in HT-1080 cells following topotecan treatment, topotecan treatment was performed and *EGFR* and *Erb2* mRNA levels were measured. Whereas topotecan treatment induces a reduction of *EGFR* expression, *Erb2* expression was not affected (I, Fig.5C). In addition, the effects of topotecan treatment on *EGFR* and *Erb2* expression were noticed on various cancer cell lines (Hela and HaCat) and normal skin fibroblasts (NSF) (I, Fig.5E). Finally, topotecan treatment did not affect transcription in general and all c-Jun target genes (I, Fig.5D). Altogether, these results show that the JNK pathway and TopoI positively regulate EGFR expression without affecting general transcription.

1.4 JNK-c-Jun pathway and TopoI stimulate cell proliferation

To study the role for c-Jun in HT-1080 cell proliferation, HT-1080 cells were transfected with wt c-Jun, dominant negative c-Jun mutant, scrambled or c-Jun shRNA. Interestingly, upon transfection of dominant negative c-Jun or c-Jun shRNA, expression of PCNA was

decreased (I, Fig.6B and C). These results indicate that c-Jun supports HT-1080 cellular proliferation.

Moreover, inhibition of JNK phosphorylation induced a clear reduction of BrdU incorporation. Interestingly, topotecan treatment induced a similar reduction of BrdU incorporation to that observed in SP600125-treated cells. More interestingly, no cumulative reduction of BrdU incorporation was observed in cells treated with both SP600125 and topotecan compared to either SP600125 or topotecan alone (I, Fig.6D). These results indicate that cells inhibited for c-Jun phosphorylation are protected from the effects of topotecan treatment. Moreover, previous results indicated that interaction between c-Jun and TopoI is dependent on the phosphorylation status of c-Jun. Therefore, these results indicate that c-Jun and TopoI interact and cooperate in the positive regulation of cancer cell proliferation.

1.5 Inhibition of EGFR expression by topotecan reduces cancer cell proliferation

To investigate whether topotecan treatment reduces cell proliferation through downregulation of EGFR expression, HT-1080 cells were treated with topotecan, a specific ErbB2 inhibitor (herceptin), and either a specific chemical EGFR tyrosine kinase inhibitor (AG1478) or thymidine as a control for proliferation inhibition independent of EGFR pathway. Topotecan and AG1478-treated cells displayed a decrease in p-EGFR, PCNA and Cyclin E expression (I, Fig.7A). However, thymidine treatment decreased PCNA and CyclinE expression but did not affect p-EGFR (I, Fig.7A). Furthermore, in cells treated with topotecan or AG1478 bromodeoxyuridine (BrdU) incorporation was decreased by 40% (I, Fig.7B). More importantly, cells treated with both AG1478 and topotecan did not exhibit cumulative inhibition, suggesting that upon EGFR activity inhibition, topotecan does not affect cell proliferation (I, Fig.7B). These results suggest that topotecan inhibition of cell proliferation is mediated by inhibition of EGFR expression. Upon overexpression of EGFR, topotecan inhibition of cell proliferation was rescued (I, Fig.7C). In the western blot analysis of these samples, a clear overexpression of EGFR was noticed in EGFR-transfected cells, and EGFR expression was decreased in control-transfected cells treated with topotecan (I, Fig.7D). Interestingly, only in control transfected cells did topotecan decrease CyclinE expression (I, Fig.7D). These results show that EGFR overexpressing cells are resistant to topotecan-elicited inhibition of proliferation.

Taken together these results show that c-Jun together with TopoI promote expression of EGFR in cancer cells. In addition, these results demonstrate that the clinically used TopoI inhibitor topotecan reduces EGFR expression. More importantly, the effect of TopoI on EGFR transcription was shown to depend on c-Jun as $Jun^{-/-}$ cells or cells treated

with JNK inhibitor SP600125 are resistant to topotecan treatment both in regulation of EGFR expression and cell proliferation.

2 c-Jun stimulates rRNA processing by supporting DDX21 rRNA binding

2.1 Identification of the interaction domain for c-Jun on DDX21

Recent report demonstrated an increased in the expression of 28S and 18S rRNA in cells transformed with v-Jun (Black et al., 2004). In addition, using the TAP purification method, c-Jun protein complex contained several protein involved in rRNA processing. Within these proteins, DDX21 was reported to directly interact with c-Jun (Westermarck et al., 2002). To pin down the binding domain for c-Jun on DDX21, His pull-down assays were conducted with various GST-tagged deletion constructs for DDX21 and a His-tagged c-Jun construct. A dichotomic analysis of the GST-DDX21 deletion constructs interacting with the His-c-Jun protein indicated that the interaction domain for c-Jun on DDX21 is contained within the amino acids 731 and 740 (II, Fig.4A). Importantly, GST control protein did not interact with His-c-Jun. Based on these results, a peptide inhibition assay was performed. His-pull down assays were performed with His-c-Jun, pre-incubated with the indicated peptides and GST-DDX21 628-783. Peptide 1 corresponds to the AA 744 to 758 on DDX21 and peptide 2 to AA 729 to 743. Whereas no significant difference was noticed in the affinity of GST-DDX21 628-783 for His-c-Jun in either the peptide 1 pre-incubated sample, or in the control sample, peptide 2 caused a clear reduction DDX21-c-Jun interaction (II, Fig.4D). In addition, random mutations within a five amino acid fragment (FRGQR to YEGIQ, AA 734-738) of the interaction domain were introduced and a His pull-down assay with His-c-Jun was performed. A clear reduction of GST-DDX21 628-783 Mut interaction with His-c-Jun compared to GST-DDX21 628-783 WT was noticed (II, Fig.4B). These results demonstrated that the four amino acids (F, R, Q and R) within the fragment (FRGQR) are essential for interaction of DDX21 with c-Jun. Moreover, protein sequence alignments of DDX21 sequences from organisms throughout the animal kingdom displayed a high level of conservation of these four amino acids, suggesting that this domain is important for DDX21 function (II, Fig.S3A).

2.2 c-Jun stimulates rRNA processing

To verify a possible function for c-Jun in rRNA processing, Hela cells were treated with c-Jun siRNA, and the steady-state abundance of 45S, 32S pre-rRNAs, 28S and 18S rRNAs was measured. We found that the accumulation of 28S and 18S rRNA species was inhibited when c-Jun expression was impeded. The dynamic measure of ^{32}P accumulation was performed, and a decrease of 18S and 28S rRNA was already noticed

in c-Jun depleted cells 2 hours after ^{32}P addition compared to scrambled transfected cells (II, Fig.1A and B). These results demonstrate that c-Jun depletion reduces 28S and 18S rRNA accumulation in Hela cells.

2.3 c-Jun regulates the nucleolar localization of DDX21

To determine whether c-Jun regulates the nucleolar localization of DDX21, the cellular localization of DDX21 was analyzed in MEFs wt and in c-Jun depleted (c-Jun$^{-/-}$) cells. In c-Jun$^{-/-}$ cells, relocalization of DDX21 to the nucleoplasm was observed (II, Fig.2A and C). To recapitulate these results, Hela cells were transfected with c-Jun siRNA, and subsequently analysis of the localization of DDX21 was performed. c-Jun siRNA-treated cells exhibited a nucleoplasmic relocalization of DDX21 (II, Fig.2B and D). In addition, transient transfections of c-Jun constructs in Hela cells were conducted. Transfected cells displayed significant nucleolar relocalization of DDX21 (II, Fig.2E and F). These results demonstrate that c-Jun positively regulates the nucleolar localization of DDX21.

To analyze the role for c-Jun interaction with DDX21 in the nucleolar localization of DDX21, peptide microinjection experiments were performed in Hela cells. When peptide 2 was microinjected, a significant nucleoplasmic relocalization of DDX21 was observed (II, Fig.4E and F). These results indicate that interaction between c-Jun and DDX21 is required for the nucleolar localization of DDX21. These results were recapitulated by transient transfections of the DDX21 construct deficient in c-Jun binding in Hela and PC-3 cells. DDX21 Mut transfected cells were significantly localized in the nucleoplasm (II, Fig.4C, III, Fig.2E). This result indicates that c-Jun interaction with DDX21 supports the nucleolar localization of DDX21. Finally, to verify that the nucleolar localization of DDX21 depends on the nucleoplasmic localization of c-Jun, c-Jun$^{-/-}$ cells were transiently transfected with a HA-Jun construct fused to the hormone-binding domain of the estrogen receptor (HA-JunER). Upon tamoxifen treatment, HA-JunER translocalized to the nucleoplasm, and DDX21 relocalized to the nucleolus (II, Fig.4G). Altogether, these results demonstrate that the direct interaction of c-Jun and DDX21 is required for the nucleolar localization of DDX21.

2.4 c-Jun stimulates DDX21 rRNA binding and rRNA processing

To study whether c-Jun directly promotes rRNA binding of DDX21, an *in vitro* RNA binding assay was developed (II, Fig.4H). FITC-RNA probes were designed and added to GST-DDX21 proteins immobilized on glutathione beads. After three washes, the residual fluorescence was measured. Upon addition of His-c-Jun proteins to GST-DDX21 and FITC-RNA, the residual fluorescence was significantly higher than the background signal and GST-DDX21 with FITC-RNA alone. These results demonstrate that c-Jun stimulates GST-DDX21 RNA binding.

To confirm these results, DDX21 rRNA immunoprecipation was performed in either scrambled or c-Jun siRNA-transfected Hela cells. Further northern blot analysis of the various rRNA species bound to DDX21 was carried out. Upon depletion of c-Jun, a clear reduction in the association of DDX21 with 45S and 32S pre-rRNA was observed (II, Fig.3E). These results indicate that c-Jun promotes DDX21 binding to 45S and 32S pre-rRNA. Moreover, c-Jun depletion did not reduce the amounts of 45S and 32S pre-rRNA but reduced the accumulation of 28S and 18S rRNA (II, Fig.1B and C). Together, these results show that c-Jun regulates rRNA binding of DDX21 and thereby the rRNA processing.

The affinity of substrate binding protein was proposed to indirectly correspond to their mobility and, to study protein mobility, FRAP method was developed. To verify that c-Jun supports the rRNA binding of DDX21 *in vivo*, wt and c-Jun$^{-/-}$ MEFs were transfected with photoswitchable cyan fluorescent DDX21 protein (PS-CFP2-DDX21) and thereafter subjected to FRAP analysis. Either the inward movement (recovery, II, Fig.3C) or the outward movement (exit, II, Fig.3D) of DDX21 occurs more rapidly in c-Jun$^{-/-}$ cells indicating that c-Jun promotes rRNA binding of DDX21.

2.5 c-Jun and DDX21 inhibition induces cell cycle block

To study the role for c-Jun-DDX21 interaction on Hela cell proliferation, flow cytometric measurements of the cell cycle distribution after depletion of c-Jun or DDX21 were performed. Upon c-Jun depletion, a G1/S block of the cell cycle was noticed (II, Fig5A). A similar result was obtained when cells were depleted for DDX21 (II, Fig5A). More interestingly, upon co-depletion of c-Jun and DDX21, comparable results were observed (II, Fig5A). Similar results were observed with BrdU incorporation assays (II, Fig.5B). These results indicate that c-Jun and DDX21 positively regulate cellular proliferation.

3 DDX21 in prostate cancer

3.1 DDX21 localization correlates with prostate cancer progression

Our previous study demonstrated the supportive role for c-Jun in the nucleolar localization and rRNA binding of DDX21 in cancer cells. To analyze the clinical role of DDX21 in prostate cancer, DDX21 was stained in primary and hormone refractory (HR) prostate tumors (III, Fig.1A). Highly metastatic HR exhibited a clearly stronger nucleolar staining of DDX21 compared to primary tumors. Quantifications of immunohistochemical stainings indicate that the number of cells displaying weak nucleolar stainings of DDX21 was decreased and the number of cells showing strong nucleolar staining of DDX21 was increased (III, Fig.1B). To study the mRNA expression level of DDX21, real-time PCR analyses were performed on primary and HR tumors (III, Fig.1C). No significant

difference of mRNA expression was detected between primary and HR tumors. Together, these results indicate that the intensity of nucleolar stainings of DDX21 correlates with prostate cancer progression and is not due to an increase of DDX21 expression.

To assess the correlation of the recruitment of DDX21 to the nucleolus and the proliferation status of tumors, primary and HR tumors were stained for the proliferation marker Ki-67 (III, Fig.1A). HR tumors displayed limpidly higher number of cells expressing Ki-67 compared to primary tumors. These results demonstrate that the nucleolar recruitment of DDX21 correlates with proliferation status of prostate tumors (III, Fig.1A). Taken together, these results describe DDX21 as a potential biomarker in the diagnosis of advanced prostate cancer.

3.2 DDX21 stimulates Cdk6 expression

DDX21 depletion in Hela cells induced a G1/S cell cycle block (II, Fig.5A). To assess the role for DDX21 in prostate cancer cell, PC-3 cells were transfected with DDX21 siRNA and cell cycle flowcytometric analysis was performed (III, Fig.3A). No significant difference between scrambled and DDX21 siRNA transfected cells could be detected. This observation indicates that DDX21 depletion does not affect PC-3 cell cycle progression. DDX21 was also described to play a role in Hela cells proliferation (II, Fig.5B). To determine the role for DDX21 in prostate cancer proliferation, PC-3 cells were transfected with DDX21 siRNA and cell proliferation was evaluated by MTT assay (III, Fig.3D). No significant difference in cellular proliferation was detected between non-treated, scrambled or DDX21 siRNA transfected cells. These results demonstrate that DDX21 does affect PC-3 cell proliferation. Thereafter cell cycle markers expression were analysed by western blot in PC-3 cells upon DDX21 depletion (III, Fig.3C). Interestingly, after multiple repetitions of the experiment, no decrease in the expression of Cyclin E, Cdk4, PCNA, Actin, Cyclin D1, Cdk1 or p15 was detected. However, in all experiments preformed, Cdk6 expression decreased upon DDX21 depletion. These results indicate that DDX21 does not regulate cell cycle or cell proliferation but stimulates Cdk6 expression in PC-3 cells.

DISCUSSION

1 Is c-Jun a human oncoprotein?

An oncoprotein is a protein encoded by a gene that, if introduced into a cell, can contribute to the transformation process (http://www.encyclopedia.com). Based on this definition and on the existing literature, I hereby question whether c-Jun can be defined as a human oncoprotein.

c-Jun was discovered more than twenty years ago as the cellular counterpart of the oncogenic avian sarcoma virus 17 (Cavalieri et al., 1985; Maki et al., 1987). Whereas established oncoproteins such as c-Myc and H-Ras exhibit mutations, amplification, and cancer-specific chromosomal translocation in human cancer, evidence for such modifications of c-Jun expression are not clearly established (Vogt, 2001). However, recent reports describe overexpression of c-Jun in breast cancer, sarcomas, myeloma, head and neck squamous cell carcinomas, gliomas, and colon cancer (Langer et al., 2006; Mariani et al., 2007; Mathas et al., 2002; Podar et al., 2007; Rangatia et al., 2003; Vleugel et al., 2006). Importantly, in aggressive undifferentiated sarcomas, the chromosomal region 1p32 containing the c-Jun locus was recently found to be amplified, and c-Jun is overexpressed both at RNA and protein levels. In addition, c-Jun overexpression blocks adipogenesis by interfering with C/EBPβ function and delays inducible adipocyte differentiation of 3T3-L1 cells (Mariani et al., 2007). However, in human multiple myeloma, c-Jun overexpression was described to induce apoptosis and reduce proliferation (Podar et al., 2007). Therefore, the causative role for c-Jun in cancer remains enigmatic.

The position of c-Jun at the end of a signaling pathway including significant oncogenes in human cancer such as Ras suggests an oncogenic role for c-Jun in human cancer. Moreover, c-Jun forms dimer in order to activate the transcription of its target genes and these dimerization partners such as c-Fos were described to exert oncogenic functions (Saez et al., 1995). c-Jun has also been described to promote cancer cell proliferation via its transcriptional activity (Chen et al., 2006; Vleugel et al., 2006). Moreover, c-Jun target genes such as *cyclin A* and *egfr* promote cancer cell growth and proliferation (Johnson et al., 2000; Katabami et al., 2005; Mialon et al., 2005). Interestingly, poorly invasive MCF-7 cells overexpressing c-Jun are able to form liver metastasis when injected to nude mice (Zhang et al., 2007). c-Jun was described to participate in the transformation process of rat, and chicken fibroblast, but there is no evidence that c-Jun can transform human cells (Bos et al., 1990; Schutte et al., 1989a). Based on these observations and the results presented in this study, c-Jun is clearly involved in cancer proliferation and growth but might not have causative role in human cancer.

However, the various roles played by c-Jun are cell types specific and depend on the dimerization partners for c-Jun. Therefore, c-Jun might exert its oncogenic role through its interaction partners. Co-activators enhance the oncogenic function of transcription factors (Huang et al., 1999). It is therefore tempting to speculate that in tumors where c-Jun and its co-activators are both overexpressed the oncogenic functions for c-Jun are enhanced. Notably, in head and neck squamous cell carcinomas and gliomas, both TopoI and c-Jun are overexpressed (http://www.oncomine.com). In addition, co-overexpression of DDX21 and c-Jun was noticed in colon cancer (http://www.oncomine.com). As shown in this study, c-Jun together with TopoI promotes cancer proliferation through its transcriptional function, whereas together with DDX21 c-Jun seems to promote cancer proliferation by supporting rRNA processing. It is therefore plausible to imagine that the cellular context, the interaction partners for c-Jun, the relative ratio of AP-1 dimers and the presence and the expression levels of oncogenic factors or tumor suppressors such as c-Myc, p53, p16 and Ras define the oncogenic activity for c-Jun. In addition, the oncogenic activity for c-Jun could involve both established transcriptional mechanisms as well as non-transcriptional effects of c-Jun (I), (II) (Leppa and Bohmann, 1999; Leppa et al., 2001). In conclusion, c-Jun could be qualified as a transcription factor with a positive role for cancer cell growth but not as an unambiguous human oncoprotein.

2 Nucleolar functions of c-Jun

The nucleolus is a non-membrane area of the nucleus where rRNA is synthesized and ribosome biogenesis occurs. However, several nucleolar proteins were demonstrated not to reside permanently in the nucleolus but shuttle constantly between the nucleoplasm and the nucleolus. Therefore, it was proposed that nucleolar proteins are retained in the nucleolus because of their function (Phair and Misteli, 2000). DDX21 is a nucleolar protein involved in ribosome biogenesis. It has an RNA binding domain on the C-terminal region of the protein and, upon RNA binding, DDX21 exerts its RNA helicase and RNA foldase activities (Valdez et al., 1997b). On the other hand c-Jun is a nuclear transcription factor (I, Fig.1C) (Sassone-Corsi et al., 1988). However, it has been noticed to localize on the peri-nucleolar area (Fang and Kerppola, 2004), where it colocalizes with TopoI and DDX21 (I, Fig.1C), (II, Fig.S1). Moreover, in our work, c-Jun was described to support (r)RNA binding of DDX21 (II).

It is puzzling to interpret how c-Jun and DDX21 molecules primarily located in separate nuclear domains could interact, and how DDX21 function could be modified upon interaction with c-Jun. The dynamic of proteins shuttling in and out of the nucleolus can be measured using FRAP analysis, and based on such analysis, DDX21 was shown to rapidly shuttle between nucleus and nucleolus (II, Fig.3C). Taken together with the

perinucleolar localization of c-Jun (II, Fig.S1), rapid exchange shuttling of DDX21 is likely to increase the probability of the interaction between c-Jun and DDX21.

In addition to its rRNA processing functions, DDX21 was described to be a co-factor for c-Jun in transcription (Westermarck et al., 2002). It is possible that c-Jun and DDX21 together drive transcription of target genes for c-Jun on the perinucleolar area. This is supported by the stimulation of c-Jun-mediated transcription by DDX21 (Westermarck et al., 2002) and the evidence that Pol II-mediated transcription occurs in the perinucleolar area (Huang et al., 1998). On the other hand, the mechanism by which c-Jun supports DDX21 binding affinity for rRNA is not yet clearly determined. Interestingly, protein sequence alignment demonstrated that the c-Jun interaction domain and the RNA binding domain are the most conserved domains within the DDX21 C-terminal tail (II, Fig.S3). Moreover structural prediction of this region suggested that both the c-Jun interaction domain and the RNA binding domain stretch out of the globular N-terminal end of DDX21. Based on these observations, it is possible to imagine that, upon c-Jun interaction, the RNA binding domain orientation on DDX21 is modified, and the rRNA binding capacity is promoted. In line with other reports demonstrating protein conformation changes upon other protein interaction, c-Jun could work as a switch to enhance DDX21 affinity for rRNA (Narayan et al., 2007; Yang et al., 2005a). The interaction between c-Jun and DDX21 might be brief but necessary as a "hit and run" mechanism. It is interesting that both the transcriptional activity of c-Jun, and the RNA binding affinity of DDX21 increases upon their interaction (II) (Westermarck et al., 2002). Whether these mutual functions of c-Jun and DDX21 are exclusive of each other remains to be studied.

Similarly to DDX21, TopoI and c-Jun colocalize on the perinucleolar area (I) and topotecan treatment inhibits c-Jun-mediated transcriptional activity. Therefore, it is possible that c-Jun together with TopoI drives the expression of EGFR in the perinucleolar area. Interestingly, whereas the interaction between c-Jun and TopoI is dependent on the phosphorylation of c-Jun (I), the interaction between DDX21 and c-Jun is not (Westermarck et al., 2002).

Figure 5 summarizes the various proposed nucleolar functions for c-Jun. All the proposed mechanisms remain to be further investigated. Nonetheless, the c-Jun mediated regulation of rRNA processing is, to date, the first report of regulation of rRNA processing by a transcription factor through direct interaction with an rRNA processing protein.

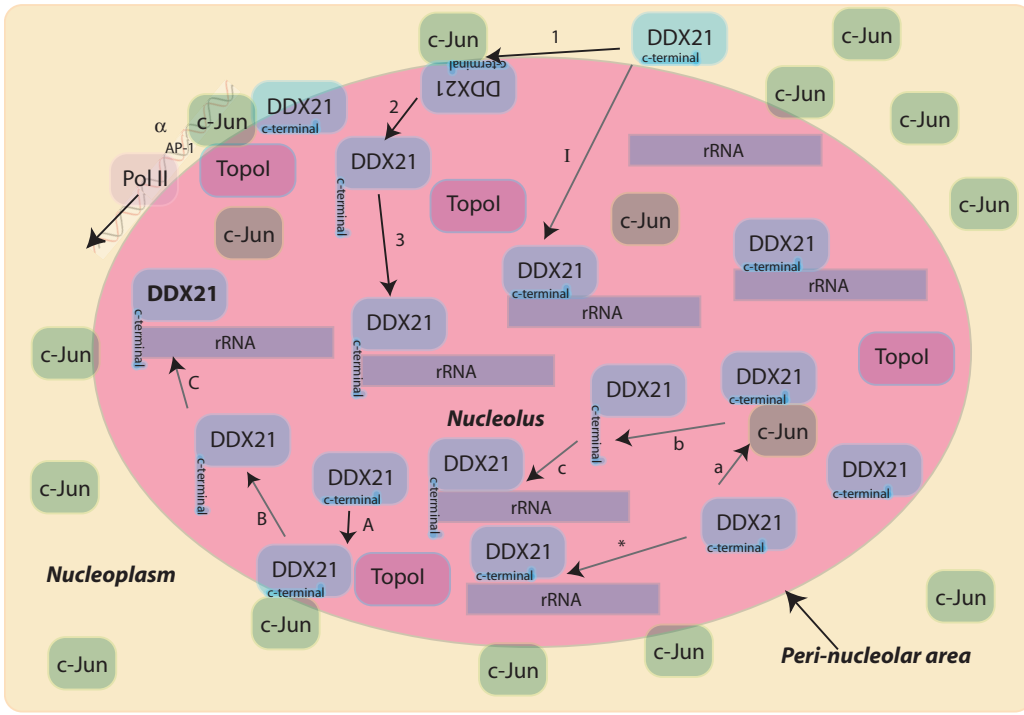

Figure 5. Nucleolar functions of c-Jun. c-Jun interacts with its nucleolar transcriptional co-activator DDX21 and TopoI in the perinucleolar area and enhances Pol II transcription (α). Regarding the RNA processing role for c-Jun, in the absence of interaction with c-Jun, DDX21 can bind to rRNA (I, *). After interaction with c-Jun in the perinucleolar area, the orientation of the c-terminal tail of DDX21 is modified, and the access to rRNA binding is increased. This can either happen during the shuttling of DDX21 from nucleoplasm to nucleolus (1-2-3) or by interaction between nucleolar DDX21 and c-Jun (A-B-C). However, c-Jun might also interact with DDX21 inside the nucleolus and thereby increase DDX21 rRNA binding affinity (a-b-c). This is based on the technical limitations of detection for c-Jun in the nucleolus which could not rule out the possibility of intranucleolar interaction between DDX21 and c-Jun.

3 Nucleolus in cancer

The nucleolus is the cellular domain where ribosomes biosynthesis is initiated by processing of pre-rRNA into ribosomal subunits. Thereafter, these subunits are assembled into ribosomes to participate in protein synthesis. Proliferating cancer cells demand a constant *de novo* protein synthesis, and in order to respond to this, more ribosomes need to be produced.

Early reports have demonstrated that the morphology of the nucleolus can be used as a marker for cellular transformation (Fischer et al., 2004; Pianese, 1896). The number of nucleoli and nucleolar enlargement are used in cancer diagnostics (Bostwick et al., 1997; Montironi et al., 1991). Moreover, a correlation between nucleolar activity, by measuring RNA polymerase I activity, and cancer proliferation has been established (Derenzini et

al., 2000). Increase of cell proliferation demands more efficient ribosome biosynthesis and nucleolar activity. It is therefore plausible that upon demand for a higher number of ribosomes, nucleolar proteins are recruited to the nucleolus. Here we demonstrate a positive correlation between recruitment of DDX21 to the nucleolus and prostate cancer progression (III, Fig.1A-C). Whereas primary tumors displayed homogeneous nuclear staining of DDX21, highly proliferative HR tumors were shown to display strong DDX21 staining in the nucleolus. In contrast to breast cancer where increased DDX21 mRNA expression correlates with poor prognosis (Cimino et al., 2008), in our study, no significant difference in the DDX21 mRNA expression between prostate cancer types was observed (III). Taking these results together, it is clear that the nucleolar recruitment of DDX21, but not its expression, correlates with prostate cancer progression. However, currently available evidence related to DDX21 in human cancers show that DDX21 hyperactivity can be exerted through either overexpression or nucleolar recruitment of the protein (II) (http://www.oncomine.com) (Cimino et al., 2008). Therefore, it is proposed here that DDX21 could be used as a diagnostic marker in the cancer types in which its overexpression or nucleolar recruitment has been established to correlate with cancer progression.

In addition to ribosome biogenesis, recent reports have demonstrated other functions for the nucleolus. The nucleolus was demonstrated to regulate cell cycle and mitosis (Boisvert et al., 2007). In addition, the nucleolus is a cellular stress sensor regulated by phosphorylation of the transcription factor TIFIA by the stress-activated protein kinase JNK2 (Mayer et al., 2005). Finally, the stability of the nucleolar structure was proposed to regulate p53 abundance, thereby protecting cells from apoptosis. Upon stress, nucleolar disruption occurs and, even in the absence of DNA damage, p53 is stabilized and subsequent apoptosis is triggered (Rubbi and Milner, 2003). Nucleolar disruption induces also interaction between the nucleolar phosphoprotein nucleophosmin and HDM2 thereby preventing HDM2-p53 interaction and increasing a subsequent stabilization of p53 (Kurki et al., 2004). Interestingly, the structural stability of the nucleolus is maintained by several proteins, such as nucleolin (Ugrinova et al., 2007). Therefore, the development of drugs targeting nucleolar stability might offer novel approaches for cancer treatment.

4 TopoI inhibition in cancer therapy

Chemotherapy and surgery, hormone therapy, radiation and biological response modifiers (interferons, interleukins, cytokines or vaccines) represent the current options for the treatment of various cancer types. Chemotherapy describes the use of small molecule drugs to treat cancer and diseases. It was initiated by the discovery of the suppressive effects of mustard gas on lymphoid and myeloid cells (Goodman et al., 1946).

Camptothecins, including its derivatives such as topotecan and irinotecan, are small molecule TopoI inhibitors currently used in the treatment of metastatic ovarian cancer, SCLC and advanced cervical cancer (Armstrong et al., 2005). However the molecular mechanism by which topotecan inhibits cancer cell growth remains obscure. TopoI causes a temporary single-strand break in DNA and the DNA religation occurs once the supercoiled DNA is relaxed (Wu et al., 1988). It has been proposed that topotecan inhibits TopoI activity by inducing an irreversible covalent complex between TopoI and DNA, thereby preventing the religation step (Horwitz and Horwitz, 1973). TopoI was described to be essential for replication and transcription elongation (Egyhazi and Durban, 1987; Mondal and Parvin, 2003; Wang, 2002). During transcription elongation, positive supercoiled DNA is formed ahead of the transcription machinery and, unless relaxed by TopoI, this torsion inhibits elongation (Mondal and Parvin, 2003). Upon inhibition of TopoI function by topotecan, it was suggested that the progression of the transcription machinery was blocked by the torsion formed a the 3'end of genes (Zhang et al., 1988). During replication, TopoI relaxes supercoiled DNA induced by the elongation of the replication machinery. It was proposed that inhibition of TopoI function by topotecan treatment induces a collision of the replication fork with the TopoI-topotecan-DNA complex (Hsiang et al., 1985). However these results were obtained by formation of an *in vitro* complex containing TopoI-topotecan-DNA; and based on x-ray crystal structure (Hsiang et al., 1985; Staker et al., 2002). Nevertheless no evidence of replication fork collision or inhibition transcription machinery elongation with a complex formed by TopoI-topotecan-DNA has been described in cell culture models. Therefore, the relative contributions of TopoI functions either in replication and or in gene regulation for cancer cell proliferation remain unclear.

The results of this study demonstrated that TopoI is a co-activator for phosphorylated c-Jun-mediated transcription of the *egfr* gene (I) (Figure 6). Importantly, c-Jun$^{-/-}$ cells or cells treated with JNK inhibitor SP600125, were found to be resistant to topotecan (I, Fig.4). Therefore, in cancers in which EGFR is overexpressed in association with constitutive JNK-mediated c-Jun phosphorylation, topotecan could be preferentially efficient in preventing cancer cell proliferation. It would be interesting to identify other target genes which their expression is positively supported by the interaction between TopoI and phosphorylated c-Jun. In line with the results presented in this study, the expression of these target genes should be impeded by topotecan. Moreover, topotecan causes side effects such as fatigue and white cell reduction. This exposes cancer patients to additional sicknesses, and it would therefore be of primary importance to use topotecan only for patients that have topotecan-sensitive tumors. It is tempting to speculate that analysis of EGFR expression and c-Jun phosphorylation in human cancers could be used to predict topotecan sensitivity.

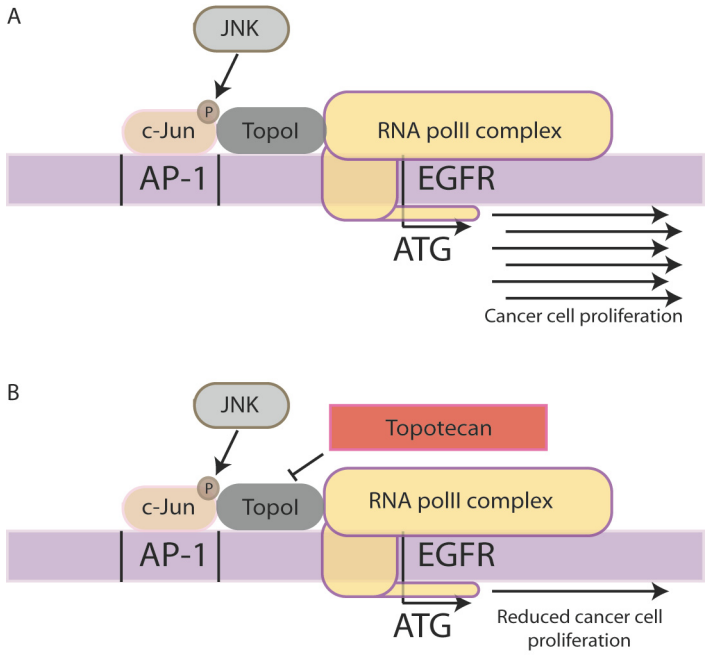

Figure 6. Proposed mechanism for the involvement of TopoI in EGFR transcription. (A) In cancer cells with a constitutively active JNK pathway, TopoI, through its co-activator function for c-Jun mediated transcription, increases EGFR expression, thereby increasing cancer cell proliferation. (B) Upon topotecan treatment, TopoI is inhibited, resulting in inhibition of c-Jun mediated transcription of *egfr* and reduction of cancer cell proliferation.

5 Targeting DDX21-c-Jun and TopoI-c-Jun interactions for cancer therapy?

Regulation of gene expression is tightly dependent on accurate protein-protein interactions (Bhardwaj and Lu, 2005). Therefore, identification of protein-protein interactions promoting cancer may provide novel possibilities for cancer treatment (Arkin, 2005; Bagnasco et al., 2007; Shangary and Wang, 2008; Yildirim et al., 2007).

However, several challenges have emerged when protein-protein interactions have been made for cancer treatment (Carter et al., 2001; McGovern et al., 2003; Wiekowski et al., 1997). Previous studies have demonstrated the power of antibodies in the inhibition of protein-protein interactions (Stockwin and Holmes, 2003). Antibodies present several advantages, such as high specificity for the target and stability in serum. Nonetheless, antibodies are unable to cross the cell membrane, they are expensive to produce, and unavailable for oral uptake. These drawbacks led investigators to focus on small-molecules and peptides as protein-protein interaction inhibitors (Berg, 2003). Several procedures can be used to develop protein-protein interaction inhibitors: *in silico* design of a chemical drug based on protein structure, and high-throughput screening of natural or

synthetic small compounds (Arkin, 2005). These molecules bind to one of the interactors and thereby prevent the interaction. The inhibition of the interaction occurs either by occupying the interaction domain or by modifying the structure of the bound protein (Fry, 2006). An encouraging example of a protein-protein interaction inhibitor used in cancer treatment is Nutlin. Nutlin is an inhibitor of the direct interaction between the tumor suppressor p53 and the oncoprotein murine double minute 2 (MDM2). Subsequent stabilization of p53 induces cell cycle arrest and apoptosis through stimulation of p21 expression and inhibition of Bcl-2 expression, respectively (Shangary and Wang, 2008; Vassilev et al., 2004). Recently, a new approach combining computational drug design and fragment based nuclear magnetic resonance technology led to the discovery of ABT-737, a small-molecule inhibitor of the anti-apoptotic Bcl-2 protein family with promising therapeutic outcome (Oltersdorf et al., 2005). Cell permeable peptides are also used to prevent *in vivo* protein-protein interactions. For example, XG-102 is a peptide inhibitor of JNK-mediated c-Jun phosphorylation, which protects from ischemic brain damage in stroke (Bonny et al., 2001; Borsello et al., 2003; Wiegler et al., 2008).

Once the inhibitor is proven to prevent the interaction between proteins *in vitro*, the most challenging task for *in vivo* application is to modify the inhibitor to have sufficient *in vivo* efficiency. In this work, the interaction domain between c-Jun and DDX21 was determined *in vitro* to be a nine amino-acid sequence at the C-terminus of DDX21 (II, Fig.4D). Efficient *in vitro* inhibition of the interaction between c-Jun and DDX21 was obtained with a peptide spanning the interaction domain. Moreover, microinjection experiments revealed relocalization of DDX21 to the nucleoplasm (II, Fig.4E). After several trials of coupling the peptide with a cell-permeable sequence (TP10), and fluorescein to verify the efficiency of cell permeability, no relocalization of DDX21 to the nucleoplasm was noticed (data not shown). This failure might be due to compound instability and intra-cellular peptide degradation. Moreover, even if fluorescein was detected in the nucleolus, it is possible that the actual blocking sequence of the peptide might not have been in contact with c-Jun in the perinucleolar area. In line with independent observations, peptide stability within cells is the bottleneck in the process of designing peptides inhibiting the *in vivo* interaction between two proteins (Walensky et al., 2004).

Recently-published evidence reveals the importance of nucleolar stability for cellular integrity (Ugrinova et al., 2007). Following these lines, the development of drugs impeding nucleolar stability could offer novel strategies for cancer treatment. In this regard, pharmacological targeting of DDX21-c-Jun interaction may present several advantages. The consequences of inhibition of DDX21-c-Jun interaction may be to induce nucleolar instability and to decrease rRNA processing due to impediment of DDX21 functions. In addition, reduction of c-Jun mediated transcription may decrease cancer proliferation. Altogether, these effects may have additive effects leading to cancer cell death.

One potential advantage of inhibition of protein-protein interactions is the selectivity of the inhibition. The inhibition of a given interaction would specifically inhibit the consequence of that interaction without interfering with the other molecular functions of the interacting proteins. Based on the results of this work, development of a specific inhibitor of TopoI-c-Jun interaction could inhibit downstream targets of TopoI-c-Jun interaction without inhibiting other cellular functions of TopoI and c-Jun. This would provide better selectivity for cancer treatment and reduce the side effects when compared to topotecan which inhibits the overall functions of TopoI.

In spite of the obvious challenges, the field of inhibition of protein-protein interactions for the treatment of cancer has provided promising examples. Several small-molecules have been developed to inhibit protein interactions involved in anti-apoptotic, growth factors, transcription activators, or cell migration signaling pathways (Arkin, 2005; Huang et al., 2006; Shangary and Wang, 2008).

SUMMARY AND CONCLUSIONS

In this study, we describe the functional role for TopoI and DDX21 in c-Jun-mediated regulation of cancer cell behavior. These two nucleolar proteins were initially identified from a c-Jun interacting protein complex isolated from HEK293 cells.

The results of this study show that c-Jun, together with DNA topoisomerase I (TopoI), positively regulates the expression of the epidermal growth factor receptor (EGFR). Furthermore, TopoI inhibition by topotecan treatment reduces EGFR expression. More importantly, c-Jun$^{-/-}$ cells and cells treated with JNK phosphorylation inhibitor SP60125 are resistant to topotecan treatment.

Analysis of c-Jun-DDX21 interaction results in the discovery of a novel non-transcriptional function for c-Jun in the regulation of DDX21 nucleolar localization. c-Jun stimulates rRNA processing by supporting DDX21 rRNA binding. In addition, we establish a positive role for DDX21 and c-Jun in Hela cell proliferation and cell cycle progression. Finally, we demonstrate a correlation between the nucleolar localization of DDX21 and prostate cancer progression. In addition, DDX21 depletion results in decreased expression of cyclin dependent kinase (Cdk) 6.

Taken together, these results describe novel interaction partner for the transcription factor c-Jun and describe novel roles for c-Jun, TopoI and DDX21 in the positive regulation of cancer cell behavior. Moreover, work presented in this thesis demonstrate the importance of protein-protein interactions in the modulation of cellular functions of proteins. Therefore, identification of protein-protein interactions with proteins involved in cancer progression may reveal novel targeting possibilities for cancer treatment.

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