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ORIGINAL ARTICLE

WS5, a direct target of oncogenic transcription factor Myc, is related to human melanoma glycoprotein genes and has oncogenic potential

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We have isolated a gene (*WS5*) that is specifically expressed at the mRNA and protein level in avian fibroblasts transformed by the v-*myc* oncogene of avian acute leukemia virus MC29. In a conditional cell transformation system, *WS5* gene expression was tightly correlated with v-*myc* activation. The *WS5* gene contains 11 exons, encoding a 733-amino acid protein with a transmembrane region and a polycystic kidney disease (PKD) domain. Near the transcriptional start site, the *WS5* promoter contains a cluster of four binding sites for the Myc–Max complex and a binding site for transcription factor C/EBPα. Electrophoretic mobility shift assays and chromatin immunoprecipitation showed that Myc, Max and C/EBPα bind specifically to these sites. Functional promoter analyses revealed that both the Myc-binding site cluster and the C/EBPα-binding site are essential for strong transcriptional activation, and that Myc and C/EBPα synergistically activate the *WS5* promoter. Ectopic expression of *WS5* led to cell transformation documented by anchorage-independent growth. The human melanoma antigen Pmel17, a type I transmembrane glycoprotein, is the mammalian protein with the highest amino acid sequence identity (38%) to WS5. The *Pmel17* gene is regulated by the MITF protein, a bHLHZip transcription factor with DNA binding specificities similar to those of Myc/Max. WS5 is also related to human glycoprotein GPNMB expressed in metastatic melanoma cells and implicated in the progression of brain and liver tumors.
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Introduction

The *myc* oncogene was originally identified as a transduced allele (v-*myc*) representing the oncogenic determinant of avian acute leukemia virus MC29 (Bister and Jansen, 1986). Deregulation of the cellular c-*myc* protooncogene by retroviral transduction, chromosomal translocation, promoter insertion, or gene amplification is involved in animal and human tumorigenesis (Nesbit *et al*., 1999). The *myc* gene encodes a bHLHZip protein (Myc) encompassing protein dimerization domains (helix-loop-helix, leucine zipper) and a DNA contact surface (basic region) (Kato and Dang, 1992). Myc forms heterodimers with the bHLHZip protein Max, binds to specific DNA sequence elements (E-boxes), and is part of a transcription factor network including additional proteins like Mad, Mnt and Mga (Grandori *et al*., 2000; Eisenman, 2001). The dimeric Myc-Max and Max-Mad transcription factors recruit co-activator or corepressor complexes that modify chromatin structure and modulate transcription. They are key regulators of genes critically involved in cellular growth, proliferation, metabolism, differentiation and apoptosis (Dang, 1999; Grandori *et al*., 2000; Eisenman, 2001; Secombe *et al*., 2004; Adhikary and Eilers, 2005).

Although the structures of Myc and Max proteins, their mode of interaction and recognition of DNA have been analysed in detail (Ferré-D'Amaré *et al*., 1993; Fieber *et al*., 2001; Nair and Burley, 2003), the dissection of the molecular events downstream of the activated Myc transcription factor proved difficult. Myc is involved in many basic cellular functions, and recent studies have suggested that it may regulate, directly or indirectly, up to 15% of all genes (Patel *et al*., 2004). For target gene validation, distinction between direct and indirect effects on gene expression is essential. Also, an important unresolved question is whether deregulated oncogenic Myc promotes tumorigenesis by aberrant regulation of its normal targets or by affecting new ones. Several Myc targets have recently been identified that display partial oncogenic potential (Ben-Porath *et al*., 1999; Zhang *et al*., 2005; O'Donnell *et al*., 2006). Notably, no target gene has been identified so far that can mimic the full oncogenic capacity of *myc* upon ectopic expression, supporting the notion that the molecular events downstream of *myc* represent a multifunctional response.

Here, we describe the isolation and complete structural dissection of a direct transcriptional target (*WS5*) of oncogenic transcription factor Myc. We show that Myc binds directly to the *WS5* promoter and activates gene expression in synergy with the C/EBPα

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transcription factor. Ectopic expression of *WS5* in avian fibroblasts leads to anchorage-independent growth, suggesting that deregulation of *WS5* contributes to *myc*-induced oncogenesis. Intriguingly, the *WS5* protein product is structurally related to type I transmembrane glycoproteins (Pmel17, GPNMB) highly expressed by human melanoma cells and implicated in the progression of tumors of several organs.

Results

Specific expression of WS5 in myc-transformed avian fibroblasts

We have previously established a conditional cell transformation system using doxycycline-controlled expression of the MC29 v-*myc* allele. Using this system, several partial cDNA clones isolated by differential screening procedures were recognized as candidate *myc* target genes (Oberst *et al*., 1999). The gene represented by one of these clones (*WS5*) is characterized here in detail. Northern analysis of RNAs from normal or transformed chicken (CEF) or quail (QEF) embryo fibroblasts using *WS5* cDNA as a probe revealed the specific expression of a 2.8-kb mRNA in v-*myc*-transformed cells, but not in normal cells or in cells transformed by v-*src*, v-*jun* or by methylcholanthrene (Figure 1a). The tight correlation of *WS5* mRNA levels with v-*myc* expression is demonstrated in the conditional cell transformation system (Q/tM_{ON}) containing a doxycycline-dependent v-*myc* allele (Oberst *et al*., 1999). In a time course experiment, *WS5* transcription starts immediately after v-*myc* activation, v-*myc* and *WS5* mRNA levels then increase in parallel, and both disappear again after v-*myc* inactivation (Figure 1b). Specific *WS5* activation by v-*myc* is also detected at the protein level (Figure 1c). By 30-min pulse labeling, a translational product with an apparent M_r of 118 000 ($p118^{WS5}$) was detected by the WS5-specific anti-peptide serum in cells freshly transformed by the MC29 v-*myc* gene and in the established v-*myc*-transformed cell line Q8, but not in normal QEF. The protein is also detectable by *in vitro* transcription-translation or by *WS5* overexpression from a retroviral vector. The minor difference in electrophoretic mobility of $p118^{WS5}$ from the different sources is possibly due to differential processing or posttranslational modification. In a chase experiment, $p118^{WS5}$ was processed into a protein with an apparent M_r of 74 000 ($p74^{WS5}$) that is also found as the major WS5 polypeptide in Q8 cells, and into a protein with an apparent M_r of 53 000 ($p53^{WS5}$). Similar aberrant electrophoretic mobility and extensive processing has been reported for the presumed human homolog of WS5 (see below). None of the proteins recognized by the WS5-specific anti-peptide serum were detected in normal cells or by a control serum (Figure 1c). The major WS5 polypeptide ($p74^{WS5}$) in Q8 cells was also readily detected by an antiserum directed against a hybrid GST-WS5 recombinant protein (not shown).

Structural organization of the WS5 gene and protein

A full-length 2355-bp *WS5* cDNA clone (accession no. AF077328) was assembled from two overlapping 3′ cDNA clones obtained by screening of a cDNA library from the MC29-transformed cell line Q8 (Bister *et al*., 1977) with the original *WS5* probe (Oberst *et al*., 1999) and from three overlapping 5′ RACE products. To determine the structural organization of the *WS5* gene, the nucleotide sequence of a 6017-bp quail genomic DNA segment (accession no. AY496440) hybridizing with a *WS5* cDNA probe was determined. The *WS5* gene contains 11 exons with the coding region extending through all of them (Figure 2a). The deduced 733-amino acid WS5 protein has a calculated M_r of 74162 (see below). The topography of the *WS5* gene locus deduced from the nucleotide sequence of cloned DNA was confirmed by Southern analysis of quail high molecular weight DNA, proving the identity of the gene isolate (Figure 2b). Within 120 nucleotides upstream of the transcriptional start site, the *WS5* promoter region displays a cluster of four putative Myc-binding sites and an adjacent binding site for transcription factor C/EBPα (Figure 2c). The cluster of Myc-binding sites contains three canonical (CACGTG or CATGTG) and one noncanonical (CACATG) sequence motives (Blackwell *et al*., 1993). The C/EBPα site perfectly conforms to the consensus TTNNNNAA and is detected with a high score by the AliBaba and TFSEARCH algorithms (Grabe, 2002; Matys *et al*., 2003). Sequence comparison of the promoter regions of *WS5* and of the presumed chicken ortholog *Mmp115* (*Gallus gallus* linkage group E22C19W28 genomic contig, NW_100947.1, nt 3231–2972) revealed conservation of the putative Myc- and C/EBPα-binding sites (Figure 2d).

An alignment of the deduced amino acid sequences of the WS5 protein and of the human melanoma antigen Pmel17 (Kwon *et al*., 1991), the mammalian protein displaying the highest amino acid sequence identity to WS5, is shown in Figure 3. The overall sequence identity between the human and the quail protein is 38%, whereas the identity between WS5 and the chicken Mmp115 protein (Mochii *et al*., 1991) is 77% (not shown). The aligned proteins display a signal sequence, a transmembrane region and a central polycystic kidney disease (PKD) domain. The PKD sequence motif was first identified in the large multimodular glycoprotein polycystin-1, the protein product of a gene (PKD1) frequently mutated in cases of autosomal dominant polycystic kidney disease (Bycroft *et al*., 1999). The amino acid sequences of WS5 and Pmel17 also contain conserved putative *N*-glycosylation sites. Similar to the WS5 protein (see above), the 668-amino acid Pmel17 protein (calculated M_r 70 992) displays a larger apparent M_r of 100 000 and is also processed into smaller polypeptides (Berson *et al*., 2001, 2003). The sequence similarities, the shared domain topography, and the common biochemical properties reported for Pmel17 and Mmp115 (Theos *et al*., 2005) suggest that Pmel17 is indeed the human homolog of the closely related avian WS5 and Mmp115 proteins. The WS5 protein also displays 24% sequence identity (not shown) with the

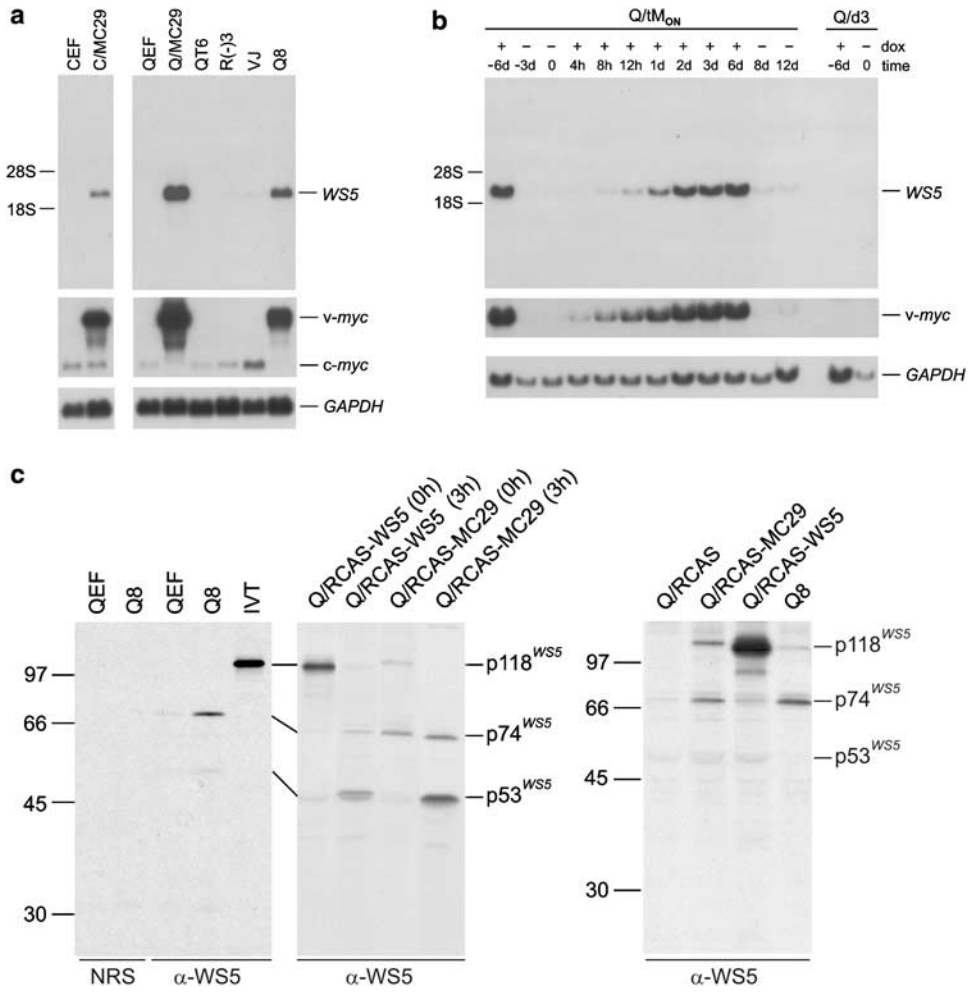

Figure 1 Specific activation of *WS5* gene expression in v-*myc*-transformed avian fibroblasts. (**a**) Northern analysis of poly(A)$^+$ RNAs from normal CEF, CEF transfected with RCAS-MC29 carrying the v-*myc* oncogene (C/MC29), normal QEF, QEF transfected with RCAS-MC29 (Q/MC29), or from the quail cell lines QT6, R(−)3, VJ, and Q8, transformed by methylcholanthrene or by the oncogenes v-*src*, v-*jun* or v-*myc*, respectively. (**b**) *WS5* gene expression in a conditional cell transformation system. Transformed Q/tM$_{ON}$ fibroblasts containing a rtTA-dependent v-*myc* allele were grown in the presence of doxycycline. At day -6, doxycycline was removed and added back at day 0. At day 6, the drug was removed again. RNA was extracted at the time points indicated and analysed. RNA from the cell line Q/d3 containing a rtTA-dependent v-*jun* allele was used a a control. Filters in **(a)** and **(b)** were successively hybridized with ^{32}P-labeled cDNA probes specific for quail *WS5*, chicken c-*myc*/MC29 v-*myc* and quail *GAPDH*. The sizes of the mRNAs are 2.8 kb (*WS5*), 2.3 kb (c-*myc*), 5.4 kb (v-*myc* containing MC29 genome), and 1.4 kb (*GAPDH*). (**c**) WS5 protein analysis. Left panel: Normal QEF and cells from the v-*myc*-transformed cell line Q8 were labeled with [^{35}S]methionine for 2.5 h, and extracts were analysed by immunoprecipitation with a WS5-specific antipeptide serum or normal rabbit serum (NRS), and subsequent SDS-PAGE (10%, wt/vol). WS5 protein produced by *in vitro* transcription-translation of vector pBS-WS5 was immunoprecipitated and analysed in parallel (IVT). Positions of molecular weight markers (in kDa) are indicated in the margin. Middle panel: processing of the WS5 protein in QEF transfected with RCAS-WS5 or RCAS-MC29. After 30-min pulse labeling with [^{35}S]methionine, the cells were incubated in unlabeled medium for a 3-h chase period, and cell extracts prepared after the pulse (0 h) or after the chase (3 h) were analysed accordingly. Right panel: coelectrophoresis of WS5 proteins from v-*myc*-transformed cells (Q/RCAS-MC29, and Q8) and from QEF transfected with RCAS-WS5. Cells were labeled for 30 min.

human transmembrane glycoprotein GPNMB that is expressed in most metastatic melanoma cells and has been implicated in the progression of several human malignancies (Weterman *et al*., 1995).

Specific binding of Myc, Max and C/EBPα to the WS5 promoter

The striking correlation between *WS5* expression and v-*myc* activation (see Figure 1) and the presence in the *WS5* promoter region of multiple potential Myc binding sites provided circumstantial evidence that Myc directly activates *WS5* transcription. This was corroborated by electrophoretic mobility shift analyses (EMSA) showing binding of recombinant protein complexes containing Myc–Max heterodimers (Fieber *et al*., 2001) to the *WS5* promoter region containing the E-box cluster (Figure 4a and b). Interestingly, increasing protein concentration led to the formation of multiple distinct protein–DNA complexes indicating binding to multiple sites in the

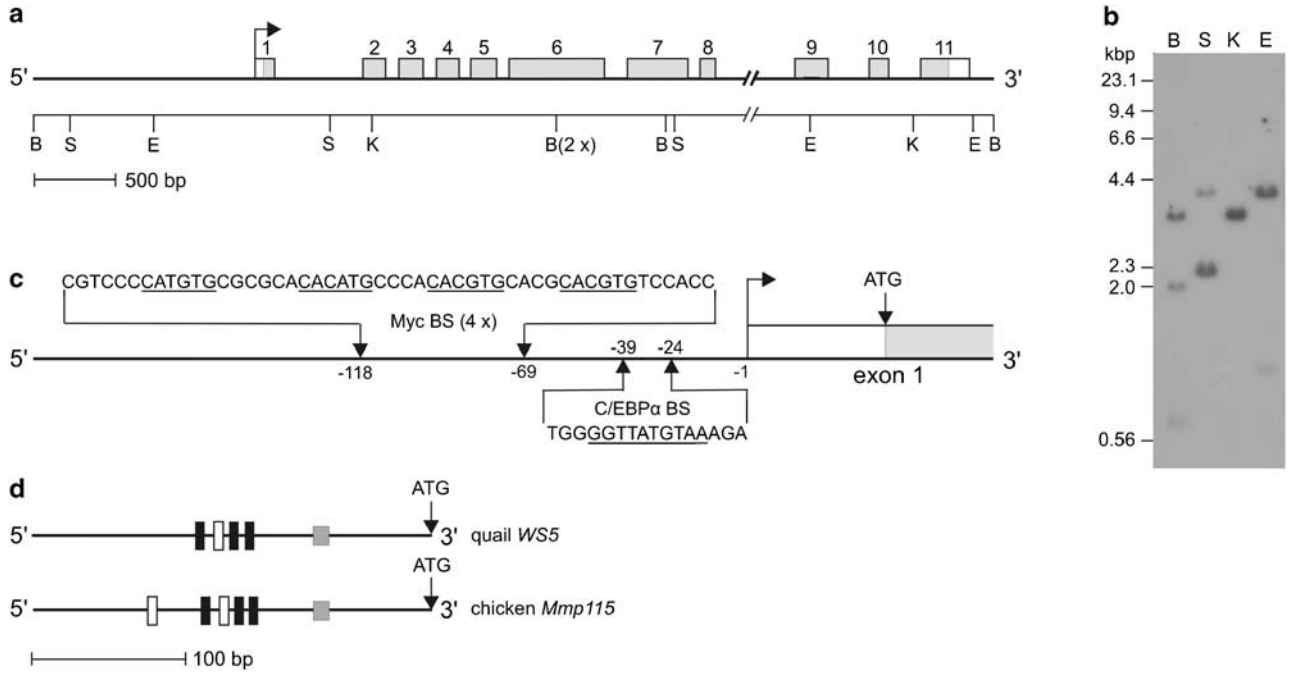

Figure 2 Structure of the quail *WS5* gene locus. (**a**) The *WS5* gene encompasses 11 exons with the open reading frame (shaded) extending through all exons. B, *Bam*HI; E, *Eco*NI; K, *Kpn*I; S, *Sac*I. (**b**) Southern analysis of high molecular weight DNA from the quail fibroblast line Q8 digested with *Bam*HI (B), *Sac*I (S), *Kpn*I (K), or *Eco*NI (E), using a ^{32}P-labeled *WS5* cDNA fragment encompassing the entire coding region as a probe. (**c**) The *WS5* promoter region immediately upstream of the transcription start site contains four putative binding sites (underlined) for Myc and one for C/EBPα. (**d**) Comparison of the quail *WS5* and the chicken *Mmp115* promoter regions aligned with respect to the ATG startcodon in exon 1. The positions of canonical or noncanonical Myc-binding sites and of C/EBPα-binding sites are indicated by black, open, or shaded boxes, respectively.

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WS5     --MRLHGAIVLLAALLALTTAQQRGGGRNPGAVQGPLWGGRPTPFRSWDATRYRPWKEGTAQQSDCWRGGDVTFDISNDAPTMAGAKATFSMLLGFPSTQ   98
Pmel17  MDLV.KRCLLH..VIG..LAVGATKVP..-----.DW.GVS.QLRTKA.NRQL.PE.T.--..RL......Q.SLKV...G..LI..N.S..IA.N..GS.   94
WS5     RALPDGRVVWNQNCAVNGTRMVQGDPVFPEQLVEGSDGVFPDGQPFPRSSWGKRGRFVYVWWTWGHYWQVVDGAASKLTVGTDGVALGSYTMEVVVYHYR   198
Pmel17  KV....Q.I.VN.TII..SQVWG.Q..Y.QETDDAC--I....G.C.SG..SQKRS.....K...Q....LG.PV.G.SI..GRAM..TH....T...R.   192
WS5     GRQKFIPIGHASTQFSITDQVPIAVDVTQLEVATGDGGRFVLNHPVAFNVRLHDPSHYLRDADISYSWDFGDQSGTLISRSPTVTHTYLQAGSFAARLVL   298
Pmel17  .SRSYV.LA.S.SA.T......FS.S.S..RALD.GNKH.LR.Q.LT.ALQ.....G..AE..L..T.....S.......A.V......EP.PVT.QV..   292
WS5     QAAIPLGSCGTSAAPVVDPTTGSVPSLGPTATEPVGPTGSGTAAASSTPTAPGTTAAPAASGAPAEPTGVSVVVPSDSAATEPIPDPVLSTGAAANTDPT   398
Pmel17  ......T...S.------.VP.TTDGHR...EA.N-----T..GQVP.TEVV...PGQ.PTAE.SGT.S.Q.PT--------------------TEVIS.   360
WS5     ADPQSPTSVSSGGDAPGTVDPTAVEGSVAAGVGAATPGATAADVEVDAAGPTAGATAGTMADSTAGIMADATAGATAQSMAGATAGATAGAIADPTAGAT   498
Pmel17  .PV.M..AE.T.-MT.EK.PVSE.M.TTL.EMSTP--------------------EAT.MTPAEVSIVVLSG.TA.QVTTTEWVETT.RELP.PE.EGPDA   440
WS5     AGAIADPTAGAIADPTAGSTAEPLLLVKRQAPESEPTGCVLYRYGSFSTELNIVQGIENVAIVQVVPAAPEGSGNSVELTVTCEGSLPEEVCTVVADAEC   598
Pmel17  SSIMSTESITGSLG.LLDG..-T.R.....V.----LD..........VT.D......SAE.L.A..SG---E.DAF....S.Q.G..K.A.MEISSPG.   533
WS5     RTAQMQTCSAVAPAPGCQLVLRQDFNQ-SGLYCLNVSLANGNGLAVASTR--VAVG-----GASPAAGGTTLTVGLLLIVAALGTAAYTYRRVKYSPLLP   690
Pmel17  QPPAQRL.QP.L.S.A.....H.ILKGG..T...I....DT.S...V..QLIMP.PGILLT.QEAGL.QVR.I..I..VLM.VVL.SLI...RLMKQDFS   633
WS5     TAPQVSRPHSWLPPGAALRLLLRQAFGGAPSGESSPLLRANAV                                                          733
Pmel17  VP--------Q..HSSSHW.R.PRI.CSC.I..N....SGQQ.                                                          668
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Figure 3 Alignment of the deduced amino acid sequences of the quail WS5 protein and the human melanocyte-specific protein Pmel17. In the Pmel17 sequence, amino acid residues are only shown at positions where they differ from the WS5 sequence, and gaps are represented by dashes. A signal sequence, a transmembrane region, and a PKD domain are indicated by yellow, blue, and green shading, respectively. Conserved putative *N*-glycosylation sites are marked by orange shading.

cluster region. Supershift analysis confirmed the presence in these complexes of the Myc and Max recombinant proteins (Figure 4b). These results were confirmed and extended by chromatin immunoprecipitation (ChIP) using chromatin from Q8 cells transformed by the MC29 p110 Gag-Myc hybrid protein (Bister *et al*., 1977) and containing high levels of *WS5* mRNA (see Figure 1). Antisera directed against Myc, Gag or Max revealed binding of p110$^{gag-myc}$ and Max to the *WS5* promoter fragment containing the E-box cluster and the adjacent C/EBPα-binding site (Figure 4c), in agreement with the direct demonstration of p110$^{gag-myc}$–Max complexes in MC29-transformed cells (Hartl *et al*., 2003). Interestingly, antiserum directed against C/EBPα revealed binding of this transcription factor to the *WS5* promoter fragment (Figure 4c), suggesting a functional role for the C/EBPα-binding site flanking the E-box cluster (see Figure 2). Using chromatin from normal QEF for control, only low level binding of Max was detected.

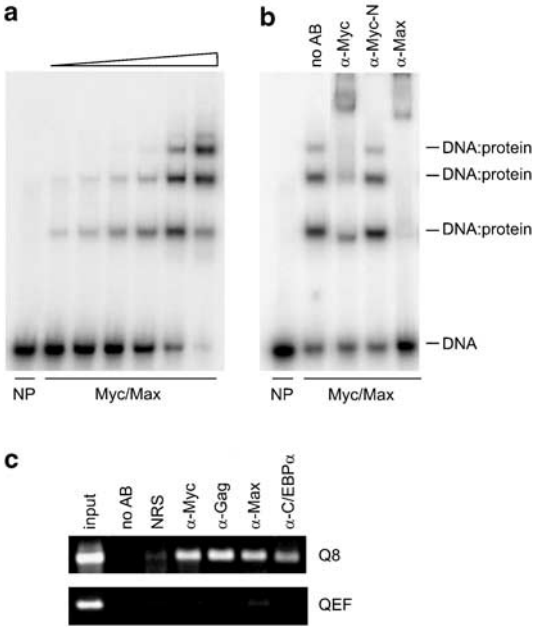

Figure 4 Binding of Myc, Max and C/EBPα to the *WS5* promoter. (**a**) A 56-bp ^{32}P-labeled double-stranded oligodeoxynucleotide containing the four E-boxes from the *WS5* promoter was used as a probe for EMSA. The probe was incubated with no protein (NP) or with increasing amounts of a mixture of recombinant avian Myc (C-terminal amino acid residues 314–416) and Max (amino acid residues 22–113) proteins. (**b**) The same probe was incubated with Myc/Max proteins in the absence of serum (no AB) or in the presence of specific antisera directed against the C-terminus of Myc (α-Myc), the N-terminus of Myc (α-Myc-N) or Max (α-Max). The reaction mixtures were analysed by native PAGE (6%, wt/vol). The positions of free DNA and protein–DNA complexes are indicated in the margin. (**c**) ChIP using chromatin from Q8 cells transformed by the p110 Gag-Myc hybrid protein or from normal QEF. Antisera directed against Myc (C-terminus), Gag, Max or C/EBPα were used for precipitation before PCR amplification of a 273-bp fragment from the *WS5* promoter containing the E-box cluster and the C/EBPα-binding site (see Figure 2). Reactions without antiserum (no AB) or with normal rabbit serum (NRS), or using total chromatin (input) were used as controls.

Transcriptional activation of the WS5 promoter by Myc and C/EBPα

To determine whether Myc-Max and C/EBPα are involved in direct activation of the *WS5* promoter, the genomic regions corresponding to nucleotides −238 through +38 (pCAT-WS5) or −1260 through +38 (pCAT-WS5-L) were tested for their capacity to direct expression of a chloramphenicol acetyltransferase (CAT) reporter gene. Transactivation of the promoter constructs was analysed in normal QEF, in QEF transformed by MC29, or in chemically transformed QT6 cells. The analysis revealed that the *WS5* promoter is efficiently activated in *myc*-transformed cells, but not in QEF or in QT6 cells, and that the short promoter construct is sufficient for full activation (Figure 5a). CAT analysis of appropriate deletion mutants of pCAT-WS5 revealed that the E-box cluster and the C/EBPα-binding site are required for efficient *WS5* promoter activation (Figure 5b). Transactivation of the pCAT-WS5 promoter construct was also tested in normal QEF that were transiently co-transfected with pRc/RSV-derived expression vectors encoding v-Myc or C/EBPα transcription factor proteins. Strongest activation was observed when vectors encoding v-Myc and C/EBPα were transfected simultaneously, and moderate activation was seen when only C/EBPα was introduced (Figure 5c). To test for equal protein expression in cells transfected with the pRc/RSV-derived expression vectors, transfected cells were labeled with [^{35}S]methionine and cell extracts were analysed by immunoprecipitation. The analysis revealed efficient and nearly equimolar expression of $p110^{gag-myc}$ or $p44^{C/EBP\alpha}$ in these cells (Figure 5d). The size heterogeneity observed for C/EBPα has been described before and was attributed to the use of alternative initiation codons (Mink *et al*., 1999). Northern analysis of normal QEF, of v-*myc*-transformed Q8 cells, or v-*jun*-transformed VJ cells revealed that the endogenous *C/EBPα* gene is activated in *myc*-transformed cells, but that mRNA levels are low or undetectable in normal QEF or in VJ cells (Figure 5e). In the co-transfection experiments (Figure 5c and d), ectopic expression of Myc is not sufficient for transactivation since normal QEF do not contain C/EBPα, whereas introduction of C/EBPα leads to moderate activation due to recruitment of endogenous c-Myc and Max proteins. Accordingly, concomitant ectopic expression of v-Myc and C/EBPα and recruitment of endogenous Max leads to strong synergistic activation.

To test for direct interaction between the MC29 Gag-Myc hybrid protein and C/EBP proteins, co-immunoprecipitation analyses were performed using extracts from MC29-transformed QEF and antisera directed against a C-terminal recombinant v-Myc protein, or against recombinant C/EBPα or C/EBPβ proteins. The analyses clearly revealed association of $p110^{gag-myc}$ with C/EBP proteins in MC29-transformed cells (Figure 5f), confirming and extending previous circumstantial evidence for possible direct interaction between these proteins (Mink *et al*., 1996; Hartl *et al*., 2003).

Overexpressed WS5 induces partial cell transformation

The strong and highly specific activation of *WS5* in v-*myc*-transformed cells prompted us to test if overexpression of *WS5* by itself would induce any parameters of the transformed phenotype. Hence, the quail *WS5* coding sequence was inserted into the replication-competent retroviral RCAS vector, and the RCAS-WS5 construct was transfected into QEF and CEF. The empty vector and the RCAS-MC29 vector (Hartl *et al*., 2003) were used as controls. Ectopic expression of *WS5* in cultured cells led to morphological alterations, more pronounced in QEF than in CEF (Figure 6a and c). Significantly, overexpression of *WS5* led to anchorage-independent growth in both cell types, monitored by colony formation in soft agar (Figure 6b and d). The number of colonies per 1000 cells seeded was 117 (QEF) and 135 (CEF) for the *WS5* expressing cells, and 488 (QEF) and 185 (CEF) for the v-*myc* transformed cells.

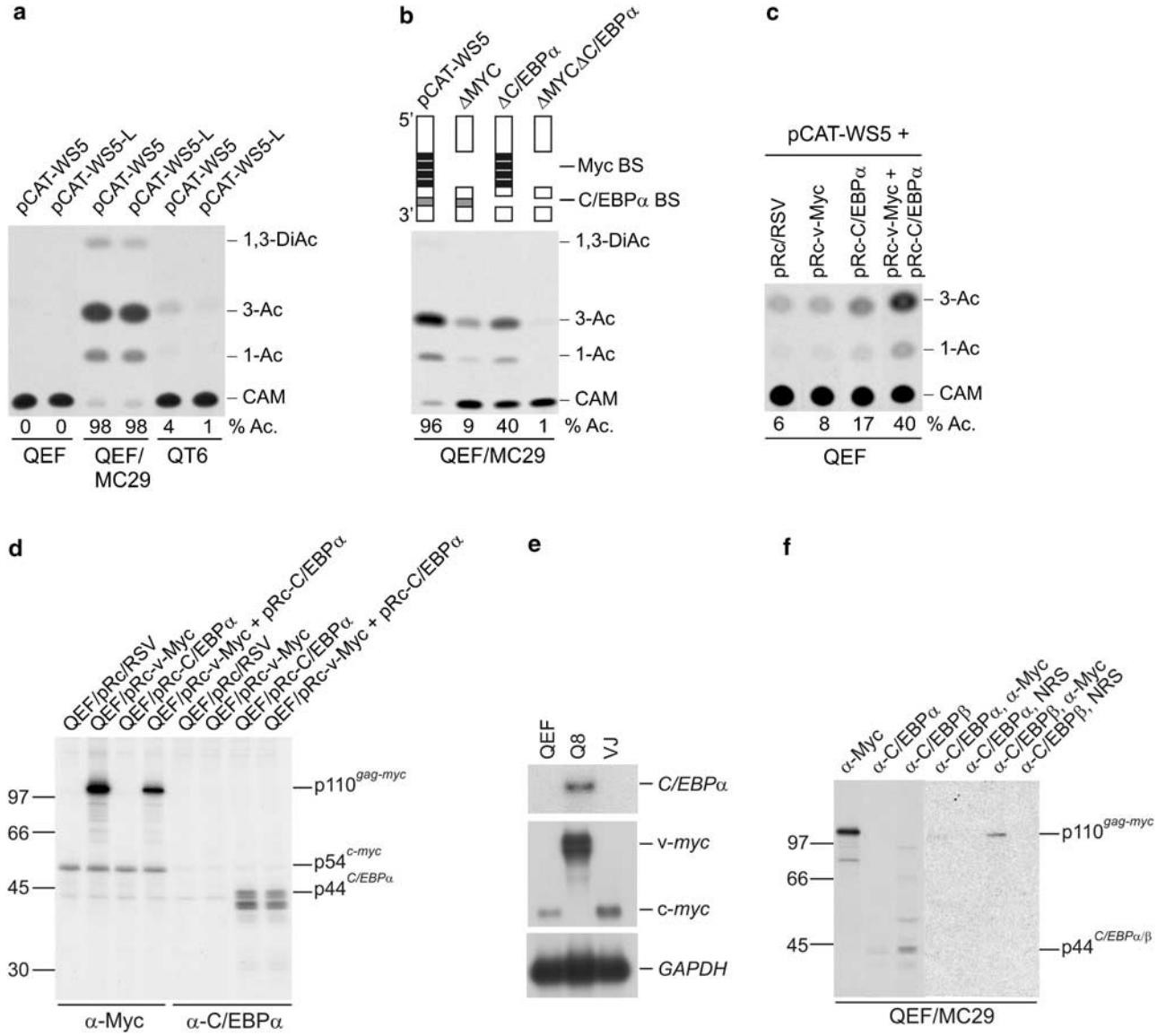

Figure 5 Regulation of the *WS5* promoter by v-Myc and C/EBPα. (**a**) DNA from CAT gene constructs containing the full-length or minimal wild-type *WS5* promoters (pCAT-WS5-L, pCAT-WS5) was transiently nucleofected into normal QEF, into QEF transformed by retrovirus MC29 encoding the Gag-Myc hybrid protein, or into the QT6 cell line chemically transformed by methylcholanthrene. Cell extracts containing equal amounts of proteins were used for acetylation of [^{14}C]chloramphenicol. Reaction products were resolved by ascending thinlayer chromatography and visualized by autoradiography. Positions of chloramphenicol (CAM), of acetylated products (1-acetyl-, 3-acetyl-, or 1,3-diacetyl-[^{14}C]chloramphenicol), and percentages of total acetylation are indicated. (**b**) Equal amounts of DNA from CAT gene constructs containing the minimal *WS5* promoter (pCAT-WS5) or derivatives thereof lacking the cluster of four Myc-binding sites (ΔMyc), the C/EBPα-binding site (ΔC/EBPα), or both sites (ΔMycΔC/EBPα) were used for nucleofection into QEF transformed by the v-*myc* oncogene (QEF/MC29), and cell extracts were analysed as described above. (**c**) pCAT-WS5 DNA was transfected by the calcium phosphate method into QEF together with equal amounts of pRc/RSV empty vector DNA, or of expression constructs carrying the coding regions of v-*myc* or *C/EBPα*, or with DNA from both vectors as indicated. Cell extracts containing equal amounts of protein were tested. (**d**) Analysis of [^{35}S]methionine-labeled proteins from QEF transfected with DNA from the empty pRc/RSV vector or from the expression constructs described under c by immunoprecipitation and SDS–PAGE (10%, wt/vol). Antisera against Myc or C/EBPα were used. Positions of molecular weight markers (in kDa) are indicated in the margin. (**e**) Northern analysis of poly(A)$^+$ RNAs from normal QEF, and from the Q8 and VJ cell lines transformed by v-*myc* or v-*jun*, respectively. Filters were hybridized using DNA probes specific for chicken *C/EBPα*, chicken c-*myc*/MC29 v-*myc*, and quail *GAPDH*. The mRNA sizes are 2.4 kb (*C/EBPα*), 2.3 kb (c-*myc*), 5.4 kb (MC29 genome carrying v-*myc*), and 1.4 kb (*GAPDH*). (**f**) Interaction of the MC29 p110$^{gag-myc}$ hybrid protein with C/EBP proteins demonstrated by co-immunoprecipitation. For immunoprecipitation, equal aliquots of extracts from [^{35}S]methionine-labeled MC29-transformed quail embryo fibroblasts (QEF/MC29) in denaturing buffer were incubated with antisera directed against recombinant v-Myc, C/EBPα or C/EBP*β* proteins. For co-immunoprecipitation, extracts prepared under nondenaturing conditions were incubated with α-C/EBPα or α-C/EBP*β* sera, and precipitates were subsequently subjected to immunoprecipitation under denaturing conditions using α-Myc or normal rabbit serum (NRS) as the second antibody. Immunoprecipitates were analysed by SDS–PAGE (10%, wt/vol). Positions of molecular weight markers (in kDa) are indicated in the margin.

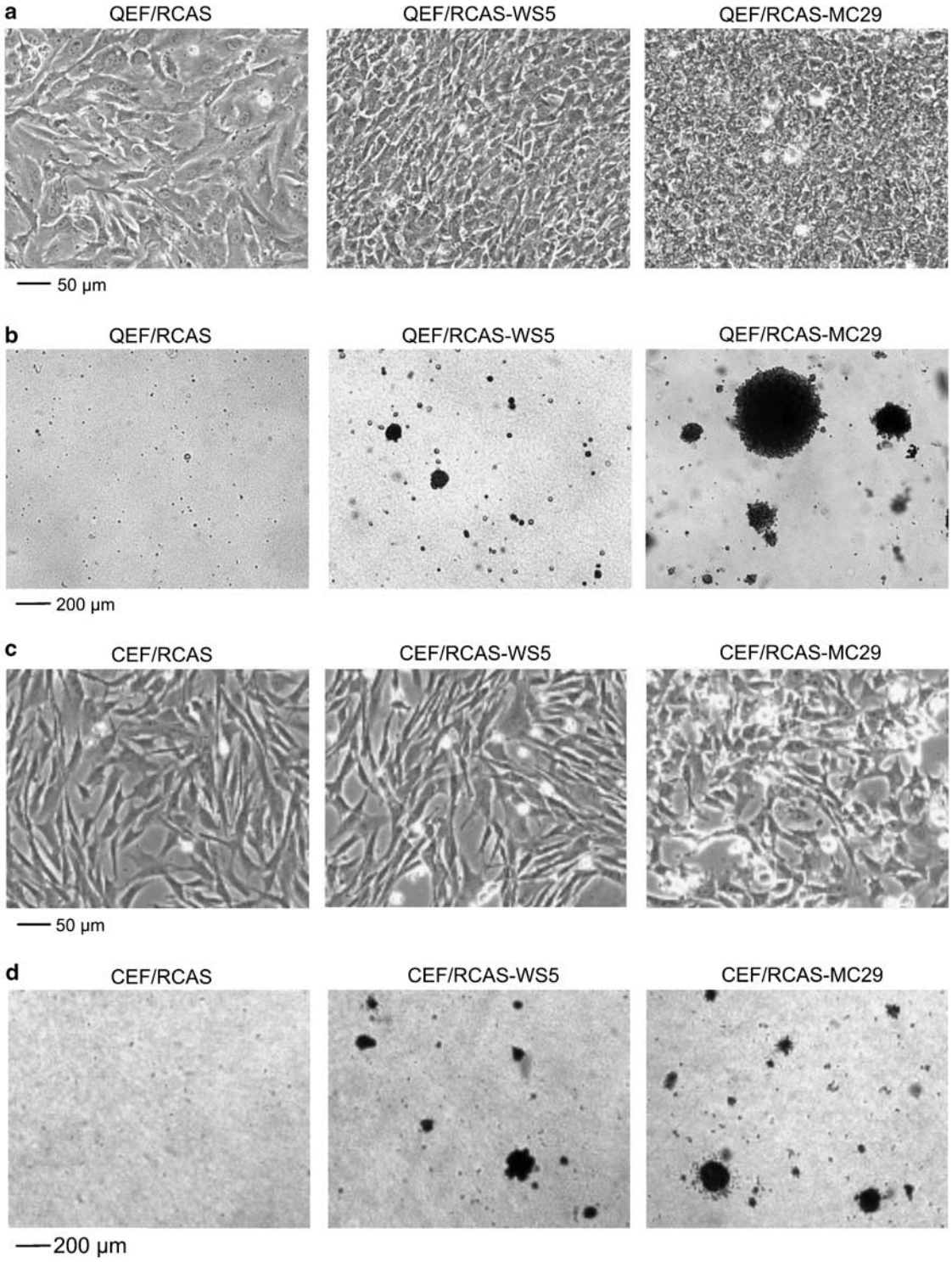

Figure 6 Biological activity of WS5. (**a**, **c**) Phase-contrast micrographs of QEF or CEF transfected with vector DNA (RCAS), or with retroviral constructs carrying the coding regions of chicken *WS5* (RCAS-*WS5*) or v-*myc* (RCAS-MC29). (**b**, **d**) Agar colony formation by cells transfected with RCAS-*WS5* or RCAS-MC29. Equal numbers of cells were seeded in soft agar. RCAS-transfected QEF or CEF were used as controls. Bright-field micrographs were taken after 14 days.

Discussion

Deregulation of c-Myc is one of the most common alterations in human cancer cells, and yet the pathways downstream of the transcription factor have not been dissected in detail. In order to understand the molecular mechanisms of carcinogenesis induced by oncogenic transcription factors, the relevant target genes have to

be identified and analysed. This is a considerable challenge since key transcriptional regulators like Myc, involved in the control of many fundamental biological processes, affect the expression of many genes (Neiman *et al*., 2001; Menssen and Hermeking, 2002; Patel *et al*., 2004). However, not all of these genes are necessarily effectors of the biological functions of Myc (Grandori *et al*., 2000; Eisenman, 2001). The major task is to distinguish direct target genes of Myc from indirect effects on gene expression. For validation of a direct target, binding of Myc *in vivo* and tight correlation of transcriptional alterations with Myc expression is required (Eisenman, 2001). Several direct Myc targets have been well defined (Dang, 1999; Grandori *et al*., 2000; Hermeking *et al*., 2000; Eisenman, 2001; Patel *et al*., 2004), most of them with biological functions in cell cycle control, metabolism or ribosome biogenesis, but only a few have so far been directly linked to the transforming function of Myc (Eisenman, 2001, Patel *et al*., 2004). These include the ornithine decarboxylase gene (*ODC*), the tumor-associated membrane glycoprotein gene *Tmp*, the metastasis-associated protein 1 (*MTA1*) gene, or the transferrin receptor 1 (*TFRC1*) gene, that were shown to have oncogenic potential (Ben-Porath *et al*., 1999; Eisenman, 2001; Zhang *et al*., 2005; O'Donnell *et al*., 2006). However, none of these genes can fully substitute for *myc* function.

The *WS5* gene characterized here meets the stringent criteria of a direct Myc target. Expression of *WS5* is tightly correlated with Myc levels, the promoter region contains a cluster of Myc-binding sites that are occupied *in vivo* by Myc and Max, and transactivation analyses of the wild-type and mutant versions of the promoter directly proved functionality of these sites. Clustering of multiple E-boxes increases the chances of recruiting multiple Myc–Max complexes to a gene, leading to synergistic activation (Hermeking *et al*., 2000; Levens, 2003). This is directly supported by our data (see Figure 4). Interestingly, the *WS5* promoter also requires binding of C/EBPα for full activation, indicating that Myc-Max and C/EBPα function synergistically (see Figure 5). Furthermore, co-immunoprecipitation revealed direct association of Myc and C/EBP proteins in MC29-transformed cells. C/EBPα is a multifunctional transcription factor that is involved in the specification of the differentiated phenotype through gene activation, but was recently also implicated in the acceleration of cell proliferation and activation of metastasis related genes (Heckman *et al*., 2003; Liu *et al*., 2004; Wang and Timchenko, 2005). Notably, functional interactions of Myc with the C/EBPβ protein have been reported previously (Mink *et al*., 1996; Hartl *et al*., 2003).

The protein product specified by the *WS5* gene displays the structural hallmarks of a transmembrane glycoprotein. In the extracellular segment, the WS5 protein contains a PKD domain, a protein module with a β-sandwich topology similar to the immunoglobulin-like fold, but defining a distinct PKD protein superfamily (Bycroft *et al*., 1999). It has been suggested that PKD domains of cell-surface proteins are involved in ligand-binding or cell–cell interactions (Bycroft *et al*., 1999, Jing *et al*., 2002). The amino acid sequence of quail WS5 shows the highest degree of identity (77%) with its presumed chicken homolog, the melanocyte-specific glycoprotein Mmp115 (Mochii *et al*., 1991). The mammalian protein displaying the highest degree of sequence identity (38%) to WS5 is encoded by the human *Pmel17* (or *SILV*) gene (Kwon *et al*., 1991). Although the melanocyte-specific type I transmembrane glycoprotein Pmel17 is known to have a central role in pigmentation, its precise function remains controversial (Theos *et al*., 2005). Pmel17 function is linked to melanosome biogenesis (Berson *et al*., 2003), and it was recently suggested that this gene has a critical role in normal mammalian development, with mutations leading to disease phenotypes (Clark *et al*., 2006). Furthermore, Pmel17 is overexpressed in human malignant melanoma at all stages of tumor progression, representing one of the most important melanoma antigens (Theos *et al*., 2005). The *Pmel17* gene is normally regulated by the melanocyte-specific bHLHZip microphthalmia-associated transcription factor (MITF) that has DNA-binding specificities similar to those of Myc-Max (Du *et al*., 2003). The WS5 protein also shows sequence identity (24%) to another human type I transmembrane glycoprotein, GPNMB (or osteoactivin) (Weterman *et al*., 1995), that is overexpressed in most metastatic melanoma cells, but also in cancers of the brain, breast, liver, stomach and pancreas. Furthermore, GPNMB was directly shown to be involved in tumor cell invasion and metastasis (Rich *et al*., 2003). Intriguingly, the presumed avian homolog of GPNMB, melanosomal glycoprotein QNR-71 displaying 25% sequence identity to WS5, had been isolated as a gene overexpressed in v-*myc*-transformed quail neuroretina cells (Turque *et al*., 1996). The identification and complete dissection of *WS5* as a direct transcriptional target of Myc displaying intrinsic oncogenic potential provides a link between the oncogenic properties of this transcription factor and a new type of target encoding a transmembrane protein.

Materials and methods

Cells and retroviruses

Cell culture and transformation of CEF or QEF was carrired out as described (Bister *et al*., 1977; Oberst *et al*., 1999; Hartl and Bister, 1995, 1998; Bader *et al*., 2000; Hartl *et al*., 2001). The following established lines of transformed QEF (Bister *et al*., 1977; Hartl *et al*., 2003) were used: VJ, transformed by ASV17 v-*jun*; Q8, transformed by MC29 v-*myc*; R(–)3, transformed by RSV v-*src*; and QT6, derived from a methylcholanthrene-induced fibrosarcoma. The quail cell lines Q/tM$_{ON}$ and Q/d3 conditionally transformed by doxycycline-dependent v-*myc* or v-*jun* alleles, and the retroviral construct RCAS-MC29 have been described (Oberst *et al*., 1999; Bader *et al*., 2000; Hartl *et al*., 2003). For construction of RCAS-WS5, a cDNA fragment containing the entire *WS5* coding region was ligated into the *Cla*I site of the RCAS-BP vector. DNA transfection and colony assays of transfected cells in 0.3%(w/v) agarose were done as described (Bister *et al*., 1977; Oberst *et al*., 1999; Hartl *et al*., 2001).

DNA cloning and nucleic acid analysis
cDNA and genomic DNA library construction, subtractive hybridization, DNA labeling, subcloning, Northern and Southern analyses, and nucleotide sequence analysis were performed as described (Hartl and Bister, 1995, 1998; Hartl *et al*., 2001, 2003). Screening of a Q8-specific cDNA library with the 919-bp insert of a clone isolated from a Q8-QEF subtractive cDNA library and encompassing the 3′-end of *WS5* cDNA (Oberst *et al*., 1999) led to the isolation of a 1398-bp 3′ cDNA clone. The full-length 2355-bp *WS5* cDNA was assembled from three overlapping 5′-RACE products and from the two cDNA clones described above. *WS5* genomic clones were isolated from a quail genomic DNA library as described (Hartl and Bister, 1998). Hybridization probes for Northern or Southern analyses (Hartl *et al*., 2003) included cDNA fragments of quail *WS5*, of chicken c-*myc*/MC29 v-*myc*, of chicken *C/EBPα* (provided by K-H Klempnauer), and of quail glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Protein analysis
In vitro transcription/translation reactions were done as described (Hartl *et al*., 2001). To construct pBS-WS5, the *Cla*I fragment of pA-WS5 containing the *WS5* coding region was inserted into the *Cla*I site of pBluescript II SK(+) (Stratagene Inc., Amsterdam, Netherlands). A 13mer peptide (NH_2-SGESSPLLRANAV-COOH) corresponding to the C-terminal residues of the WS5 protein was used to generate a rabbit polyclonal WS5-specific antiserum. Immunoprecipitations of [^{35}S]methionine-labeled proteins from cell lysates or from *in vitro* transcription/translation reactions, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were performed as described (Hartl *et al*., 2001, 2003). Co-immunoprecipitation of Myc-C/EBP protein complexes from lysates of [^{35}S]methionine-labeled cells using antisera directed against a C-terminal recombinant v-Myc protein (see below), or against recombinant C/EBPα or C/EBP*β* proteins (provided by K-H Klempnauer; Mink *et al*., 1999) was as described (Hartl *et al*., 2003).

Protein–DNA interaction analysis
EMSA was carried out as described (Hartl and Bister, 1998). A ^{32}P-labeled 56-bp double-stranded oligodeoxynucleotide corresponding to nucleotide positions −121 to −66 in the *WS5* promoter including the four E-boxes was used. A recombinant v-Myc protein (corresponding to the C-terminal residues 314–416 of chicken c-Myc), and a recombinant chicken Max protein (residues 22–113) were prepared as described (Fieber *et al*., 2001). For supershift analysis, the following antisera were used: α-Myc, directed against the C-terminal Myc recombinant protein; α-Max, directed against the chicken Max recombinant protein; and α-Myc-N, directed against a recombinant v-Myc protein (corresponding to the N-terminal residues 1–140 of chicken c-Myc).

ChIP was carried out as described (Hartl *et al*., 2006). The antisera α-Myc and α-Max (see above), α-Gag directed against avian retroviral Gag proteins (Hartl *et al*., 2001), and α-C/EBPα (Mink *et al*., 1999) were used. PCR was carried out with the primer pair 5′-CCGCAGCACCATCGCTGTGC-3′ (nt positions −238 through −219 in the *WS5* promoter) and 5′-GTGCTCCGACTCCGGGAGAGG-3′ (+35 through +15) flanking the Myc- and C/EBPα-binding sites.

Transactivation analysis
For construction of the CAT reporter plasmids pCAT-WS5 and pCAT-WS5-L, DNA fragments from the *WS5* promoter region with sizes of 276 and 1298 bp corresponding to nucleotides 1124–1399 and 102–1399 in the 6017-bp genomic *WS5* sequence (accession no. AY496440) were amplified by PCR and inserted into the *Hind*III/*Xba*I sites of the pCAT-Basic vector (Promega Inc., Mannheim, Germany). For construction of pCAT-WS5ΔMYC, a 38-bp fragment encompassing the four Myc-binding sites (nucleotides 1250–1287) was deleted from pCAT-WS5 by PCR mutagenesis using internal primers with complementary 5′-regions. Site-directed mutagenesis to delete the C/EBPα-binding site was performed as described (Hartl *et al*., 2001, 2003) using pCAT-WS5 or pCAT-WS5ΔMYC as templates and mutator oligodeoxynucleotides yielding pCAT-WS5ΔC/EBPα and pCAT-WS5ΔMYCΔC/EBPα, respectively. The pRc/RSV (Invitrogen Inc., Karlsruhe, Germany)-derived eukaryotic expression vector pRc-v-Myc containing the coding region of v-*myc* has been described (Hartl *et al*., 2003). To generate the expression vector pRc-C/EBPα, a 1419-bp *Eco*RI-*Xba*I fragment encompassing the coding region of the chicken *C/EBPα* gene was excised from the plasmid pcDNA3-chC/EBPα (Mink *et al*., 1999) and ligated into *Hind*III/*Xba*I-digested pRc/RSV. DNA transfer into cells by the calcium phosphate method, CAT-analysis, and quantification of radioactive signals were done as described (Hartl *et al*., 2001, 2003, 2006). DNA transfer into cells by the nucleofection technology (Amaxa Biosystems Inc., Cologne, Germany) was performed as described (Hartl *et al*., 2001, 2003). For nucleofections, liposome solution V was applied in combination with the electroporation programs T20 for gene transfer into transformed QEF (Q/MC29, QT6), or program A23 for transfer into normal QEF.

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References

Adhikary S, Eilers M. (2005). Transcriptional regulation and transformation by Myc proteins. *Nat Rev Mol Cell Biol* **6**: 635–645.

Bader AG, Hartl M, Bister K. (2000). Conditional cell transformation by doxycycline-controlled expression of the ASV17 v-*jun* allele. *Virology* **270**: 98–110.

Ben-Porath I, Yanuka O, Benvenisty N. (1999). The *Tmp* gene, encoding a membrane protein, is a c-Myc target with a tumorigenic activity. *Mol Cell Biol* **19**: 3529–3539.

Berson JF, Harper DC, Tenza D, Raposo G, Marks MS. (2001). Pmel17 initiates premelanosome morphogenesis within multivesicular bodies. *Mol Biol Cell* **12**: 3451–3464.

Berson JF, Theos AC, Harper DC, Tenza D, Raposo G, Marks MS. (2003). Proprotein convertase cleavage liberates a fibrillogenic fragment of a resident glycoprotein to initiate melanosome biogenesis. *J Cell Biol* **161**: 521–533.

Bister K, Hayman MJ, Vogt PK. (1977). Defectiveness of avian myelocytomatosis virus MC29: isolation of long-term nonproducer cultures and analysis of virus-specific polypeptide synthesis. *Virology* **82**: 431–448.

Bister K, Jansen HW. (1986). Oncogenes in retroviruses and cells: biochemistry and molecular genetics. *Adv Cancer Res* **47**: 99–188.

Blackwell TK, Huang J, Ma A, Kretzner L, Alt FW, Eisenman RN *et al*. (1993). Binding of Myc proteins to canonical and noncanonical DNA sequences. *Mol Cell Biol* **13**: 5216–5224.

Bycroft M, Bateman A, Clarke J, Hamill SJ, Sandford R, Thomas RL *et al*. (1999). The structure of a PKD domain from polycystin-1: implications for polycystic kidney disease. *EMBO J* **18**: 297–305.

Clark LA, Wahl JM, Rees CA, Murphy KE. (2006). Retrotransposon insertion in *SILV* is responsible for merle patterning of the domestic dog. *Proc Natl Acad Sci USA* **103**: 1376–1381.

Dang CV. (1999). c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol Cell Biol* **19**: 1–11.

Du J, Miller AJ, Widlund HR, Horstmann MA, Ramaswamy S, Fisher DE. (2003). MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. *Am J Pathol* **163**: 333–343.

Eisenman RN. (2001). Deconstructing Myc. *Genes Dev* **15**: 2023–2030.

Ferré-D'Amaré AR, Prendergast GC, Ziff EB, Burley SK. (1993). Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. *Nature* **363**: 38–46.

Fieber W, Schneider ML, Matt T, Kräutler B, Konrat R, Bister K. (2001). Structure, function, and dynamics of the dimerization and DNA binding domain of oncogenic transcription factor v-Myc. *J Mol Biol* **307**: 1395–1410.

Grabe N. (2002). AliBaba2: context specific identification of transcription factor binding sites. *In Silico Biol* **2**: S1–15.

Grandori C, Cowley SM, James LP, Eisenman RN. (2000). The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annu Rev Cell Dev Biol* **16**: 653–699.

Hartl M, Bister K. (1995). Specific activation in *jun*-transformed avian fibroblasts of a gene (*bkj*) related to the avian β-keratin gene family. *Proc Natl Acad Sci USA* **92**: 11731–11735.

Hartl M, Bister K. (1998). Structure and transcriptional regulation of *BKJ*, a novel AP-1 target gene activated during *jun*- or *fos*-induced fibroblast transformation. *Oncogene* **17**: 2901–2913.

Hartl M, Karagiannidis AI, Bister K. (2006). Cooperative cell transformation by Myc/Mil(Raf) involves induction of AP-1 and activation of genes implicated in cell motility and metastasis. *Oncogene* **25**: 4043–4055.

Hartl M, Matt T, Schüler W, Siemeister G, Kontaxis G, Kloiber K *et al*. (2003). Cell transformation by the v-*myc* oncogene abrogates c-Myc/Max-mediated suppression of a C/EBPβ-dependent lipocalin gene. *J Mol Biol* **333**: 33–46.

Hartl M, Reiter F, Bader AG, Castellazzi M, Bister K. (2001). *JAC*, a direct target of oncogenic transcription factor Jun, is involved in cell transformation and tumorigenesis. *Proc Natl Acad Sci USA* **98**: 13601–13606.

Heckman CA, Wheeler MA, Boxer LM. (2003). Regulation of Bcl-2 expression by C/EBP in t(14;18) lymphoma cells. *Oncogene* **22**: 7891–7899.

Hermeking H, Rago C, Schuhmacher M, Li Q, Barrett JF, Obaya AJ *et al*. (2000). Identification of *CDK4* as a target of c-MYC. *Proc Natl Acad Sci USA* **97**: 2229–2234.

Jing H, Takagi J, Liu JH, Lindgren S, Zhang RG, Joachimiak A *et al*. (2002). Archaeal surface layer proteins contain β propeller, PKD, and β helix domains and are related to metazoan cell surface proteins. *Structure* **10**: 1453–1464.

Kato GJ, Dang CV. (1992). Function of the c-Myc oncoprotein. *FASEB J* **6**: 3065–3072.

Kwon BS, Chintamaneni C, Kozak CA, Copeland NG, Gilbert DJ, Jenkins N *et al*. (1991). A melanocyte-specific gene, Pmel 17, maps near the silver coat color locus on mouse chromosome 10 and is in a syntenic region on human chromosome 12. *Proc Natl Acad Sci USA* **88**: 9228–9232.

Levens DL. (2003). Reconstructing MYC. *Genes Dev* **17**: 1071–1077.

Liu YN, Kang BB, Chen JH. (2004). Transcriptional regulation of human osteopontin promoter by C/EBPα and AML-1 in metastatic cancer cells. *Oncogene* **23**: 278–288.

Matys V, Fricke E, Geffers R, Gößling E, Haubrock M, Hehl R *et al*. (2003). TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res* **31**: 374–378.

Menssen A, Hermeking H. (2002). Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. *Proc Natl Acad Sci USA* **99**: 6274–6279.

Mink S, Mutschler B, Weiskirchen R, Bister K, Klempnauer KH. (1996). A novel function for Myc: inhibition of C/EBP-dependent gene activation. *Proc Natl Acad Sci USA* **93**: 6635–6640.

Mink S, Jaswal S, Burk O, Klempnauer KH. (1999). The v-Myb oncoprotein activates C/EBPβ expression by stimulating an autoregulatory loop at the C/EBPβ promoter. *Biochim Biophys Acta* **1447**: 175–184.

Mochii M, Agata K, Eguchi G. (1991). Complete sequence and expression of a cDNA encoding a chicken 115-kDa melanosomal matrix protein. *Pigment Cell Res* **4**: 41–47.

Nair SK, Burley SK. (2003). X-ray structures of Myc-Max and Mad-Max recognizing DNA: molecular bases of regulation by proto-oncogenic transcription factors. *Cell* **112**: 193–205.

Neiman PE, Ruddell A, Jasoni C, Loring G., Thomas SJ, Brandvold KA *et al*. (2001). Analysis of gene expression during *myc* oncogene-induced lymphomagenesis in the bursa of Fabricius. *Proc Natl Acad Sci USA* **98**: 6378–6383.

Nesbit CE, Tersak JM, Prochownik EV. (1999). *MYC* oncogenes and human neoplastic disease. *Oncogene* **18**: 3004–3016.

Oberst C, Hartl M, Weiskirchen R, Bister K. (1999). Conditional cell transformation by doxycycline-controlled expression of the MC29 v-*myc* allele. *Virology* **253**: 193–207.

O'Donnell KA, Yu D, Zeller KI, Kim JW, Racke F, Thomas-Tikhonenko A *et al*. (2006). Activation of transferrin receptor 1 by c-Myc enhances cellular proliferation and tumorigenesis. *Mol Cell Biol* **26**: 2373–2386.

Patel JH, Loboda AP, Showe MK, Showe LC, McMahon SB. (2004). Analysis of genomic targets reveals complex functions of MYC. *Nat Rev Cancer* **4**: 562–568.

Rich JN, Shi Q, Hjelmeland M, Cummings TJ, Kuan CT, Bigner DD *et al*. (2003). Bone-related genes expressed in advanced malignancies induce invasion and metastasis in a genetically defined human cancer model. *J Biol Chem* **278**: 15951–15957.

Secombe J, Pierce SB, Eisenman RN. (2004). Myc: A weapon of mass destruction. *Cell* **117**: 153–156.

Theos AC, Truschel ST, Raposo G, Marks MS. (2005). The *Silver* locus product Pmel17/gp100/Silv/ME20: controversial in name and in function. *Pigment Cell Res* **18**: 322–336.

Turque N, Denhez F, Martin P, Planque N, Bailly M, Bègue A *et al*. (1996). Characterization of a new melanocyte-specific gene (*QNR-71*) expressed in v-*myc*-transformed quail neuroretina. *EMBO J* **15**: 3338–3350.

Wang GL, Timchenko NA. (2005). Dephosphorylated C/EBPα accelerates cell proliferation through sequestering retinoblastoma protein. *Mol Cell Biol* **25**: 1325–1338.

Weterman MA, Ajubi N, van Dinter IM, Degen WG, van Muijen GN, Ruitter DJ *et al*. (1995). Nmb, a novel gene, is expressed in low-metastatic human melanoma cell lines and xenografts. *Int J Cancer* **60**: 73–81.

Zhang XY, DeSalle LM, Patel JH, Capobianco AJ, Yu D, Thomas-Tikhonenko A *et al*. (2005). Metastasis-associated protein 1 (MTA1) is an essential downstream effector of the c-MYC oncoprotein. *Proc Natl Acad Sci USA* **102**: 13968–13973.