MXD/MIZ1 transcription regulatory complexes activate the expression of MYC-repressed genes
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Received 13 March 2021, revised 31 March 2021, accepted 1 April 2021, available online 12 May 2021
doi:10.1002/1873-3468.14097
Edited by Ivan Sadowski

MXDs are transcription repressors that antagonize MYC-mediated gene activation. MYC, when associated with MIZ1, acts also as a repressor of a subset of genes, including p15 and p21. A role for MXDs in regulation of MYC-repressed genes is not known. We report that MXDs activate transcription of p15 and p21 in U2OS cells. This activation required DNA binding by MXDs and their interaction with MIZ1. MXD mutants deficient in MIZ1 binding interacted with the MYC-binding partner MAX and were active as repressors of MYC-activated genes but failed to activate MYC-repressed genes. Mutant MXDs with reduced DNA-binding affinity interacted with MAX and MIZ1 but neither repressed nor activated transcription. Our data show that MXDs and MYC have a reciprocally antagonistic potential to regulate transcription of target genes.

Keywords: MIZ1; MNT; MXD; MYC; ZBTB17

MYC-associated factor X (MAX) and its binding partners comprise a family of basic helix–loop–helix leucine zipper (bHLH-Zip) transcription factors, which are implicated in the regulation of cell growth, proliferation, differentiation, apoptosis, and tumorigenesis [1–3]. Complexes of MAX with MYC and its homologs MYCN and MYCL bind to enhancer box motifs (E-boxes) and promote the expression of target genes [1].

Under physiological conditions, MYC is expressed in response to mitogens and promotes cell growth and proliferation [2,4–7]. Elevated expression or activation of MYC is associated with uncontrolled cellular growth and proliferation and supports the development of cancer, and MYC or its homologues are over-expressed, amplified, or deregulated in many cancer types [8,9]. MYC has been reported to function as a regulator of specific target genes [10–13] and/or as a general amplifier of transcription of active genes on a genome-wide scale [14–17]. MYC interacts not only with several co-activators and RNA-polymerase II (Pol II)-associated factors including chromatin modifiers, transcription initiation, and elongation factors that are implicated in transcription activation, but also with several complexes and assemblies involved in transcriptional repression [3,10,14]. MYC has been shown to facilitate the release of promoter-proximally paused Pol II [18], enhance mRNA capping [19], facilitate the transfer of PAF1 to Pol II [15,20], and enhance rate and processivity of transcription elongation by loading SPT5 onto Pol II [14], and thereby support transcription of most active genes. In contrast, rapid depletion of MYC in leukemia and colon cancer cell lines affects transcription of a small subset of genes, suggesting that the expression of a rather limited set of activated genes might depend on the presence of MYC [11].

The activation of genes by MYC/MAX is antagonized by the MAX dimerization (MXD) proteins, MXD1–4, and MAX network transcriptional repressor (MNT), henceforth collectively referred to as MXDs, and by MGA [2]. MXDs and MGA are bHLH

Abbreviations
bHLH-Zip, basic helix–loop–helix leucine zipper; CDK, cyclin-dependent kinase; MAX, MYC-associated factor X; MXD, MAX dimerization.
transcription factors that form complexes with MAX and bind to the same E-boxes as MYC/MAX [1,2]. MXDs, via their SID domain, recruit mSin3-HDAC1/2 co-repressor complexes, and repress transcription [21,22].

In contrast to MYC, MXDs support cell cycle arrest and differentiation [23–25]. Genetic studies in mice confirmed the antagonism between MYC and MXDs. MXD1−/− mice show increased proliferation and delayed differentiation of granulocyte precursors [26]. Mice lacking MXD2/MXI1 display multiple histological abnormalities due to increased cell proliferation in several tissues, and are more susceptible to spontaneous and induced cancerogenesis [27]. Depletion of MNT triggers increased cell proliferation [28,29]. Mice bearing a deletion of Mnt in mammary glands develop spontaneous tumors with increased frequency, pheno-copying transgenic overexpression of MYC [30]. Finally, human MNT, MXD1, and MXD2 genes are located in regions that are frequently mutated in different cancer types [31–35].

MYC, in particular in oncogenic or overexpressed conditions, has also the potential to repress transcription. The underlying mechanisms are not fully understood, and a comprehensive set of bona fide MYC-repressed genes is not known. This is in part due to the fact that MYC supports cell growth and proliferation, and, thus, directly or indirectly promotes expression of all genes when compared to the transcription rates of resting cells. Hence, upon normalization, lower than average activation of genes may appear as relative repression even though the genes are actually activated [36]. However, MYC has been shown to interact with the zinc-finger transcription factor MIZ1 (ZBTB17) and the related transcription factors, SP1 and YY1 [3]. MIZ1 regulates embryonic development and differentiation [13,36–38]. MYC in association with MIZ1 has been shown to repress genes, including the cyclin-dependent kinase (CDK) inhibitor genes p15 (CDKN2B), p21 (CDKN1A), and p27 (CDKN1B) and the circadian transcription factor genes BMAL1 (ARNTL), CLOCK, and NPAS2 [13,39–42]. Mutations compromising the interaction of MYC with MIZ1 specifically affect the repressing but not the activating potential of MYC [39,43,44], indicating that MYC together with MIZ1 has the potential to directly repress transcription. In oncogenic conditions, overexpressed MYC may recruit MIZ1 to a larger number of genes and attenuate their transcription [13,36,45]. A low ratio of MYC versus MIZ1 occupancy [45] and/or low relative affinity of promoters for MYC [46] appear to correlate with repression of transcription, suggesting that the ratio of activating MYC/MAX versus repressing MYC/(MAX)/MIZ1 complex determines transcriptional outcome.

The development of lymphoma in mice is critically dependent on the interaction of MYC with MIZ1 [47]. When the MYC/MIZ1 interaction is challenged by mutation, the repressive capacity of MYC is decreased, its pro-proliferative functions are reduced, and self-renewal of stem cells is compromised [39,48].

In this study, we addressed specifically the question whether and how MXDs have the potential to impact expression of genes that are repressed by MYC together with MIZ1. Using U2OS cells, which endogenously express MXDs, we report the surprising observation that MNT, MXD1, and MXD2 activate transcription of specific MYC-repressed genes, in parallel to their known function as transcriptional repressors of MYC-activated genes. We show that the activation of transcription by MXDs relies on their physical interaction with MIZ1 and requires functional DNA-binding and co-repressor recruitment domains. We show that MXDs inhibit U2OS cell growth and proliferation through activation of MYC-repressed genes, and MXD-dependent activation of p21 was particularly crucial.

Materials and methods

Cell culture and transfections

U2OStx and HEK293 cells [39] were maintained in DMEM with 10% FBS and 1 × PenStrep. Cell culture reagents were obtained from Life Technologies (Thermo Fisher Scientific, Carlsbad, CA, USA) unless indicated differently. Inducible U2OStx cells overexpressing different transgenes were obtained by stable transfection with AhdI-linearized pcDNA4/TO vector using Xfect (Clontech, Mountain View, CA, USA). Resistant clones were selected with 50 µg·mL−1 hygromycin and 100 µg·mL−1 zeocin (InvivoGen, Toulouse, France) for 2 weeks and pooled together. For siRNA transfections, U2OS cells were seeded on 24- or 96-well plates and next day transfected with the siRNAs (sequences are given in Table S1) using Lipofectamine RNAiMAX reagent. Cells were kept in the siRNA transfection mix minimum 24 h before further applications. For luciferase reporter assays, HEK293 cells were transfected with the indicated plasmids using Lipofectamine 2000. Next day, luciferase expression was measured using Dual-Luciferase Reporter Assay (Promega, Walldorf, Germany) and an EnSpire Reader (Perkin Elmer, Waltham, MA, USA).

Plasmid constructs

6xEbox-luc, p15-luc, and p21-luc reporters were used previously [39]. Vectors containing the ORFs of MNT and MXD1 were kindly provided by B. Lüscher. The MXD2
ORF was amplified from U2OS cDNA. To produce inducible constructs, V5-tagged ORFs of MXDs were cloned in pcDNA4/TO vector. Subsequent L-D, RRDD, and ΔSΔD mutagenesis, respectively, was performed using DF-Pfu polymerase (Bioron, Römerberg, Germany). Cloning and mutagenesis primers are available upon request.

Bioluminescence measurements

For bioluminescence measurements, transgenic U2OStx cells, untreated or pretreated with siRNA, were transiently transfected with luciferase reporters using Xfect (Clontech). After 24 h, the growth medium was replaced with prewarmed luminescence medium (DMEM w/o Phenol Red (Cat. No. 21063-029) supplemented with 10% FBS, 1× Normocin (Invitrogen), and 0.125 µM luciferin (BioSynth Carbosynth, Compton, UK), 10 ng·mL⁻¹ doxycycline, or PBS) and plates were measured with an EnSpire Reader (Perkin Elmer).

Gene expression analysis

Total RNA from U2OStx cells was extracted with Tri Fast (GenEon, University Ouk, San Antonio, TX, USA), and cDNA synthesis was performed with Maxima First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). qPCR was performed on LightCycler 480 (Roche, Basel, Switzerland) using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and gene expression was quantified using a DΔCt method relative to GAPDH. Primer sequences are listed in Table S1.

ChIP

U2OStx cells overexpressing different transgenes were incubated with doxycycline for 24 h and then cross-linked in 1% formaldehyde for 10 min. Chromatin was prepared as described previously [39]. Sheared chromatin was incubated overnight at 4 °C with 40 µL of salmon sperm DNA-blocked anti-FLAG (A220; Sigma-Aldrich, Munich, Germany) and anti-V5 (A7345; Sigma-Aldrich) beads. Subsequently, precipitated chromatin was washed and recovered as previously described [39]. Samples were then analyzed by qPCR, and values were normalized to percentage of input. Primer sequences are listed in Table S1.

Cell confluence and fluorescent microscopy

For proliferation assays, siRNA-transfected or untreated transgenic U2OStx cells were seeded on transparent 96-well plates (~ 3000 cells per well) and next day induced with standard growth medium containing 10 ng·mL⁻¹ doxycycline or PBS for control. To quantify apoptosis, growth medium was supplemented with Caspase-3/7 Green Reagent (4440; Sartorius - Essen BioScience, Sartorius, Göttingen, Germany). Cell confluence and apoptosis were measured with an Incucyte ZOOM Reader (Sartorius - Essen BioScience, Sartorius) using in-built software.

Co-immunoprecipitation and western blotting

Protein lysates of U2OS cells were prepared by incubation with ice-cold lysis buffer [39] and subsequent sonication in the ultrasonic bath (Merek, Darmstadt, Germany) for 10 min. Precleared lysates (centrifugation at 16 000 g for 10 min at 4 °C) were boiled with 4× Laemmli buffer, separated using 12% SDS/PAGE, and transferred on nitrocellulose membranes. Membranes were decorated with anti-FLAG (1 : 5000, M2; Sigma-Aldrich), anti-V5 (1 : 5000, 46-0705; Life Technologies, Thermo Fisher Scientific, Carlsbad, CA, USA), and anti-Tubulin (1 : 1000, WA3) antibodies in TBS 5% milk at 4 °C overnight. Next day, membranes were incubated with respective HRP-conjugated secondary antibodies and exposed to X-ray films. For co-IP experiments, transfected HEK293 cells were collected and prepared as described above. Then, cell lysates (500 µg total protein) were incubated with 40 µL of PBS-washed anti-FLAG M2 beads (Sigma-Aldrich) at 4 °C overnight. Next day beads were washed three times with PBS supplemented with 500 mM NaCl and 1% Triton X-100, and precipitated proteins were eluted by boiling in 4× Laemmli buffer and loaded on 12% SDS/PAGE gels.

Results

Endogenous MXDs activate the p21 core promoter in U2OS cells

MXDs repress genes that are activated by MYC, but a role for MXDs in the regulation of genes that are repressed by MYC has not been investigated. Since MXDs belong, like MYC, to the bHLH-ZIP family (Fig. 1A), we analyzed available ENCODE ChIP-seq data of MXD2 (GSM935498), MNT (GSE91968), and MYC (GSM822286, GSM822301). As expected, the data of MXD2 (GSM935498), MNT (GSE91968), and MYC (GSM822286, GSM822301). As expected, the analysis revealed that MXD2 and MNT binding overlaps with MYC binding (Fig. 1B). MXD2 and MNT were also recruited to MIZ1 binding sites in genes that are repressed by MYC such as p21 and VAMP4 (Fig. 1B). A genome-wide comparison of the cistromes of MNT and MYC (encode data from MCF7 cells) and of MXD2 and MYC (encode data from HEla cells) with the cistrome of MIZ1 in U2OS cells [13] revealed a significant overlap of MNT and MXD2 with MYC and with MIZ1 binding sites (Fig. S1A). It should be noted that due to low sequence coverage we pooled binding sites of native MIZ1 from several ChIP-seq experiments published by Walz et al. Although the published ChIP-seq analyses are from
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Fig. 1. MXDs bind MIZ1/MYC sites and modulate MIZ1-dependent transcription. (A) Schematic of the domain structure of MXDs, MYC, and MIZ1. MXDs contain a central bHLH-Zip DNA-binding domain (bHLH) and an N-terminal SID domain. SID domains are short segments of ~20 aa required for the recruitment of mSIN3-HDAC co-repressor complexes. MYC contains a C-terminal bHLH domain and MYC boxes (MB) I, II, and III. MIZ1 contains an N-terminal BTB (POZ) dimerization domain and 13 Zn-finger domains. (B) SNHG15, VAMP4, and p21 (CDKN1A) loci with ChIP-seq signals of native MYC (GSM822286, GSM822301), MXD2 (GSM935498), and MNT (GSE91968) in HELA and MCF7 cells (based on data from ENCODE). (C) Relative expression (combined datasets GSM1632189, GSM1632191, GSM2341646, and GSM2341647 normalized to MYC) of MXDs, MIZ1, MAX, and MYC in U2OS cells quantified by RNA-seq [50,51]. It should be noted that MAX protein is about an order of magnitude more stable than MYC, and hence believed to be present in excess over its binding partners MYC and MXDs [61]. (D) The ratio of MXDs versus MYC rather than their levels determines p21-luc expression. Quantification of bioluminescence (24 h) from p21-luc reporter transfected in U2OSx cells pretreated with siRNAs against MXDs (MNT, MXD1, and MXD2), MYC, or MIZ1, as indicated. (E) MXDs support expression of p21-luc. Relative bioluminescence of p21-luc with and without induction of FLAG-MIZ1 (24 h) in U2OSx cells transfected with siRNAs against MXDs (MNT, MXD1, and MXD2) or negative siRNA (n = 3). Data are presented as mean ± SEM. *P < 0.05; one-way ANOVA with Bonferroni post-test.
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To analyze the impact of MXDs on MYC-repressed genes, we generated stable U2OS cells expressing V5-tagged MXD1, MXD2, and MNT under control of a doxycycline-inducible promoter (Fig. S3D). ChIP from these cells and from U2OS cells expressing MYC:V5 and FLAG:MIZ1, respectively, revealed that tagged MYC, MXDs, and MIZ1 bound to the promoter of the NCL gene, which is a MYC-activated gene, suggesting that the tagged TFs were functional in DNA binding (Fig. 2A). In agreement with the published ChIP-seq data shown in Fig. 1B, tagged MYC, MXDs, and MIZ1 were also recruited to the p21 promoter (Fig. 2A), which is repressed by MYC.

We have previously shown that doxycycline-induced overexpression of MYC supported the expression of a 6xEbox-luc reporter and inhibited the expression of p15-luc and p21-luc reporters [39]. Doxycycline-induced overexpression of MNT, MXD1, and MXD2 attenuated the expression of the 6xEbox-luc reporter, while they supported elevated expression of p15-luc and p21-luc reporters (Fig. 2B–D). Activation and repression of the reporters were strongest for MNT and rather moderate for MXD2, yet even these moderate increases in expression were significant and highly reproducible. Interestingly, MNT, MXD1, and MXD2 did not support elevated expression of p21-luc when MIZ1 was depleted by siRNA (Fig. 2D and Fig. S2A). These data indicate that the activation of the reporter required endogenous MIZ1.

In accordance with the reporter assays, MXDs also impacted the expression levels of endogenous genes. Thus, induction of MNT, MXD1, and MXD2 attenuated the expression of NCL and SNHG15, confirming the repressive role of MXDs on MYC-activated genes (Fig. S2B). In contrast, MNT, MXD1, and MXD2 supported expression of endogenous p15, p21, p27, and CEBPA (Fig. S2B). As observed with the gene reporters, MNT was the strongest activator under our conditions, while the activating potential of MXD2 was rather modest. The activation of p15 by MXDs was pronounced, while the activation of p27 was significant but rather small. Together, the reporter assays and the measurement of the expression levels of the corresponding endogenous genes indicate that overexpressed MXDs have the potential to activate the CDK inhibitor genes p15 and p21, and p27 as well as CEBPA in U2OS cells. The extent of activation by the individual MXDs differed between genes and was dependent on MIZ1. Furthermore, overexpression of ectopic MXDs and downregulation of endogenous MXDs, respectively, had the opposite effect on p21-luc expression. Thus, our data indicate that MXDs (endogenous and ectopic) have the potential to activate these MYC-repressed genes in U2OS cells under conditions where they are active as repressors of MYC-activated genes.

The above measurements were done with confluent U2OS cells, which were growing rather slowly. We
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chose these conditions to avoid or minimize potential indirect effects on gene expression that could be associated with MXD-dependent differences in cell number (due to growth or apoptosis). Colorimetric cell counting by WST-8 staining indicated that induction of MXDs had little impact on cell growth and viability under such conditions (Fig. S2C), and quantification of GAPDH expression from the entire cultures confirmed the results of the WST-8 assay (Fig. S2D).

Hence, the MXD-dependent increase in expression levels of p15, p21, and p27 (luc reporters and endogenous genes) was due to transcriptional regulation.

**MXDs interact with MIZ1 and directly activate MYC-repressed genes**

The bHLH domains of MYCs and MXDs are highly conserved (Fig. 3A) [55]. In order to analyze whether MXDs physically interact with MIZ1, we expressed in HEK293 cells V5-tagged versions of MXD1, MXD2, and MNT together with FLAG-tagged MIZ1. Pull-down assays revealed that FLAG-tagged MIZ1 formed complexes with MXD1, MXD2, and MNT, respectively (Fig. 3B and Fig. S3A). We then set out to generate MXD mutants that are compromised in their ability to interact with MIZ1. The interaction of MYC with MIZ1 is critically dependent on V393 and V394 in the bHLH domain of MYC [39,43]. A sequence comparison revealed that V393 is conserved in MYC proteins, while MXDs carry a leucine residue in the corresponding position (Fig. 3A). To assess whether MXDs interact with MIZ1 via a similar interface as MYC, we expressed in HEK293 cells V5-tagged versions of MNT, MXD1, and MXD2 with leucyl-to-aspartyl (L-D) substitutions in the position corresponding to V393 of MYC. Subsequent pull-down assays revealed that the mutation abolished the interaction of MNTL258D with MIZ1 (Fig. 3C). MXD1L95D and MXD2L106D were substantially compromised in their capacity to interact with MIZ1 (Fig. 3D and Fig. S3B). The potential of the mutant MXDs to bind MAX was not affected (Fig. S3C). The data suggest that MYC and MXDs interact with MIZ1 in corresponding manner.

We then generated U2OS cells expressing doxycycline-inducible L-D versions of MXDs (Fig. S3D). The capacity of MNTL258D, MXD1L95D, and MXD2L106D to repress 6xEbox-luc was not affected (Fig. 3E and Fig. S3E), indicating that the mutant proteins were functional repressors. However, the L-D versions of MXDs did not support elevated expression of p21-luc (Fig. 3F and Fig. S3F), suggesting that the capacity to activate this promoter was dependent on their interaction with MIZ1. Furthermore, MNTL258D, MXD1L95D, and MXD2L106D attenuated the expression of endogenous NCL and SNHG15 genes as efficiently as the corresponding WT proteins but showed reduced activation of p15, p21, p27, and CEBPA genes (compare Fig. S3G and Fig. S2B). Together, the data suggest that the activation of these MYC-repressed genes by MXDs was supported by their interaction with MIZ1. The data are not compatible with an indirect activation of transcription by sequestration of MAX and thereby relieving MYC-dependent repression.

**MYC and MXDs require DNA binding to modulate MIZ1 activity**

To test whether MYC and MXDs require an intact bHLH domain in order to associate with MIZ1 target genes, we replaced in the basic region of the DNA-binding domain two critical neighboring arginine residues (RR) by aspartyls (DD) (Fig. 3A). Recruitment of the corresponding mutants, MYCRR367DD, MNTRR232DD, MNTL258DD, MXD1RR68DD, and MXD2RR79DD, to MYC-activated and MYC-repressed genes was significantly reduced (Fig. 4A). However, the RR-to-DD substitutions in the DNA-binding domains of MYC and MXDs did not affect their ability to interact with MIZ1 in a pull-down assay (Fig. S4A).

Overexpression of MYCRR367DD in U2OS cells (Fig. S4B) neither activated 6xEbox-luc nor repressed p21-luc (Fig. 4B, upper panels) [39], suggesting that the DNA-binding domain of MYC is required for both activation and repression of genes. Similarly, RR-to-DD substitutions in the DNA-binding domains of MXDs impaired their potential to repress 6xEbox-
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luc and their capacity to activate p21-luc (Fig. 4B). These data suggest that the DNA-binding domains of MXDs contribute to the repression and activation of genes. Since the RR-to-DD substitutions did not affect the interaction of MXDs with MIZ1, the data also indicate that MXDs did not indirectly activate p21-luc by squelching MIZ1 away from MYC/MIZ-repressed promoters.

MXDs harbor a SID domain by which they recruit co-repressor complexes [21]. The SID domains of MXDs are short N-terminal segments of about 20 amino acid residues (aa). We deleted the SID domains of MNT (Δaa 2–16), MXD1 (Δaa 2–20), and MXD2 (Δaa 2–20) (Fig. 1A). It has been previously shown that the deletion of the SID domain does not affect the interaction of MXDs with MAX [56]. ChIP analysis revealed that binding of MXDΔSID mutants to the promoters of NCL and p21 was not compromised (Fig. 5A). Previous data [55] had shown that MXDΔSID mutants failed to repress MYC-activated genes. In agreement with these data, MXDΔSID mutants failed to repress the 6Ebox-luc reporter in HEK293 cells (Fig. 5B). MNTΔSID even activated 6Ebox-luc. Surprisingly, however, the ΔSID versions of MXDs were compromised in their ability to activate p21-luc (Fig. 5C), suggesting that the SID domain is also required for MIZ1-dependent transactivation of MYC-repressed genes.

MXDs inhibit cell growth in a MIZ1-dependent manner

Expression of MXDs reduces cell growth and proliferation in various cellular models [55,57,58]. Doxycycline-induced overexpression of MXDs also reduced proliferation of growing U2OS cells that were seeded at low density (Fig. 6A, upper panels). To analyze whether MXDs inhibit growth in MIZ1-dependent fashion, we overexpressed the MIZ1 interaction mutants, MNTL258D, MXD1L95D, and MXD2L106D, which are functional repressors of MYC-activated genes (see Fig. 3E and Fig. S3E,G). The capacity of MNTL258D, MXD1L95D, and MXD2L106D to inhibit cell growth and proliferation was severely blunted (Fig. 6A lower panels and Fig. S5A), suggesting that the inhibition of growth and proliferation of U2OS cells relies critically on the interaction of MXDs with MIZ1.

We then asked whether the reduced apparent kinetics of cell growth was due to an increase in apoptosis of U2OS cells or a reduction in cell cycle frequency. Cell cycle stage-specific FACS sorting of cells stained with propidium iodide revealed that the overexpression of MXDs reduced the fraction of cells in G2 and M phases, while the overexpression of L-D mutants of MXDs had no significant effect on the cell cycle (Fig. 6B). The measurement of caspase activity revealed that cells gradually started undergoing apoptosis only after about 60 h of growth, long after cells reached confluence (Fig. S5B). The overexpression of MXDs reduced apoptosis, while the overexpression of MYC increased apoptosis, as reported previously [59] (Fig. S5B). Together, the data demonstrate that MXDs inhibit proliferation of U2OS cells by inhibiting the cell cycle.

Finally, we addressed the role of MXDs in regulating growth of U2OS cells via p21. When p21 was depleted in growing U2OS cells, the growth rate did not further increase, suggesting that growth was limited by other factors. Overexpression of MXDs attenuated the proliferation of U2OS cells but failed to do so when MXD-induced accumulation of p21 was prevented by siRNA treatment (Fig. S5C,D). Hence, the induction and/or repression of other genes by MXDs could obviously not compensate for the lack of p21. These data indicate that the regulation of the CDK inhibitor gene, p21, is a major pathway by which MXDs and MIZ1 impact proliferation of U2OS cells.

Discussion

MXD proteins encompass a group of transcriptional repressors that antagonize the activation of genes by MYC [1]. The aim of this work was to investigate
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whether and how MXDs impact on genes that are not activated but repressed by MYC. We therefore focused specifically on the regulation of a selected group of gene promoters (mainly \( p15 \), \( p21 \), and \( p27 \)) that were previously shown by various means to be directly repressed by MYC in a MIZ1-dependent manner \([13,39–42,44]\), and we restricted our analyses to U2OS cells, which express MXDs and MYC at a functional level. We report the unexpected and surprising finding that MXDs activated transcription of this validated group of MYC-repressed genes. Thus, the antagonizing role of MXDs appears to extend to the genes that are repressed by MYC, that is, genes that are activated by MYC are repressed by MXDs, and genes that are repressed by MYC are activated by MXDs. Hence, just like MYC, also MXDs have the capacity to

\[ Eq. \text{Fig. 4. MYC and MXDs require DNA binding to regulate MIZ1 targets. (A) Binding of MYC and MXDs to DNA requires an intact bHLH domain. ChIP-qPCR of MYC, MNT, MXD1, and MXD2 (WT and RRDD versions) at promoters of } NCL \text{ and } p21 \text{ in U2OSx cells 24 h after DOX induction (} n = 3). *P < 0.05; Student’s t-test. (B) Transcription regulation by MYC and MXDs requires an intact bHLH domain. Bioluminescence of } p21\text{-luc and } 6\times\text{Ebox-luc reporters measured from U2OSx cells 24 h after DOX induction of WT and RRDD versions of MYC and MXDs (} n = 3). Ctrl: noninduced control. *P < 0.05; one-way ANOVA with Bonferroni post-test. Data are presented as mean ± SEM. \]

\[ Eq. \text{Fig. 5. MXDs require SID domains to activate and repress genes. (A) DNA binding of MXDs does not require SID domains. ChIP-qPCR analysis of showing that DOX-induced (24 h) WT and } \Delta\text{SID versions of MNT, MXD1, and MXD2 bind to } NCL \text{ and } p21 \text{ genes in U2OSx cells (} n = 3). (B, C) The SID domains of MXDs are required for repression of } 6\times\text{Ebox-luc} \text{ and activation of } p21\text{-luc. (B) Luciferase reporter assay of } 6\times\text{Ebox-luc} \text{ in HEK293 cells transfected with wild-type or } \Delta\text{SID MNT, MXD1, and MXD2 (} n = 3). (C) } p21\text{-luc} \text{ expression in HEK293 cells transfected with MIZ1 and co-transfected with wild-type or } \Delta\text{SID versions of MXDs (} n = 3). *P < 0.05; one-way ANOVA with Bonferroni post-test. Data are presented as mean ± SEM. \]
activate and repress genes in a context-specific manner. Since the activating function of MYC is antagonized by MXDs, it seems conceivable that also the repressing function of MYC requires regulatory counterbalance, in particular since MYC-repressed genes include crucial inhibitors of the cell cycle.

Several lines of evidence support that transcription of genes such as p15, p21, and p27 was directly activated by MXDs. Most importantly, MXDs are functionally expressed in U2OS cells and their downregulation resulted in elevated expression of the selected MYC-repressed genes. We controlled by WST-8 staining that the apparent induction of these MYC-repressed genes was not artificially due to differences in number and size of living cells (biomass) in MXD-induced versus control-treated cultures (e.g., by less apoptosis of MXD-induced cells).

In addition to the analysis of endogenous gene expression, we measured the impact of MXDs on luciferase reporter genes to assess the transcriptional control of these genes. The results are shown in Figure 6B, which illustrates the changes in cell cycle stage induced by MXD1 and MXD2 WT and L-D alleles. The data are presented as mean ± SEM.
regulation of the core promoters. The p21-luc reporter contains a short, truncated core promoter with binding sites for SP1, MIZ1, and MYC [41], and also for MXDs. Thus, analysis of p21-luc minimizes the potential impact of other transcription factors implicated in the complex regulation of the endogenous p21 gene. Induction of MXDs in confluent cultures of U2OS cells triggered an increase in p21-luc bioluminescence, while the number of viable cells did not increase relative to control cultures. Moreover, depletion of endogenous MXDs and overexpression of MXDs had the opposite effect, that is, reduced and elevated expression of p21-luc, respectively. Together, these data show that MXDs have the potential to activate in U2OS cell transcription of selected MYC-repressed genes.

We then analyzed the molecular basis underlying the activation of transcription by MXDs. These analyses were based on the functional comparison of overexpressed WT and mutant MXD versions defective in MIZ1 interaction, DNA binding, and co-repressor recruitment, respectively. In principle, overexpressed MXDs could induce MYC-repressed genes indirectly by sequestering MAX, and thereby reducing the levels of repressive MYC complexes. However, DNA-binding mutants and MIZ interaction mutants of MXDs interacted with MAX as efficiently as WT MXDs but did not induce MYC-repressed genes, which is not compatible with indirect gene activation by sequestration of MAX.

Potentially, MXDs could (via the canonical pathway) repress putative MYC-activated genes that encode (co)repressors of MYC-repressed genes, and thereby indirectly, that is, by repression of a (co)repressor, induce transcription. However, the MIZ interaction mutants of MXDs were active as repressors of MYC-activated genes but failed supporting expression of the selected MYC-repressed genes, excluding indirect activation via repression of a (co)repressor.

Together, our data suggest that MXDs activated MYC-repressed genes in a direct manner in addition to their function as repressors of MYC-activated genes. The dual function of MXDs is not unprecedented as MYC itself also acts as activator and repressor, and interacts via its MYC boxes with factors associated with transcription activation and repression [3,10,14,60]. The conditions and mechanisms specifying MYC as either (general) activator or repressor of particular genes are only partially understood.

MXDs repress MYC-activated genes by recruiting, via their short (~20 aa) N-terminal SID domains, mSIN3-HDAC co-repressor complexes [21], but MXDs do not contain known domains for the recruitment of co-activators. SID deletions do not affect the recruitment of MXDs to their target genes but compromised repression of 6xEbox-luc, and, surprisingly, also activation of p21-luc. The underlying mechanism remains obscure and awaits further investigation.

Together, these data indicate that MXDs (at normal and overexpressed levels in U2OS cells) have the potential to directly activate in a MIZ1-dependent fashion selected genes that are repressed by MYC/MIZ1. While MIZ1 may activate transcription by default, it is limiting in U2OS cells and its regulatory potential in the presence of endogenous levels of MXDs and MYC is determined by the functional ratio of MXDs versus MYC. In growing U2OS cells, MYC may dominate and hence MIZ1 is predominantly repressing, while in nongrowing U2OS cells MDXs may functionally dominate and MIZ1 is predominantly activating. The physiological relevance and contribution of MXDs to the expression of MYC-repressed genes in the context of a living organism are beyond the scope of this manuscript and remain to be investigated.

MYC and MXDs are regulators of cell proliferation. We show here that the overexpression of MXDs attenuated proliferation of growing U2OS cells (nonconfluent cultures), consistent with their reported role as antagonists of MYC, which stimulated proliferation under corresponding conditions. Mutant versions of MXDs with reduced affinity for MIZ1 were compromised in their capacity to inhibit cell proliferation, just as a corresponding MIZ1 interaction mutant of MYC was recently shown to be compromised in its capacity to stimulate cell proliferation [39,47,48]. Hence, at least in U2OS cells MXDs antagonized MYC’s pro-proliferative function predominantly in MIZ1-dependent manner via the regulation of MYC-repressed genes. The overexpression of MXDs failed to attenuate cell growth when the expression of p21 was suppressed by siRNA, indicating that regulation of p21 was of particular importance in U2OS cells.

In summary, our results reveal that transcription factors of the MXD family, which are characterized repressors of MYC-activated genes, show transcription-activating properties at genes that are repressed by MYC together with MIZ1. Thus, our results suggest that MXD proteins have the potential to antagonize activation and repression of genes by MYC.

Acknowledgements

We thank Bernhard Lüscher (RWTH Aachen University) for plasmid constructs and Bianca Ruppert and Maria Luisa Möller-Winheim for technical assistance. The work was supported by the Collaborative...
Research Centre TRR186 of the Deutsche Forschungsgemeinschaft (DFG).

Author contributions
AS performed the experiments, and GS did the bioinformatic analysis. AS, AD, and MB planned and designed the experiments and wrote the manuscript.

Data accessibility
The data that support the findings of this study are available in the figures and the supplementary material.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. (A) Venn diagram showing the overlap between native MYC (GSM82286 for HELA and GSM822301 for MCF7), MIZ1, and MNT (GSE91968) or MXD2 (GSM935498) binding sites in MCF7 and HELA cells, respectively. MIZ1 binding sites (GSM1088664, GSM1231600, GSM1231599) were pooled from several ChIP-seq experiments (Walz et al. 2014). (B) Downregulation of MXDs by siRNA. qPCR quantification of MNT, MXD1, and MXD2 transcripts in U2OStx cells transfected with the respective siRNAs (n = 3). Total RNA was isolated 24 h after siRNA transfection. Data are presented as mean ± SEM. * P < 0.05; Student’s t-test. (C) Western blot analysis of U2OStx cell lysates expressing DOX-induced FLAG:MIZ1.

Fig. S2. (A) qPCR quantification of MIZ1 mRNA in U2OStx cells transfected with siRNA against MIZ1 (n = 3). (B) Expression of MYC-activated and MYC-repressed genes (24h) determined by qPCR in U2OStx with DOX-induced MXDs (n = 3). (C) Growth rate of U2OStx cells expressing MNT, MXD1, and MXD2 determined by WST-8 assay. Cells were treated with DOX or PBS (Ctrl) for 24 h. (D) GAPDH mRNA abundance in U2OStx cells 24 hours after induction of MXDs. Data are presented as mean ± SEM. * P < 0.05; Student’s t-test.

Fig. S3. (A) Co-immunoprecipitation of V5-tagged MXD1 and MXD2 with FLAG-tagged MIZ1 from HEK293 lysates. (B) FLAG:MIZ1 pulldown and co-immunoprecipitation of WT and L-D versions of MXD1 and MXD2 expressed in HEK293 cells. MIZ1 and MXD2 were tagged with FLAG and V5 epitopes, respectively. (C) Co-immunoprecipitation of WT and L-D versions of MNT, MXD1, and MXD2 with MAX expressed in HEK293 cells. MXDs and MAX were tagged with V5 and FLAG epitopes, respectively. Ctrl: Flag-IPs were performed from cells transfected with only V5-tagged MXDs. cells. (D) WT and L-D mutants of MNT, MXD1, and MXD2 are expressed at similar levels. Western blot analysis of U2OStx cell lysates expressing DOX-induced WT or L-D versions of MXDs. Repression of 6x Ebox-luc (E) and activation of p21-luc (F) in U2OS cells overexpressing WT or L-D mutants of MXD1 and MXD2 after induction with DOX after 24 hours (n = 3). (G) qPCR quantification of MYC-activated and MYC-repressed genes...
(24 h) in U2OS cells expressing L-D versions of MXDs (n = 3). Data are presented as mean ± SEM. * P < 0.05; one-way ANOVA with Bonferroni post-test (E and F) and Student’s t-test (G).

**Fig. S4.** (A) Co-immunoprecipitation of WT and RRDD versions of V5 tagged MYC, MNT, MXD1, and MXD2 with FLAG:MIZ1 expressed in HEK293 cells. Ctrl: Flag-IPs were performed from cells expressing only indicated V5-tagged MXDs. (B) Western blot analysis of DOX-induced MYC, MNT, MXD1, and MXD2 in U2OS cells. Tubulin (TUB) is shown for control.

**Fig. S5.** (A) L-D versions of MXDs do not inhibit proliferation. Relative confluence (AUC) of U2OS cells expressing DOX-induced WT and L-D versions MXDs over several days is shown (n = 3). For raw data see Fig. 6A. (B) Normalized Caspase 3/7 activity (solid lines) and confluence (dashed lines) in U2OS cells expressing inducible MNT, MXD1, MXD2, and MYC. Cells, seeded at 50-60% confluency one day before the experiment, were treated with DOX or PBS (Ctrl) at the time point 0 and green fluorescence was measured using the Incucyte ZOOM system (n = 3). (C) qPCR analysis showing downregulation of p21 by siRNA in U2OS cells (n = 3). * P < 0.05; Student’s t-test. (D) Inhibition of cell proliferation by MIZ1 and MXDs requires p21. Growth curves of indicated transgenic cell lines treated with siRNA against p21 (n = 3). * P < 0.05; AUC comparison of DOX vs. Ctrl curves by two-way ANOVA with Bonferroni post-test. Data are presented as mean ± SEM.

**Table S1.** Primer sequences and siRNAs.