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Specific c-Jun target genes in malignant melanoma

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ABSTRACT

A fundamental event in the development and progression of malignant melanoma is the de-regulation of cancer-relevant transcription factors. We recently showed that c-Jun is a main regulator of melanoma progression and, thus, is the most important member of the AP-1 transcription factor family in this disease.

Surprisingly, no cancer-related specific c-Jun target genes in melanoma were described in the literature, so far. Therefore, we focused on pre-existing ChIP-Seq data (Encyclopedia of DNA Elements) of 3 different non-melanoma cell lines to screen direct c-Jun target genes. Here, a specific c-Jun antibody to immunoprecipitate the associated promoter DNA was used. Consequently, we identified 44 direct c-Jun targets and a detailed analysis of 6 selected genes confirmed their deregulation in malignant melanoma. The identified genes were differentially regulated comparing 4 melanoma cell lines and normal human melanocytes and we confirmed their c-Jun dependency. Direct interaction between c-Jun and the promoter/enhancer regions of the identified genes was confirmed by us via ChIP experiments. Interestingly, we revealed that the direct regulation of target gene expression via c-Jun can be independent of the existence of the classical AP-1 (5′-TGA(C/G)TCA-3) consensus sequence allowing for the subsequent down- or up-regulation of the expression of these cancer-relevant genes.

In summary, the results of this study indicate that c-Jun plays a crucial role in the development and progression of malignant melanoma via direct regulation of cancer-relevant target genes and that inhibition of direct c-Jun targets through inhibition of c-Jun is a potential novel therapeutic option for treatment of malignant melanoma.

Introduction

The family of AP-1 (activating protein-1) transcription factors includes the JUN, JDP, FOS/FRA and MAF subfamilies.[1] They share a conserved basic DNA-binding domain and a leucine zipper domain (bZIP). The DNA-binding domain determines the spectrum of genes that are controlled by the protein that binds to it, and the transactivation domain (delta domain) is responsible for the regulation of transcriptional activation.[2] AP-1 proteins are known to bind to the classical palindromic recognition sequence 5′TGA(C/G)TCA-3′ to regulate target gene expression. In the promoter/enhancer regions, the sequences of the AP-1 regulatory elements often deviate from the classical AP-1 recognition sequence. This variation may contribute to the differential functions of different AP-1 family dimers[3]; however, a detailed analysis has not yet been performed. AP-1 transcription factor dimers are known to play a crucial role in the development and progression of different cancer types, particularly malignant melanoma.[1,4-6] Though, it is still unclear which direct target genes of AP-1 transcription factors cause the functional effects that support the development of melanoma.

Malignant melanoma is the most aggressive skin cancer, and its incidence is growing faster than for any other cancer entity. In the past few decades, many mechanisms of regulation of the development and progression of melanoma and the high migratory and invasive potential of melanoma cells have been identified, but the detailed molecular causes of this disease remain elusive.

Due to the critical influence of the activity of AP-1 transcription factors in malignant melanoma, DNA-protein interactions have been investigated using a variety of biochemical and genomic approaches. In addition to the traditionally used *in vitro* techniques, such as electrophoretic mobility shift assays (EMSA) and DNase I footprinting assays, chromatin immunoprecipitation (ChIP) has become a very popular technique for identifying DNA-protein interactions *in vivo*. Apart from identifying interactions between specific proteins and DNA in living cells, the localization of proteins at a specific genomic region can also be determined. ChIP assays can further be combined with sequencing (ChIP-Seq) to allow for a genome-wide analysis of DNA-protein interactions and, thus, the identification of specific DNA-binding sites and direct target genes of individual transcription factors,[7,8] such as c-Jun. Recent studies have indicated that the AP-1 family member c-Jun is a main regulator of melanoma progression [6,9,10] and that it acts by up-regulating pro-oncogenic genes and down-regulating anti-oncogenic target genes, whose activation promotes the malignant phenotype. We have previously shown that the microRNA miR-125b directly regulates the transcription factor c-Jun and, thus,

effects the proliferative and migratory potential of melanoma cells.[11] Moreover, we identified an alternative regulatory pathway of c-Jun in melanoma cells that leads to an upregulation of c-Jun activity via the loss of the cell-adhesion molecule E-cadherin.[12,13] Although the de-regulation of the transcription factor c-Jun is known to be one of the most important events in the development and progression of malignant melanoma, the specific c-Jun target genes that contribute to the functional effects of c-Jun up-regulation in melanoma cells and their molecular relevance have not been determined.

Consequently, a detailed analysis of directly regulated target genes of the transcription factor c-Jun and their expression level is necessary to determine the role of c-Jun in malignant melanoma. We speculate that targeting transcription factors may be a novel and effective therapeutic approach.

Results

In this study, we used pre-existing ChIP-Seq data of the human cervical adenocarcinoma cell line *HeLa S3*, the human liver carcinoma cell line *HepG2* and the human umbilical vein endothelial cell line HUVEC archived in the *Encyclopedia of DNA Elements (ENCODE; http://genome.ucsc.edu/ENCODE/)* for *in silico* analysis to identify genome-wide, general c-Jun target genes. In these data, we screened for enriched DNA sequences (peaks) after ChIP-Seq with a c-Jun antibody (sc-1694). By examining the data in ENCODE, we were able to identify 44 c-Jun binding sites in the promoter/enhancer regions of a variety of target genes in all 3 cell lines (Table 1). First, we scanned for known or predicted associations between the 44 identified genes and the transcription factor c-Jun by *STRING (Search Tool for the Retrieval of Interacting Genes/Proteins).*[14] STRING analysis showed known or predicted associations between the transcription factor c-Jun and many of the newly identified c-Jun target genes. The level of confidence of the associations is represented by the thickness of the lines (Fig. 1 (ai)). Some identified c-Jun targets are not described yet (e.g. SGCE, DKK3 or HPSE), hence STRING analysis showed no relation between those identified c-Jun targets. Then, we analyzed the 44 identified general c-Jun target genes using the *Functional Annotation Tool* of the **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (**DAVID** v6.7).[15,16] Functional annotation clustering exemplarily using the category GOTERM_BP_FAT resulted in 7 different significantly enriched annotation clusters (ACs) (Table S1). The analysis of other categories resulted in similar functional ACs. The ACs contained genes that could be summarized according to their molecular function: adhesion

(AC 1; enrichment score: 2.76), cell motion (AC 2 and 4; enrichment score: 2.42 and 1.70), phosphorus metabolic process (AC 3; enrichment score: 1.84), cytoskeleton organization (AC 5; enrichment score: 1.62), response to oxidative stress (AC 6; enrichment score: 1.46) and positive regulation of cell proliferation (AC 7; enrichment score: 1.14) (Fig. 1 (aii)).

For the subsequent analysis, we focused on 6 of the 44 target genes that have previously been described as cancer-relevant genes. The enrichment of DNA fragments displayed as peaks in the promoter/enhancer regions of specific target genes (ENCODE) were localized to the promoter/enhancer regions of FosB, NFATC2, WEE1, PVR, MAP1LC3B and LGALS3[17-22] (Fig. 2, Table 2). Furthermore, we confirmed the presence of DNA-motifs identical or similar to the AP-1 recognition site within the enriched ChIP-Seq sequences of the 6 detected potential target genes of c-Jun via the *Multiple Em for Motif Elicitation (MEME) Suite 4.10.2* database[23] (Fig. 1(b)). We revealed that 4 of the 6 identified potential target genes of the transcription factor c-Jun contain a classical AP-1 recognition sequence (5′-TGA(C/G)TCA-3′) within the enriched DNA fragments. In the promoter/enhancer regions of FosB, WEE1, PVR and LGALS3, we observed the classical AP-1 binding sequence with 100% accordance (5′-TGA (C/G) TCA-3′). In the promoter/enhancer regions of NFATC2 and MAP1LC3B, the potential AP-1 binding sequence contained one base pair change compared to the the classical sequence (NFATC2: 5′-TGA C ACA-3′; MAP1LC3B: 5′-TGA T TCA3-′).

We next validated the results of the *in silico* analysis of the ENCODE data by performing ChIP in the melanoma cell line Mel Juso, to confirm the direct interaction between c-Jun and the identified promoter/enhancer regions in the new target genes. We performed ChIP experiments with a c-Jun antibody (sc-1694), a RNA polymerase II (Pol II) antibody as a positive control and an IgG antibody as a negative control and analyzed the precipitated DNA fragments by PCR with different primer pairs to amplify specific promoter regions: *GAPDH* as a positive control for ChIP experiments with the Pol II antibody, *negative control* primers, *ChIP_FosB, ChIP_NFATC2, ChIP_WEE1, ChIP_PVR, ChIP_LGALS3 and ChIP_MAP1LC3B* (Table S2). Total DNA of the melanoma cell line Mel Juso served as the *Input* DNA for the ChIP experiments. Gel electrophoresis of the amplified precipitated DNA fragments after PCR showed a significant enrichment of the promoter/enhancer sequences of the predicted direct c-Jun target genes FosB, NFATC2, WEE1, PVR, LGALS3 and MAP1LC3B (Fig. 3, Fig. S1).

Further, we analyzed whether these newly defined c-Jun target genes (FosB, NFATC2, WEE1, PVR, MAP1LC3B and

Table 1. General, potential c-Jun target genes identified via ENCODE. The list includes the 44 identified general c-Jun target genes, their gene description and their localization on the human genome (hg19).

Gene Product	Detected Regulator	Described Activity	Reference in terms of Gene Product Activity
FosB	c-Jun (up-regulates)	proliferation, differentiation, transformation	6
NFATC2	c-Jun (up-regulates)	apoptosis	18
WEE1	c-Jun (up-regulates)	proliferation, survival	33
PVR	c-Jun (up-regulates)	invasiveness	37
MAP1LC3B	c-Jun (down-regulates)	survival	40
LGALS3	c-Jun (down-regulates)	progression, metastasis	42

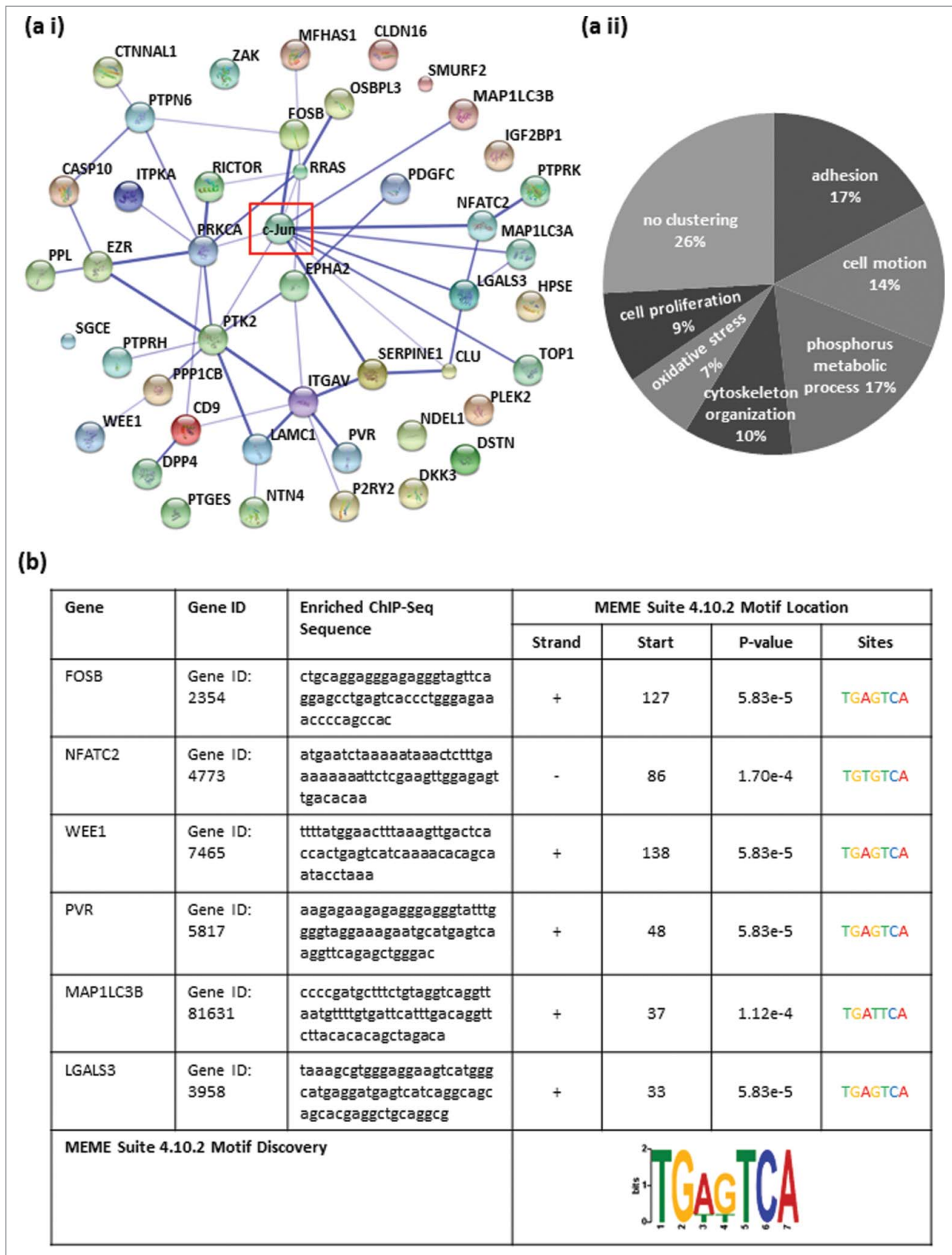| Gene	Gene ID	Enriched ChIP-Seq Sequence	MEME Suite 4.10.2 Motif Location			
			Strand	Start	P-value	Sites
FOSB	Gene ID: 2354	ctgcaggagggagagggtagttca ggagcctgagtcaccctgggagaa accccagccac	+	127	5.83e-5	TGAGTCA
NFATC2	Gene ID: 4773	atgaatctaaaaataaactctttga aaaaaaattctcgaagttggagagt tgacacaa	-	86	1.70e-4	TGTGTCA
WEE1	Gene ID: 7465	ttttatggaactttaaagttgactca ccactgagtcatcaaaacacagca atacctaaa	+	138	5.83e-5	TGAGTCA
PVR	Gene ID: 5817	aagagaagagagggagggtatttg gggtaggaaagaatgcatgagtca aggttcagagctgggac	+	48	5.83e-5	TGAGTCA
MAP1LC3B	Gene ID: 81631	ccccgatgctttctgtaggtcaggtt aatgttttgtgattcatttgacaggtt cttacacacagctagaca	+	37	1.12e-4	TGATTCA
LGALS3	Gene ID: 3958	taaagcgtgggaggaagtcatggg catgaggatgagtcatcaggcagc agcacgaggctgcaggcg	+	33	5.83e-5	TGAGTCA
MEME Suite 4.10.2 Motif Discovery						

Figure 1. All identified potential c-Jun target genes share cancer-relevant molecular functions and contain classical or non-classical AP-1 recognition sequences in their promoter/enhancer regions. (a i) Predicted relation between the 44 identified target genes and the transcription factor c-Jun via *STRING*. (a ii) Functional annotation clustering using the category GOTERM_BP_FAT resulted in 7 different significantly enriched annotation clusters (ACs). The ACs contained genes that could be summarized according to their molecular function: adhesion (enrichment score (ES): 2.76), cell motion (ES: 2.42), phosphorus metabolic process (ES: 1.84), cytoskeleton organization (ES: 1.62), response to oxidative stress (ES: 1.46) and positive regulation of cell proliferation (ES: 1.14). (b) The promoter/enhancer regions of FosB, WEE1, PVR and LGALS3 contained the classical AP-1 binding sequence with 100% agreement (FosB: 5′-TGA G TCA-3′, WEE1: 5′-TGA(C/G)TCA-3′, PVR: 5′-TGAGTCA-3′, and LGALS3: 5′-TGA G TCA-3′). In the promoter/enhancer regions of NFATC2 and MAP1LC3B, the AP-1 binding motifs each contained a one base pair exchange (NFATC2: 5′-TGA C ACA-3′ and MAP1LC3B: 5′-TGA T TCA-3′). The potential c-Jun binding sites were validated in all 6 detected potential target genes as DNA-motifs, identical or similar to the AP-1 recognition sequence, using the Motiv Discovery program *MEME*.
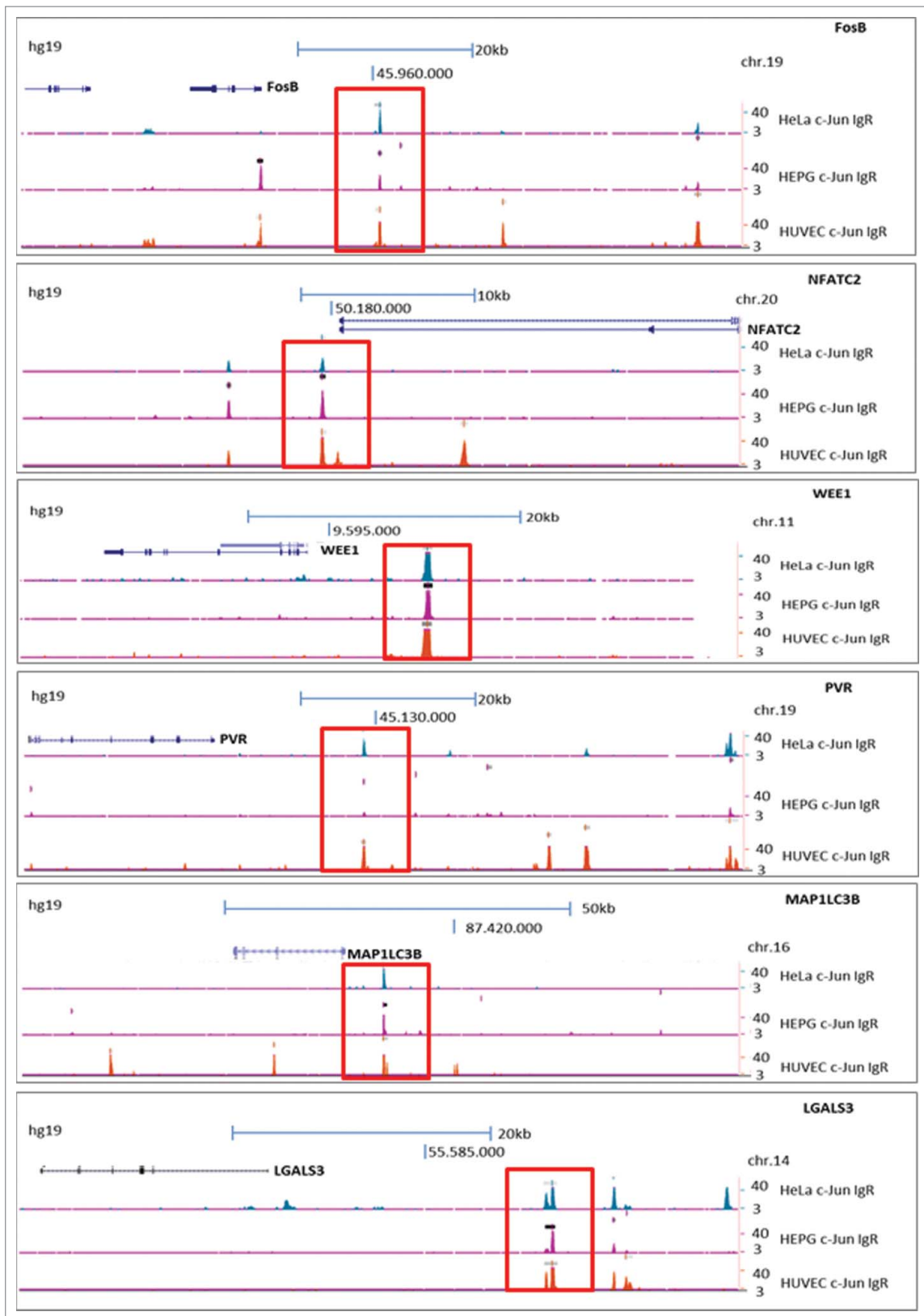
Figure 2. Analysis of archived ChIP-Seq data (ENCODE; hg19) showing enrichment of DNA fragments after ChIP with a c-Jun antibody (sc-1694) in the cell lines HUVEC, HepG2 and HEK. The enrichments of DNA fragments are displayed as peaks in the promoter/enhancer regions of specific target genes (labeled in red). The peaks were localized to the promoter/enhancer regions of FosB, NFATC2, WEE1, PVR, LGALS3 and MAP1LC3B.

LGALS3) also are deregulated in malignant melanoma. We performed mRNA expression analysis by qRT-PCR in 4 different melanoma cell lines (Mel Juso, A375, Mel Ei, and Mel Ju) and in primary melanocytes (NHEM) and detected de-regulation of the potential c-Jun target genes in all melanoma cell lines. We observed upregulation of FosB, NFATC2, WEE1 and PVR and

Table 2. Direct c-Jun target genes and described activities. The identified direct c-Jun target genes in malignant melanoma and their recently described cancer-relevant activities.

Gene	Gene description	location
RRAS	related RAS viral (r-ras) oncogene homolog	19q13.33
PLEK2	pleckstrin 2	14q23.3
MAP1LC3B	microtubule-associated protein 1 light chain 3 β	16q24.2
WEE1	WEE1 homolog (S. pombe)	11p15.3-p15.1
EPHA2	EPH receptor A2	1p36
ITGAV	integrin, α V	2q31-q32
DPP4	dipeptidyl-peptidase 4	2q24.3
PVR	poliovirus receptor	19q13.2
LGALS3	lectin, galactoside-binding, soluble, 3	14q22.3
CLU	Clusterin	8p21-p12
ITPKA	inositol-trisphosphate 3-kinase A	15q15.1
NTN4	NTN4 – netrin 4	12q22
HPSE	heparanase	4q21.3
IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	17q21.32
TOP1	topoisomerase (DNA) I	20q12-q13.1
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	7q22.1
PRKCA	protein kinase C, α	17q22-q23.2
PDGFC	platelet derived growth factor C	4q32
CASP10	caspase 10, apoptosis-related cysteine peptidase	2q33-q34
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	17q22-q23
PPP1CB	protein phosphatase 1, catalytic subunit, β isozyme	2p23
NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	20q13.2
PTPN6	protein tyrosine phosphatase, non-receptor type 6	12p13
CTNNAL1	catenin (cadherin-associated protein), α-like 1	9q31.2
PTK2	protein tyrosine kinase 2	8q24.3
PTPRH	protein tyrosine phosphatase, receptor type, H	19q13.4
LAMC1	laminin, gamma 1 (formerly LAMB2)	1q31
CLDN16	claudin 16	3q28
RICTOR	RPTOR independent companion of MTOR, complex 2	5p13.1
PPL	Periplakin	16p13.3
DSTN	Destrin	20p12.1
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	19q13.32
NDEL1	nudE neurodevelopment protein 1-like 1	17p13.1
P2RY2	purinergic receptor P2Y, G-protein coupled, 2	11q13.5-q14.1
PTGES	prostaglandin E synthase	9q34.3
MFHAS1	malignant fibrous histiocytoma amplified sequence 1	8p23.1
ZAK	sterile α motif and leucine zipper containing kinase AZK	2q24.2
EZR	Ezrin	6q25.3
CD9	CD9 molecule	12p13.3
DKK3	dickkopf WNT signaling pathway inhibitor 3	11p15.2
SGCE	sarcoglycan, epsilon	7q21.3
PTPRK	protein tyrosine phosphatase, receptor type, K	6q22.2-q22.3
MAP1LC3A	microtubule-associated protein 1 light chain 3 α	20q11.22
OSBPL3	oxysterol binding protein-like 3	7p15

down-regulation of MAP1LC3B and LGALS3 in all melanoma cell lines compared to NHEMs (Fig. 4).

To further ensure that the predicted target genes are regulated by the transcription factor c-Jun in malignant melanoma, we transfected a melanocyte-resembling cell clone lacking c-Jun expression (HMB2-5[24]) with a c-Jun expression construct (*HA-JunMut1*[11]) and confirmed an increase in c-Jun accumulation by Western blotting with a transfection efficiency of about 80% (Fig. 5 (a)). qRT-PCR of mRNA from the transfected cells revealed that the increase in c-Jun expression was accompanied by an increase in expression of FosB, NFATC2, WEE1 and PVR compared to the pcDNA3 (negative control)-transfected Hmb2-5 cells (Fig. 5 (a)). *Vice versa*, we analyzed the c-Jun dependent regulation of MAP1LC3B and LGALS3 in Mel Juso after knockdown of c-Jun via siRNA. The successful knockdown with a transfection efficiency of about 85% of c-Jun was confirmed by Western blot analysis (Fig. 5 (b)). We observed up-regulation of MAP1LC3B and LGALS3 mRNA expression

in the si c-Jun cells compared to the control transfected cells (si c) (Fig. 5 (b)).

Discussion

Malignant melanoma is an aggressive cancer derived from melanocytes and is resistant to most current therapeutic approaches. In recent studies with melanoma cells, we demonstrated that the transcription factor c-Jun plays a crucial role in the development and progression of this cancer type.[5,11,12] However, the detailed molecular mechanism of c-Jun's influence on melanoma progression and development remains elusive, as only a subset of target genes is known. Previous studies have shown that cancer-relevant genes, such as cyclin D1, p53, and INK4A[25-27] are regulated by c-Jun. In the present study, we identified 40 4 general c-Jun target genes in non-melanoma cell lines by *in silico* analysis of pre-existing ChIP-Seq data (ENCODE). The identified c-Jun target genes within this study
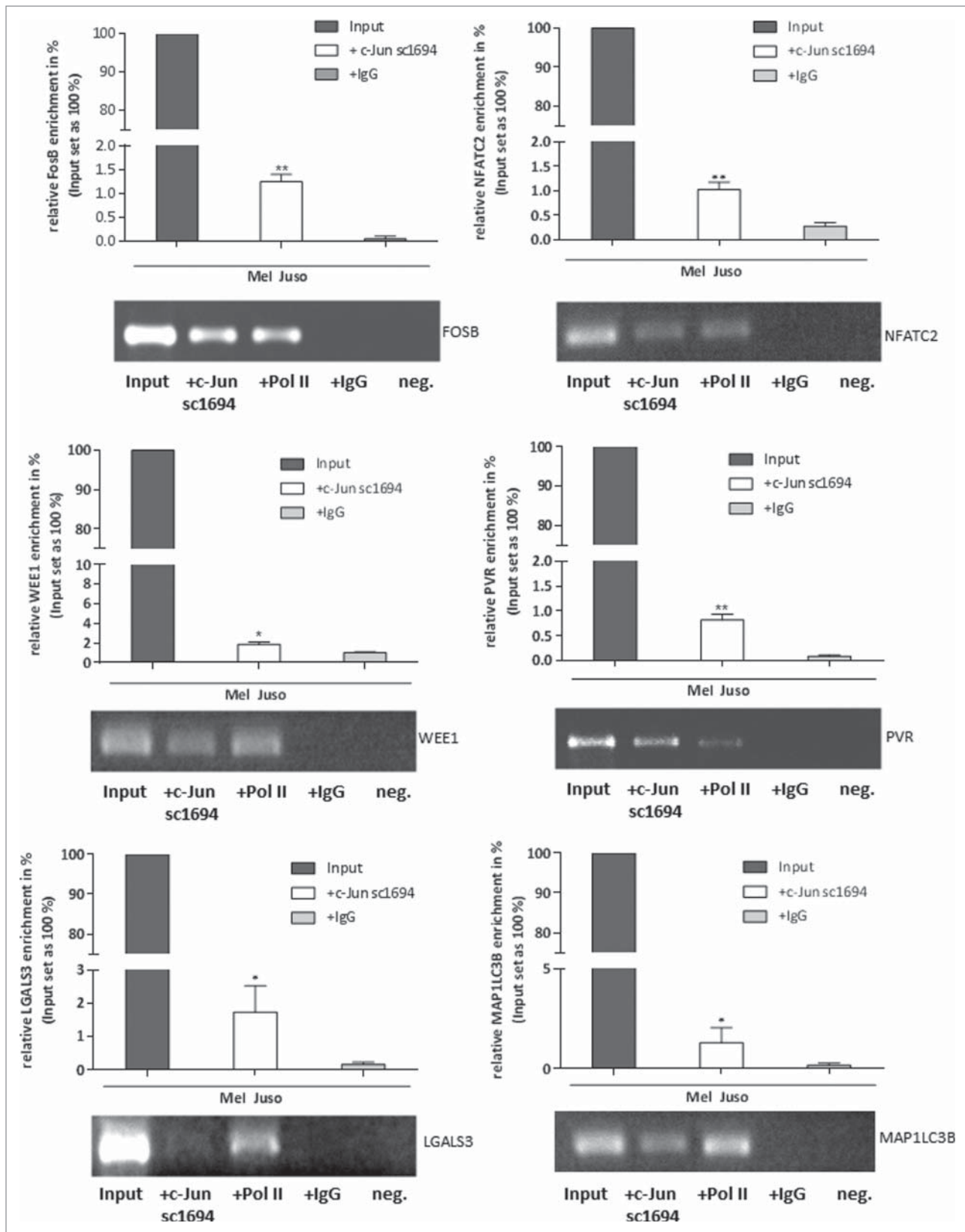
Figure 3. Direct binding of c-Jun to the promoter/enhancer regions of FosB, NFATC2, WEE1, PVR, LGALS3 and MAP1LC3B. ChIP assays demonstrate direct binding of c-Jun to the promoter/enhancer regions of the detected c-Jun target genes. DNA samples from the ChIP reactions (Pol II, IgG, and c-Jun) and input DNA were used for PCR with different primer pairs to amplify specific promoter regions (*ChIP_FosB, ChIP_NFATC2, ChIP_WEE1, ChIP_PVR, ChIP_LGALS3 and ChIP_MAP1LC3B*). All PCR fragments were detected in the input DNA sample. The bars show the means±s.d. of three independent experiments; measurements were performed in triplicate (**P < 0.01; *P < 0.5).

did not include the genes cyclin D1, p53, and INK4A described as c-Jun regulated genes in previous studies, indicating that these genes are potentially indirectly regulated by the transcription factor c-Jun, but not by direct binding in the promotor-/enhancer region of these genes. However, we observed that these via ENCODE identified c-Jun target genes also share
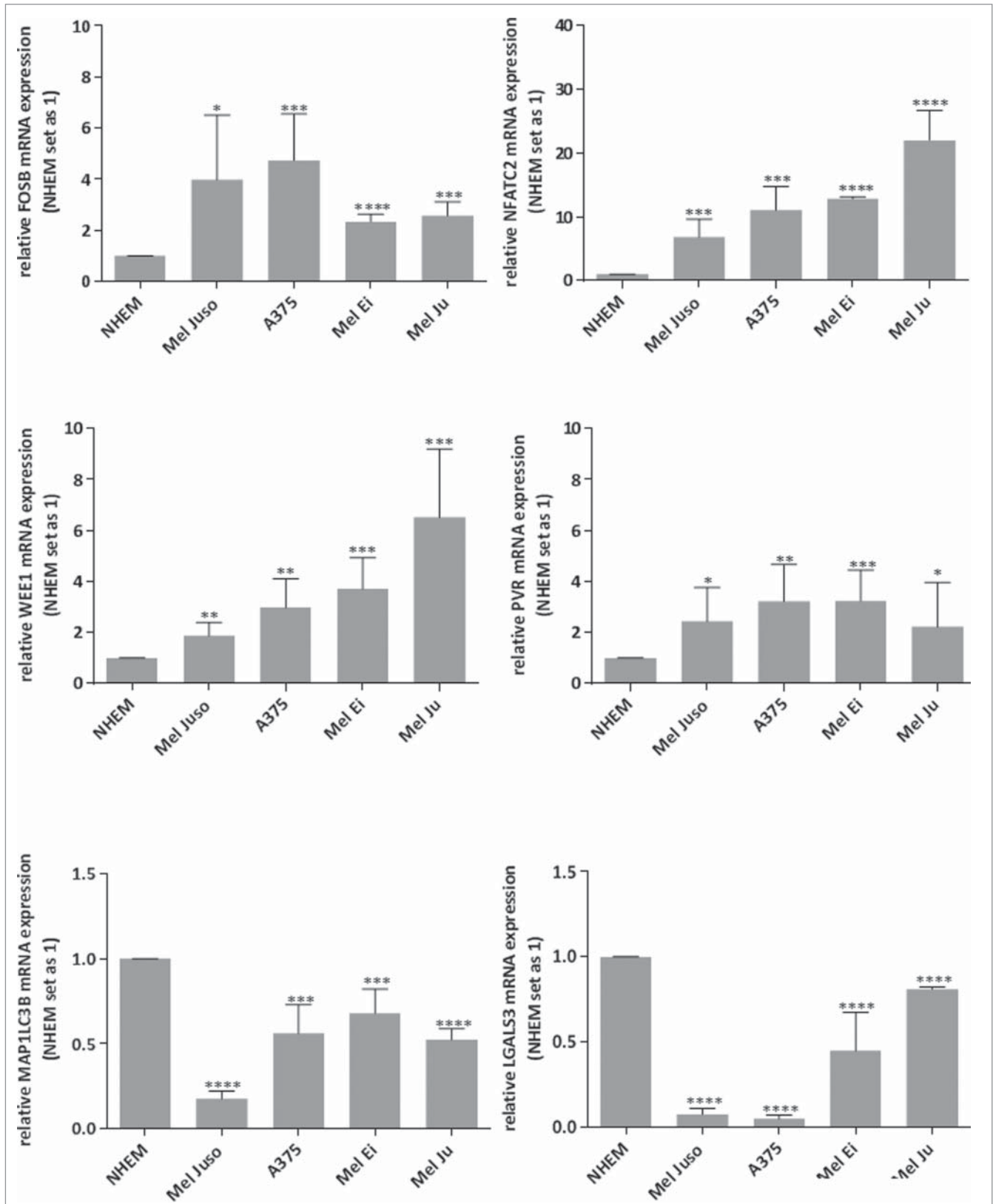

Figure 4. Relative FosB, NFATC2, WEE1, PVR, LGALS3 and MAP1LC3B mRNA expressions in melanoma cell lines. qRT–PCR of FosB, NFATC2, WEE1, PVR, LGALS3 and MAP1LC3B in 4 different melanoma cell lines shows up-regulation of FosB, NFATC2, WEE1 and PVR and down-regulation of LGALS3 and MAP1LC3B compared to in NHEMs. The bars indicates the means ± s.d. of three independent experiments; measurements were performed in triplicate (****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.5$).
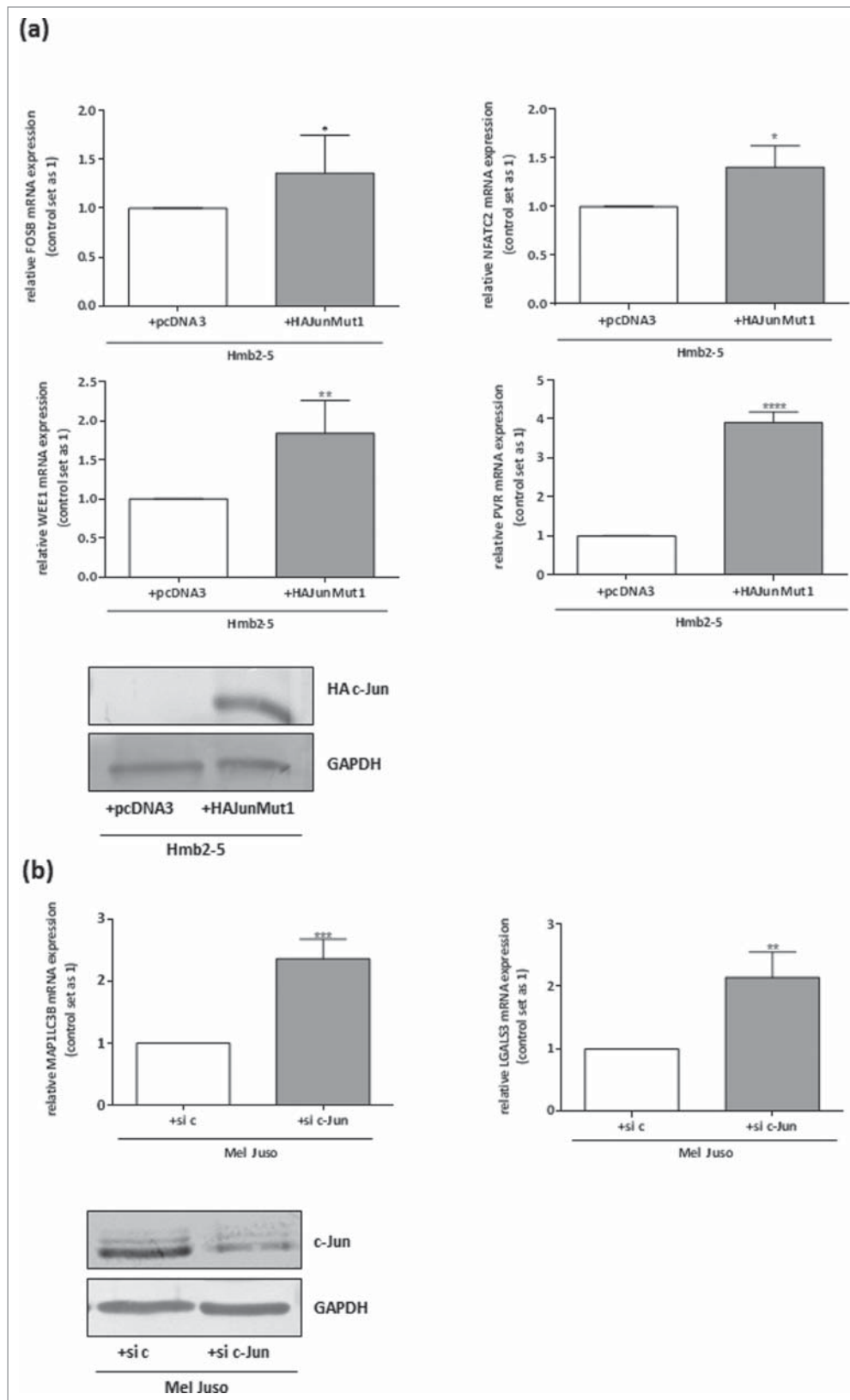
Figure 5. FosB, NFATC2, WEE1, PVR, LGALS3 and MAP1LC3B are regulated by the transcription factor c-Jun. (a) qRT-PCR after c-JUN re-expression with a HA-tagged-c-Jun expression construct (HA-Jun Mut1) [11] in the melanocyte-resembling cell clone Hmb2-5 lacking c-Jun expression [24] showed upregulation of FosB, NFATC2, WEE1 and PVR. pcDNA3 served as a negative control. Transfection efficiency after transfection with the HA-tagged-c-Jun expression construct was verified by Western Blot analysis. (b) qRT-PCR after transfection of siRNA against c-Jun in the melanoma cell line Mel Juso revealed downregulation of MAP1LC3B and LGALS3. Transfection experiments with a control siRNA served as the negative control. Transfection efficiency after transfection with the siRNA against c-Jun was verified by Western Blot analysis. The bars show the means ± s.d. of three independent experiments; measurements were performed in triplicate (****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.5).

known cancer-relevant molecular functions, such as adhesion, cell motion and positive regulation of cell proliferation (GO terms), indicating that c-Jun supports cancer development. Moreover, for the first time, we confirmed the direct regulation of 6 selected target genes by the transcription factor c-Jun in melanoma cells. Based on the results of this study, the c-Jun target genes FosB, WEE1, PVR, LGALS3 contain the classical AP-1 consensus sequence, whereas MAP1LC3B and NFATC2 contain variations of the classical AP-1 DNA binding site. Thus, interactions between c-Jun and DNA are not strictly dependent on the classical AP-1 consensus sequence and, consequently, we suppose an interaction between c-Jun and 2 novel DNA binding sites most similar to the consensus sequence. Moreover, our research showed that the newly identified c-Jun target genes are de-regulated in melanoma cell lines compared to melanocytes: FosB, NFATC2, WEE1, PVR are up-regulated, whereas MAP1LC3B and LGALS3 are down-regulated. It is possible that the detected up- and downregulation of c-Jun target genes depends on the observed differences in the c-Jun binding sites in the different target genes. Additionally, we suggest a mechanism resulting in the up- or downregulation of the potential c-Jun target genes. One possible mechanism is that the binding site of c-Jun overlaps a binding site of an activator and prevents its binding. Such a mechanism was previously suggested for the crosstalk between c-Jun and the glucocorticoid receptor in the regulation of some genes.[28]

Previous studies suggest that the newly identified direct c-Jun target genes FosB, WEE1, PVR, MAP1LC3B and LGALS3 have crucial roles in tumorigenesis in several cancer types. Moreover, according to the The Human Protein Atlas database (http://www.proteinatlas.org/)[29] the immunohistochemistry stainings with antibodies against the identified potential c-Jun target genes of malignant melanoma tissue samples and normal melanocytes show a similar expression pattern in melanoma patients as suggested by our data. FOS proteins have been implicated as regulators of cell proliferation, differentiation and transformation. Kim and colleagues[30] detected under-expression of FosB in pancreatic cancer tissues with lymph node metastasis compared to pancreatic cancer tissues without lymph node metastasis and showed that decreased expression of FosB is associated with reduced survival. In contrast, other studies showed a bipolar FosB expression pattern in breast cancer,[31] whereas our study indicated high FosB expression in melanoma cell lines compared to NHEMs. Moreover, the gene product of NFATC2 was found to be functionally important in malignant melanoma, as it inhibits apoptosis.[18] Braeuer and colleagues[32] showed a decrease in tumor growth and the number of experimental lung metastasis after silencing NFATC2 in A375SM melanoma cells, which indicates a crucial role in melanoma growth and metastasis. Furthermore, several studies have confirmed the functional relevance of WEE1 overexpression in multiple cancer types, such as breast cancer,[33] ovarian carcinoma,[34] medulloblastoma[35] and melanoma.[36] Other studies have shown overexpression of PVR in lung adenocarcinoma and cutaneous melanoma, which influences invasive activity and, thus, the development of a malignant phenotype.[37,38] In contrast to our melanoma data, Wu and colleagues described MAP1LC3B overexpression in hepatocellular carcinoma and its correlation with malignant progression and poor prognosis.[39] Interestingly, our study showed down-regulation of MAP1LC3B in melanoma cells, which was previously and concordantly with our findings observed in hypopharyngeal squamous cell carcinoma (HSCC) correlating with poor prognosis.[40] Therefore, we speculate that the status and role of MAP1LC3 expression vary across cancer types. Some other studies confirmed our expression data in case of LGALS3, which is known to be associated with melanoma[41, 42] and has been reported to play an essential role in the acquisition of vasculogenic mimicry and angiogenic properties associated with melanoma progression. Thus, c-Jun target genes seem to have both pro-oncogenic and anti-oncogenic functions, which possibly depend on cancer type and/or variations in DNA binding sites recognized by c-Jun. The identification of melanoma-specific, direct c-Jun target genes by ChIP-Seq would offer detailed insight into the molecular role of c-Jun in the development of the malignant phenotype. Moreover, inhibiting direct c-Jun target genes whose expressions and/or activities support the development and progression of malignant melanoma, by inhibiting the transcription factor c-Jun itself is a potential novel therapeutic option.

In summary, the results of this study indicate that the transcription factor c-Jun plays a crucial role in the development and progression of malignant melanoma through direct regulation of cancer-relevant target genes. Moreover, the expression of c-Jun can lead to upregulation and downregulation of specific target genes; thus, we hypothesize that c-Jun enhances the expression of pro-oncogenic target genes and inhibits the expression of anti-oncogenic target genes. A further detailed analysis of direct c-Jun target genes and their possible functions in the development and progression of malignant melanoma is warranted to determine whether c-Jun could serve as a therapeutic target for malignant melanoma.

Material and methods

In silico *analysis*

ChIP-Seq data archived in the Encyclopedia of DNA Elements (ENCODE; http://genome.ucsc.edu/ENCODE/) were used for *in silico* analysis. The data were screened for c-Jun binding regions within the human genome (hg19) of HUVEC, HepG2 and HeLa-S3.

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database was used for the identification of known and predicted protein associations, including indirect (functional) associations.

To discover novel, ungapped c-Jun binding motifs in nucleotide sequences the database MEME Suite 4.10.2 (Multiple Em for Motif Elicitation) was utilized.

Cell culture

Melanoma cell lines Mel Juso, Mel Ju, A375, Mel Ei and normal human epidermal melanocytes (NHEMs) were described previously.[43] The cell lines Mel Juso, Mel Ei and A375 were derived from primary cutaneous melanomas; Mel Ju was derived from metastasis of melanoma. Cells were maintained in DMEM supplemented with penicillin (400 units/ml), streptomycin (50mg/

ml), L-glutamine (300 mg/ml), 10% FCS (Sigma-Aldrich, Steinheim, Germany) and split at a 1:5 ratio every 3 d. NHEMs (PromoCell, Heidelberg, Germany) were derived from neonatal foreskin and were used between passages 2 and 6. HMB2-5 is a cell clone in our laboratory resembling melanocytes.[24] Once a week this cell line is treated with G418 (2 mg/ml) to ensure clone selection.

Analysis of gene expression by quantitative PCR

cDNAs of total RNA fractions were generated using SuperScript II Reverse Transcriptase Kit (Invitrogen, Groningen, The Netherlands). qRT-PCR was performed on a Lightcycler (Roche, Mannheim, Germany). cDNA template (500 ng), 0.5 μl (20 μM) each of forward and reverse primers and 10 μl of SybrGreen LightCycler Mix in a total of 20 μl were applied to the PCR program as described previously.[11] Annealing and melting temperatures were optimized for each primer set (Table S2). The PCR reaction was evaluated by melting curve analysis and determining the PCR products on agarose gels. β-Actin was used for normalization.

Chromatin Immunoprecipitation

ChIP assays were performed following the manufacturer's instructions (ChIP-IT Express; Active Motif, Carlsbad, CA, USA) as described previously.[44] Mel Juso cells grown to 70–80 % confluence on 3 T-175 flasks were used for one chromatin isolation (20 million cells/ ChIP). Samples were immunoprecipitated using a specific c-Jun antibody (3 μg of anti-c-Jun (sc-1694; Santa Cruz Biotechnology, Heidelberg, Germany). A RNA polymerase II antibody was used as a positive, and an IgG antibody as a negative control, following the protocol provided with the control kit (ChIP-IT control Kit-human; Active Motif). DNA samples from the ChIP experiments were used for analysis by PCR utilizing the real-time PCR LightCycler system (Roche) as described previously.[12,45] PCR was performed on 4 DNA templates: the input DNA (1: 10), DNA isolated through RNA polymerase II ChIP (Pol II), DNA isolated through the negative control IgG ChIP (IgG), and DNA isolated through the c-Jun ChIP (c-Jun). A control reaction (H$_2$O) with no DNA template was also performed. Sets of primer pairs amplifying a specific promotor region of a control gene and of each predicted target gene were used: the positive control (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) and the negative control (Active Motif; amplifies a 78 base pair fragment from a gene desert on human chromosome 12) - primer pairs provided by the kit, and primer pairs spanning the c-Jun-binding site within the promoter regions of the predicted c-Jun target genes (ChIP Primers; Table S2). DNA fragments precipitated with the c-Jun and Pol II antibodies were analyzed on a 1.5 % agarose gel compared to the *Input* DNA and the fragments precipitated with the IgG antibody.

Transfection experiments

Two times 10^5 cells were seeded each well in 6-well plates and transfected with 0.5 mg of HA-tagged c-Jun (*HAJunMut*) or pcDNA3 constructs using Lipofectamine Plus (transfection

efficiency of 80%) (Invitrogen). The HA-tagged c-Jun expression vector was generated in our laboratory and results in a complete loss of repression by miR-125b. Exogenous expression of HA-tagged wt c-Jun (HAJun wt) in the HMB2-5 cell clone (expressing miR-125b) did not lead to c-Jun protein expression, whereas the mutated version (HAJun Mut miR-125b) led to strong induction of protein expression in this cell type.[11] Twenty-four h after transfection, the cells were used for further analysis.

siRNA transfection experiments

siRNA transfection of Mel Juso cells was performed using the reverse transfection protocol of the Lipofectamine RNAiMAX reagent (transfection efficiency of 85%) (Invitrogen) according to the manufacturer's instructions. Eight times 10^4 cells were transfected with 10 nM of c-Jun (JUNVHS40918; Invitrogen) or negative control siRNA (Qiagen, Hilden, Germany) for 16 h, respectively. Each experiment was repeated at least 3 times.

Western blotting

The transfected cells were resuspended in 200 ml RIPA buffer (Roche) and lysed for 15 min at 4°C. Insoluble fragments were removed by centrifugation at 13.000 r.p.m. for 10 min and the supernatant was stored at −20°C. Twenty μg of RIPA complete cell lysates was loaded per lane and separated on SDS–PAGE gels (Invitrogen) and subsequently blotted onto a PVDF membrane. After blocking for 1 h blocking with 5 % MP/TBST the membrane was incubated for 16h with one of the following antibodies: anti-c-Jun (1 in 1000 dilution; Cell Signaling, Frankfurt am Main, Germany), anti-GAPDH (1 in 3000 dilution; Cell Signaling) and anti-HA-tag (1 in 1000 dilution; Cell Signaling). After three washing steps with TBS-T, the membrane was incubated for 1 h with an alkaline phosphate-coupled secondary anti-mouse (1 in 3000 dilution in TBS-T) or anti-rabbit (1 in 3000 dilution in TBS-T) IgG antibody (Chemicon, Hofheim, Germany) and then washed again for 3 times in TBS-T. Finally, immunoreactions were visualized by NBT/BCIP (Sigma-Aldrich) staining.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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