The Chromatin Remodeling Factor Mi-2α Acts as a Novel Co-activator for Human c-Myb*

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The c-Myb protein belongs to a group of early hematopoietic transcription factors that are important for progenitor generation and proliferation. These factors have been hypothesized to participate in establishing chromatin patterns specific for hematopoietic genes. In a two-hybrid screening we identified the chromatin remodeling factor Mi-2α as an interaction partner for human c-Myb. The main interacting domains were mapped to the N-terminal region of Mi-2α and the DNA-binding domain of c-Myb. Surprisingly, functional analysis revealed that Mi-2α, previously studied as a subunit in the NuRD co-repressor complex, enhanced c-Myb-dependent reporter activity. Consistently, knock-down of endogenous Mi-2α in c-Myb-expressing K562 cells, led to down-regulation of the c-Myb target genes NMU and ADA. When wild-type and helicase-dead Mi-2α were compared, the Myb-Mi-2α co-activation appeared to be independent of the ATPase/DNA helicase activity of Mi-2α. The rationale for the unexpected co-activator function seems to lie in a dual function of Mi-2α, by which this factor is able to repress transcription in a helicase-dependent and active in a helicase-independent fashion, as revealed by Gal4-tethering experiments. Interestingly, desumoylation of c-Myb potentiated the Myb-Mi-2α transactivational co-operation, as did co-transfection with p300.

The c-Myb transcription factor plays a central role in the regulation of cell growth and differentiation, in particular in hematopoietic progenitor cells (1). Studies in mice have shown that c-Myb is essential for normal hematopoiesis to take place (2). The transcription factor controls crucial steps at several key stages of hematopoiesis and/or histone tails and/or remodeling nucleosomes, which influence the chromatin dynamics. Conflicting evidence exists regarding the composition of the NuRD complex (17), probably reflecting its heterogeneity as well as its tissue and target specificity. In short, the consensus NuRD complex consists of the core subunits: HDAC1 and CHD3 was originally identified as an autoantigen in the human connective tissue disease, dermatomyositis (14, 15). This protein and the highly related Mi-2β/CHD4 belong to the CHD family (16). Mi-2α and -β are integral components of the NuRD (nucleosome remodeling and histone deacetylase) co-repressor complex and responsible for its chromatin remodeling activity. Mi-2α/CHD3 is originally identified as an autoantigen in the human connective tissue disease, dermatomyositis (14, 15). This protein and the highly related Mi-2β/CHD4 belong to the CHD family (16). Mi-2α and -β are integral components of the NuRD (nucleosome remodeling and histone deacetylase) co-repressor complex and responsible for its chromatin remodeling activity. Conflicting evidence exists regarding the composition of the NuRD complex (17), probably reflecting its heterogeneity as well as its tissue and target specificity. In short, the consensus NuRD complex consists of the core subunits: HDAC1 and CHD3 was originally identified as an autoantigen in the human connective tissue disease, dermatomyositis (14, 15). This protein and the highly related Mi-2β/CHD4 belong to the CHD family (16). Mi-2α and -β are integral components of the NuRD (nucleosome remodeling and histone deacetylase) co-repressor complex and responsible for its chromatin remodeling activity. Conflicting evidence exists regarding the composition of the NuRD complex (17), probably reflecting its heterogeneity as well as its tissue and target specificity. In short, the consensus NuRD complex consists of the core subunits: HDAC1 and -2, RbAp46 and -48, Mi-2α and/or -β (18–20), one or more MTA proteins (MTA1, -2, or -3, or splice variants of these) (21), MBD2 or MBD3 (22), and often p66S and/or -β (23).

In the present work, we report the identification of Mi-2α as an interaction partner of human c-Myb. The association has been confirmed by several interaction assays. We also provide evidence for a dual function of Mi-2α. Depending on context,
Mi-2α is able to repress transcription in a helicase-dependent fashion and activate in a helicase-independent fashion, as revealed by Gal4-tethering experiments. In a c-Myb context, the latter dominates, causing Mi-2α to act as a co-activator of c-Myb, as seen both in transfection experiments and after knock-down of Mi-2α. Desumoylation of c-Myb as well as co-transfection with p300 further potentiate the Myb-Mi-2α transactivation co-operation.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The low-copy number bait plasmid pDBT-hcM was used for expression of full-length human c-Myb in the two-hybrid screening (10). Different regions of c-MYB (designated DBD, TAD, FAETL, TP, EVES, TAC, FAC, and TPC; Table 1 and Fig. 2A) were amplified by PCR and inserted into pGEX-6P-2 (GE Healthcare) or pGEX-KG (24) for expression of GST fusion proteins. The pGEX-2TK-SUMO-1 was kindly provided by Prof. G. Del Sal.

The expression plasmid for full-length FLAG-tagged Mi-2α (pCIneoB-3FLAG-Mi2α) was made from three partial Mi-2α cDNA clones (hZEH clones 37-1 and 37-8 (25), covering the cDNA of Mi-2α from bp 945 to bp 6005, relative to ATG and a commercially bought construct pUC57-NTD-Mi-2α (GeneScript) covering the cDNA of Mi-2α from bp 1 to bp 974 relative to ATG) and the vector pCIneoB-3FLAG (unpublished construct; pCIneo from Promega). The mammalian expression plasmid for FLAG-tagged N-terminal domain (amino acid residues 1–315) of Mi-2α (pCIneoB-3FLAG-Mi2α-NTD) was made by subcloning the region encoding the N-terminal Mi-2α (GeneScript) from pUC57-NTD-Mi-2α into pCIneoB-3FLAG. To generate a plasmid for expression of the C-terminal domain, (c-MYB interaction domain) in mammalian cells (pCIneoB-3FLAG-Mi2α-CTD), the region encoding the amino acid residues 1655–2000, was amplified by PCR and inserted into pCIneo-3FLAG. The plasmid encoding the Mi-2α helicase-dead mutant K767A was generated using the QuikChange Site-directed Mutagenesis kit (Stratagene) on a subfragment of human Mi-2α.

The mammalian expression vectors pCIneo-hcM-HA and pCIneo-hcM-HA-2KR (encoding wild type and sumoylation-deficient c-Myb, respectively) have been described (10). The pCIneo-hcM-SUMO-1 was constructed by PCR amplification and modification of SUMO-1 from the appropriate IMAGE clone, followed by subcloning in-frame into pCIneo-hcM-HA (between PshAI and Sall), losing the HA tag, but gaining SUMO-1 in the expressed product.

The p300 expressing mammalian vector pCMVβ-NHA-p300 is a kind gift from Prof. David Livingston and has been described previously (26), while the PML IV-expressing vector was received as a kind gift from Prof. G. Del Sal (27).

The mammalian expression vectors for Gal4-DBD fused to Mi-2α wild-type and K767A were made by PCR amplification of Gal4-DBD from the yeast vector pDBT (28) and subcloning into pCIneo (Promega), generating pCIneo-GBD2. Thereafter, Mi-2α wild-type and K767A were cloned in-frame into pCIneo-GBD2 yielding pCIneo-GBD2-Mi-2α and pCIneo-GBD2-Mi2α-K767A. The Gal4-tethering constructs pCIneo-GBD2-Mi2α-NTD and pCIneo-GBD1-Mi2α-NTD encoding amino acid residues 1–315 and 316–2000, respectively, were made from pCIneo-GBD2-Mi2α by removing the PshAI-NotI fragment, filling in the gaps with Klenow and religating (Mi2α-NTD), or by subcloning the PshAI-BamHI fragment into pCIneo-GBD1 (alternative MCS) between Smal and BamHI (Mi2α-ΔNTD). pCIneo-hcM-VP16–2KR was made by PCR amplifying the region encoding the VP16 transactivation domain (TAD) from pBD11 described previously (29) and cloning it in-frame into pCIneo-hcM-HA-2KR, loosing the HA tag, but gaining VP16 TAD in the expressed product. The TRHR reporter, pGL2b-TRHR-1250 covering the area −1250 to +1 from the thyrotropin-releasing hormone receptor promoter has been described (30). The reporter plasmid harboring the RAG-2 (−279 to +419) promoter was kindly provided by Prof. R. J. Aguilera (31). The SNRPN-driven Gal4 responsive luciferase reporter was made by PCR amplification of the SNRPN promoter (−942 to −426) from genomic human DNA. This promoter region was subcloned into pcG3 Basic (E1751; Promega). Thereafter, the 5×Gal4 responsive element (5×GRE) region was PCR-amplified from pGAL-tk (kind gift from Prof. U. Moens) and subcloned into pGL3b-SNRPN generating pGL3b-5GRE-SNRPN. The SV-40-driven Gal4 responsive luciferase reporter was made by PCR amplification of the SV-40 promoter from pGL3 promoter vector (E1761; Promega) and subcloning into pGL3b-5GRE-SNRPN between Nhel and HindIII. This concomitantly removed the SNRPN promoter.

All cloned fragments generated by PCR or by oligo-insertion were verified by sequencing. Primer sequences are available upon request.

**Two-hybrid Screening**—The yeast two-hybrid screening has been described (10). Proteins interacting with full-length human c-Myb were identified by growth on -Histidine plates from 10-cm dish with 5 mM 3-aminotriazole (3-AT). The interaction between the Mi-2α (amino acid residues 1655–2000) and c-Myb was confirmed by retransformation, controlling reporter activation and assaying for β-galactosidase activity in a liquid β-Gal assay.

**Protein Expression**—GST and GST fusion proteins were expressed in *Escherichia coli* as previously described (32). The regions of human c-Myb expressed, spanned the amino acid residues given in Table 1. Some of the proteins (GST-TAD, GST-FAETL, GST-TAC, and GST-FAC) came in the insoluble fraction and had to be solubilized from the pellet with urea. To renature the proteins, the urea was removed by dialysis.

**GST Pulldown Assays**—GST fusion proteins prebound to glutathione–Sepharose beads (GE Healthcare) were incubated for 1 h at 4°C with total cell extract from a 10-cm dish with transfected COS-1 cells lysed in 300 μl of F-buffer (20 mM Hepes pH 7.4, 10% glycerol, 0.2% Triton X-100, 50 mM NaCl, 30 mM Na3P04, 50 mM NaF, 2 mM iodoacetate, 5 μM ZnCl2, pH adjusted to 7.10 Complete Protease Inhibitor mixture, Roche Applied Science). Beads were washed three times in 500 μl of F-buffer, and the proteins eluted in 20 μl of 3× SDS loading buffer. Proteins were separated by SDS-PAGE and detected with immunoblotting.

**Cell Culture and Transfection, Luciferase Assays and Immunoblotting**—COS-1, CV-1, and HD11 cells were grown as described (33, 34). All three cell lines were transiently transfected with the indicated plasmids using FuGENE6 (Roche).
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Applied Science). For the luciferase assays, transfected CV-1 cells were harvested 24 h after transfection and lysed in Passive Lysis Buffer (Promega). Luciferase assays were performed in triplicate (24 well trays; 2 × 10^4 cells/well) using Luciferase Assay Reagent (Promega) and data from at least three independent transfection experiments are presented. In parallel transfected CV-1 cells were lysed in Nonidet P-40 buffer and subjected to immunoblotting.

**Immunoprecipitation**—Transfected COS-1 or CV-1 cells (15-cm dishes; 2.25 × 10^6 cells/dish; 1 dish per IP) were harvested 24 h after transfection in 300 μl of Nonidet P-40 buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris pH 8.0, Complete Protease Inhibitor mixture), debris was removed by centrifugation and the cleared lysate was diluted 1:1 in 50 mM Tris, pH 8.0. Then 500 μl of diluted lysate (75 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris, pH 8.0) was subjected to immunoprecipitation with indicated antibodies and protein G-Sepharose beads. Immunoprecipitation was performed on a roller at 4 °C overnight. The beads were washed three times in 300 μl of wash buffer (75 mM NaCl, 0.5% Nonidet P-40, 50 mM Tris, pH 8.0), and the proteins eluted in 70 μl Laemmli buffer with 5% SDS for 10 min at 65 °C. Proteins were separated by SDS-PAGE and detected with immunoblotting.

**Antibodies**—As immunoprecipitation antibodies we used anti-FLAG M2 monoclonal antibody (F3165, Sigma), anti-GST (27-4577-01, GE Healthcare) and protein G-Sepharose 4 Fast Flow (17-0618-01, GE Healthcare). For immunoblot detection the following antibodies were used: the murine Myb 5e11 antibody (35), anti-FLAG (described above), mouse anti-human p300 (33-7600, Zymed Laboratories Inc.), anti-PML, (H238; sc-5621, Santa Cruz Biotechnology), anti-GAPDH-HRP (H86504P Biodesign, International), anti-mouse IgG-HRP (NA931, GE Healthcare) and anti-rabbit IgG-HRP (NA934, GE Healthcare).

**RNA Interference**—K562 cells were cultured in RPMI 1640 with GlutaMAX™ (Invitrogen) to a density of ~6 × 10^5 cells/ml. 1 × 10^6 cells were then nucleofected using the Cell Line Nucleofector™ Kit V (Amamax) and the Amaxa Nucleofector (program T-16), following the online protocol optimized for K562 nucleofection (Amaxa). The following modifications were made: After nucleofection the cells were directly transferred to 5 ml of preheated medium. The suspension was then centrifuged at 125 × g, 5 min to remove the medium. The cells were then grown in 5 ml of RPMI 1640 with GlutaMAX™ for 24 h before subjected to RNA isolation and immunoblotting. The K562 cells were either mock-transfected or transfected with siRNA at a final concentration of 2 μM. A pool of three Mi-2α siRNAs (sICHD3; ID 9877, 216241, 216242; 16708A, Ambion) was used for specific knock-down of Mi-2α, while siCONTROL Non-Targeting siRNA (d-001210-02-05, Dharmacon) was used as control.

**RNA Isolation and Quantitative Real Time PCR**—Total RNA was extracted from K562 cells using TRIzol Reagent (Invitrogen) followed by purification with RNeasy (Qiagen). For HD11 cells total RNA was extracted using Absolutely RNA™ RT-PCR Miniprep kit (Stratagene). Total RNA (1–2 μg) was reverse-transcribed using SuperScript™ III (Invitrogen), and the resulting cDNA was diluted 1:5, 1:50, 1:500, and 1:5000 prior to real-time PCR on a LightCycler (Roche Applied Science). Reactions were performed in 20-μl volumes with 0.5 μM primers and LightCycler DNA MasterPlus SYBR Green kit (Roche Applied Science). The PCR conditions were optimized for annealing at 58–60 °C for 5 s and elongation at 72 °C for 10–14 s. The efficiency of each primer pair was calculated from the slope of the standard curve (E = −1/slope), and each standard curve was developed using the LightCycler Data File Editor (Roche Applied Science). The quantification was performed using the second derivative maximum method, and the amount of each target gene was calculated relative to the reference genes HPRT (HD11) or the POLR2A (K562). Primer sequences are available upon request.

**FIGURE 1. Isolation of Mi-2α as an interacting protein for human c-Myb.** The C-terminal region of Mi-2α encoded by the library plasmid was isolated in a yeast two-hybrid screening with full-length human c-Myb as bait. The c-Myb-Mi-2α interaction was verified as shown. A, empty library vector (pACT2) and plasmids encoding the C-terminal domain of Mi-2α fused to Gal4-AD (pACT2-Mi2α-CTD) were transformed into the yeast two-hybrid strain PJ69–4a. Similarly, empty bait vector (pDBT), and full-length human c-Myb fused to Gal4-DBD (pDBT-hcM) were transformed into the same strain of opposite mating type (PJ69–4a). Mating was performed to make the diploid combinations indicated in the figure. These were subjected to X-gal overlay assay to reveal activation of the LacZ reporter gene as blue color. B, yeast cells were transformed and mated as in A, as indicated in the figure. The activity of the LacZ reporter was measured by a liquid β-galactosidase assay. The results are shown as mean values ± S.E. of four independent experiments.
RESULTS

Isolation of Mi-2α as a c-Myb-interacting Protein—To identify novel gene products that interact with c-Myb, we used full-length human c-Myb as bait in a yeast two-hybrid screening of a mixed cDNA library (human bone marrow and human erythroleukemia cell line K562) as described previously (10). Among the positively interacting clones, we identified a sequence identical to the C-terminal region of the human zinc finger helicase (hZFH) mRNA (GenBankTM accession no. U91543), encoding a Snf2-like helicase. The hZFH cDNA was by alignment and comparison with genomic sequences found to be identical to the C-terminal region of the human zinc finger helicase (hZFH) mRNA (GenBankTM accession no. U91543), encoding a Snf2-like helicase. The hZFH cDNA was by alignment and comparison with genomic sequences found to be identical to the human chromodomian helicase DNA-binding protein 3 (CHD3) (GenBankTM accession no. AF006515), except for a 102-bp insert present in the C-terminal part of hZFH and a slightly extended C terminus. The clone we identified started in the 102-bp insert, which has been shown to be an optional exon slightly extended C terminus. The clone we identified started in the 102-bp insert present in the C-terminal part of hZFH and a

Mapping of the Mi-2α-interacting Domain of c-Myb—To map the Mi-2α-interacting domain in c-Myb, we performed GST pulldown experiments. From partial clones and gene synthesis, we assembled a cDNA encoding full-length Mi-2α (Fig. 2A; "Experimental Procedures"). Lysates from COS-1 cells expressing 3FLAG-Mi-2α were then analyzed for binding to a series of different GST-Myb fusion proteins (Table 1 and Fig. 2A). Strong binding was detected to GST-DBD and weaker interaction was seen with GST-FAETL, GST-TAC, and GST-FAC, the latter having in common the leucine-rich c-Myb interaction was seen with GST-FAETL, GST-TAC, and GST-FAC, the latter having in common the leucine-rich c-Myb binding domain and the FAETL region of c-Myb. To further investigate which part(s) of Mi-2α that binds to c-Myb, we used the C-terminal c-Myb-interacting domain of Mi-2α (amino acid residues 1655–2000), identified in the two-hybrid screening and an N-terminal clone, used in the full-length assembly (amino acid residues 1–315), and compared those to full-length Mi-2α. All three constructs were FLAG-tagged and lysates from COS-1 cells were analyzed for binding to a subset of GST-Myb fusion proteins (Fig. 2C). Comparison of the three Mi-2α constructs showed that the N-terminal domain of Mi-2α bound GST-DBD, while the C-terminal

Table 1

| c-Myb regions cloned into GST expression vectors and expressed as GST fusion proteins |
|---------------------------------|-----|-----|
| Abbreviation | Start | End |
| DBD | 1 | 192 |
| TAD | 259 | 337 |
| FAETL | 352 | 417 |
| TP | 410 | 526 |
| EVES | 489 | 566 |
| TAC | 259 | 640 |
| FAC | 352 | 640 |
| TPC | 410 | 640 |
domain mainly bound to GST-FAETL. Hence, c-Myb and Mi-2α seem to have two interaction surfaces; one linking c-Myb DBD and the N-terminal region of Mi-2α, and another linking the C-terminal region of Mi-2α with the FAETL region of c-Myb, with the former interaction being the strongest.

**c-Myb and Mi-2α Interact in Vivo**—Association between human c-Myb and Mi-2α was then tested by co-immunoprecipitation in lysates from transfected COS-1 cells. A band of 75 kDa corresponding to c-Myb was detected with a Myb specific monoclonal antibody (5e11) after immunoprecipitation with anti-FLAG antibodies (reactive toward 3FLAG-Mi-2α). Only background levels of c-Myb were detected when Mi-2α was not present, or when immunoprecipitating with irrelevant antibodies (Fig. 3A).

The interaction between full-length Mi-2α and full-length c-Myb was further validated in a functional context by performing mammalian two-hybrid analysis in CV-1 cells (Fig. 3B). Gal4-DBD fused Mi-2α repressed the transcription of luciferase about 30% from an SNRPN-driven Gal4-responsive reporter construct, but activated the reporter to 215% upon co-transfection with c-Myb fused to VP-16 transactivation domain (Fig. 3B). We preferred to use the more active SUMO-conjugation negative mutant of c-Myb (designated 2KR, harboring the K503R and K527R mutations) in these experiments to get a stronger readout. The two mutated amino acid residues lie outside the interaction surface determined above. These experiments verify that c-Myb interacts with Mi-2α in vivo.

To study the interaction at the endogenous level, we searched extensively for antibodies specific to Mi-2α. Seven different commercially available antibodies were tested, but unfortunately, five of them did not pick up any Mi-2 at all, one detected only Mi-2β, and one did not discriminate between Mi-2α and Mi-2β. This left us without the necessary tools to perform endogenous CoIP and ChIP.

**Co-expression of Mi-2α Enhances c-Myb-dependent Reporter Activation in a Helicase-independent Fashion**—We expected the Mi-2α protein to exert its function as part of the NuRD complex, which possesses both chromatin remodeling and histone deacetylase activity and is involved in repression of gene expression (17). To examine how Mi-2α influences c-Myb-dependent transactivation, we performed effector-reporter assays in CV-1 cells using the c-Myb responsive TRHR promoter (30). Contrary to our expectations, co-expression of increasing amounts of Mi-2α resulted in a gradual increase of c-Myb dependent reporter activation, causing a 3-fold enhancement of c-Myb activity (Fig. 4). These results were also verified on another reporter harboring the RAG-2 promoter (data not shown).

Because Mi-2α is a protein with enzymatic activity we reasoned that its co-regulator function would be tightly linked to its ATPase/helicase activity. Hence, we introduced the classical lysine to alanine mutation in the conserved helicase domain (36). We then carefully compared the wild-type and the K767A mutant of Mi-2α in the effector-reporter assay in CV-1 cells, using the c-Myb responsive TRHR promoter. Notably, we found that Mi-2α K767A enhanced the Myb-dependent reporter activation to exactly the same extent as did Mi-2α.
wild-type (Fig. 5) suggesting that its co-activation effect on c-Myb in fact is independent of its helicase activity.

It might be argued that these observations are only artifacts associated with transiently transfected reporter-activation, given that the activity of a remodeling factor can only be properly observed on a fully chromatinized target. To directly test this, we analyzed how Mi-2α influenced c-Myb-dependent transcriptional activity in a more physiological setting, using an established model for Myb-dependent activation of a chromatin-embedded gene. This model monitors activation of the resident mim-1 gene in the chicken macrophage cell line HD11, which expresses the c-Myb collaborating factor C/EBPβ/NF-M, but not c-Myb (37, 38). When assayed by real-time PCR, co-expression of c-Myb and Mi-2α resulted in a similar increase in c-Myb-dependent activation of the resident mim-1 gene (Fig. 6) as was seen in the TRHR transfection assay (Fig. 5). Moreover, the helicase-dead mutant enhanced c-Myb activation to the same extent (or slightly more) compared with wild-type Mi-2α. This strongly suggests that the Mi-2α-induced enhancement of c-Myb activation is not restricted to transiently transfected reporters, but is equally well operating on a chromatin embedded target gene.

These experiments also allowed us to exclude the more trivial explanation that the activation might be caused by a general enhancement of all transcriptional activity by the helicase activity introduced, because Mi-2α appeared to have little or no effect by itself on the background expression levels (Figs. 5 and 6).

A final possibility was that Mi-2α could display both activating and repressive activities depending on its context as some reports seem to indicate, and that c-Myb exploits the first rather than the latter property. To address this hypothesis we used an approach where Mi-2α in a wild-type and a helicase-dead form were fused to Gal4-DBD. This allowed us to observe how activation of a reporter gene is affected by the recruitment of Mi-2α variants directly to its promoter (Fig. 7A). Hence, we expressed Gal4-DBD-Mi-2α wild-type and K767A fusions together with a luciferase-reporter construct driven by the constitutively active SNRPN promoter into which multiple Gal4-responsive elements had been inserted (similar to (39)). As expected, Mi-2α wild-type repressed the transcription of the luciferase reporter as compared with the Gal4-DBD control (Fig. 7A). This repression was relieved by the helicase-dead Mi-2α, which in fact activated at higher concentration, as can be seen in Fig. 7A. To support these findings, the experiments were repeated on an SV-40-driven Gal4-responsive reporter. Also on this reporter the same reciprocal relationship was observed, with a repressive effect of the wild-type Mi-2α and reporter activation with the helicase-dead mutant (data not shown), indicating that the dual behavior cannot be attributed to a particularity of the SNRPN promoter.

Mi-2β has previously been shown to have an activating domain in the N terminus (N-term-PHD: amino acid residues 1–360) (40). To examine if the same activating domain could be found in Mi-2α, we made deletion constructs, fused to Gal4-DBD, and tested them on the SV-40-driven Gal4-responsive reporter. The Mi-2α-NTD (amino acid residues 1–315) activated the reporter (Fig. 7B), and was indeed twice as active as the full-length, helicase-dead mutant (data not shown). When deleting the N-terminal region, the reporter output was repressed as in the case of wild type Mi-2α. Taken together, these data support the notion of Mi-2α harboring two activities;
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A

![Graph A](image)

B

![Graph B](image)

FIGURE 7. Mi-2α displays a dual function depending on its helicase activity. A, CV-1 cells were transfected with 0.2 μg (gray bars) or 0.4 μg (black bars) Gal4-DBD fused to full-length Mi-2α wild-type or K767A (helicase-dead). The reporter output from the SNRPN-driven Gal4-responsive reporter plasmid (0.2 μg) was normalized to the effect of Gal4-DBD, which was set to 100. B, CV-1 cells were transfected with 0.1 μg of (gray bars) or 0.2 μg of (black bars) Gal4-DBD fused to wild-type, full-length Mi-2α, Mi-2α-NTD (amino acid residues 1-315), or Mi-2α-DNTD (amino acid residues 316-2000). The reporter output from the UV-40-driven Gal4-responsive reporter plasmid (0.2 μg) was normalized to the effect of Gal4-DBD, which was set to 100. The results are presented as relative luciferase units (RLU). The results represent the mean RLU ± S.E. of at least three independent assays performed in triplicates.

one repressive, helicase-dependent and one activating helicase-independent, with the latter activity probably localized at the N-terminal domain. In the Gal4-tethering experiment with wild-type Mi-2α, the repressive function dominates, and the activation function is first disclosed when the helicase activity is eliminated by mutation or deletion.

Having found evidence for dual activities in Mi-2α, it appears that in the context of c-Myb-dependent activation, the activating function of Mi-2α dominates, as no difference was seen between wild-type and helicase-dead mutant. We conclude that Mi-2α in two experimental settings behaves as a co-activator for c-Myb and that the helicase activity is dispensable for this function of Mi-2α.

Mi-2α Activates the Expression of Endogenous c-Myb Target Genes in Hematopoietic Cells—The implication of our findings is that Mi-2α seems to be a conditional co-regulator, able to co-repress in the context of the NRD complex and to co-activate transcription together with c-Myb. The latter implies that at least some endogenous c-Myb target genes should be affected by removal of Mi-2α. To directly test this, we specifically knocked down Mi-2α in c-Myb expressing human erythroleukemia K562 cells, and monitored the expression of a set of established c-Myb target genes. The cells were nucleofected with a pool of three siRNAs directed against Mi-2α, or a control siRNA (siCONTROL), and then grown for 24 h. The specific siRNAs gave a clear knock-down of Mi-2α, to ~25% of the normal mRNA levels (Fig. 8A). Furthermore, there was no effect of the control siRNA (Fig. 8A). Because Williams and co-workers (41) had shown a compensatory up-regulation of Mi-2α in Mi-2β knock-out mice, we paid particular attention to the Mi-2β expression in these experiments. However, neither Mi-2β (Fig. 8A) nor c-Myb levels (Fig. 8B) changed in response to the Mi-2α RNA interference. Hence, we were confident that the down-regulation of Mi-2α was both effective and specific.

When examining the expression of c-Myb target genes, we focused on three previously reported targets: NMIU, MYC, and ADA (42–44), which we have verified respond to c-Myb knockdown in K562 cells.3 As shown in Fig. 8C, two of three targets did respond to the Mi-2α knock-down with detectable reduced mRNA levels. The mRNA expression of neuromedin U (NMIU) and adenosine deaminase (ADA) were both reduced, while the MYC mRNA levels remained unchanged (Fig. 8C). Taken together, this supports the hypothesis that Mi-2α is able to co-activate the transcription of endogenous, bona fide c-Myb target genes. However, the results also underline the context dependence of this co-activation since we observed a clear promoter-to-promoter variation. Given the level of Mi-2α knock-down and the fact that many Myb-controlled promoters are under complex regulation, such a context dependence is not unexpected.

Desumoylation of c-Myb Potentiates the Myb-Mi-2α Trans-activational Co-operation—We have previously established that c-Myb is sumoylated in its negative regulatory region (NRD; K503 and K527), and that this makes an important contribution to the NRD negative influence on c-Myb transactivational properties (10). Even though Mi-2α does not bind to NRD (GST-TPC; see Fig. 2B), conformational changes, recruitment of co-repressors, or relocation of c-Myb as a result of sumoylation could potentially influence the Myb-Mi-2α interaction. We therefore studied the functional consequence of c-Myb sumoylation on Myb-Mi-2α transactivational co-operation. Effector-reporter assays were performed in CV-1 cells using the c-Myb responsive TRHR promoter, co-transfcting c-Myb wild-type, c-Myb 2KR (mutated in both SUMO-acceptor lysines (10), or c-Myb-SUMO-1 (SUMO-1 fused C-termi-

3 E. M. Brendeford, unpublished results.
whereas the PML-SUMO-1 association used as positive control, showed strong interaction. This indicates that Mi-2α does not interact with SUMO-1, which supports the notion that Mi-2α co-activation is exerted primarily on non-sumoylated c-Myb.

**Mi-2α Enhances c-Myb-p300 Transactivation Activity**—CBP and p300 are well established co-activators of c-Myb (48–50). Therefore, the physical interaction between p300, Mi-2β, and HEB on the CD4 promoter reported by Williams et al. (41) inspired us to check if Mi-2α and p300 were able to cooperate in Myb-dependent reporter activation. As can be seen in Fig. 10 co-transfection of c-Myb and increasing amounts of p300 resulted in the expected gradual increase of c-Myb dependent reporter activation, from 3.6-fold in the absence to 14.1-fold in the presence of p300, which is only slightly higher (~50%) than what was seen for Mi-2α (Fig. 4). Complementing the c-Myb-p300 co-transfection with a small input of Mi-2α caused the c-Myb-dependent reporter activation to increase even more (to 20.6-fold; Fig. 10), implying that Mi-2α is able to enhance the c-Myb-p300 transactivational activity. To further support the idea that Mi-2α and p300 cooperate and interact with c-Myb, enhancing its transactivational potential, we tried to pull down the tripartite complex from COS-1 transiently transfected with c-Myb, Mi-2α, and p300. However, we were only able to detect minor amounts of p300 in the Mi-2α precipitate (data not shown). We therefore do not believe that c-Myb in solution forms a strong complex with both co-activators at the same time. This does not, however, exclude that they may functionally co-operate on Myb responsive promoters. It is also noteworthy that the Mi-2α-c-Myb interaction seemed to be as strong in the presence as in the absence of overexpressed p300 (data not shown).

**DISCUSSION**

In this work we have identified the helicase Mi-2α as a novel c-Myb-interacting protein. Mi-2α mainly interacts with c-Myb via its N-terminal domain binding to the DBD of c-Myb, while an additional contact is formed between Mi-2α C-terminal region and the leucine-rich FAETL region of c-Myb. This suggests a complex interaction surface. In addition to GST pull-down experiments used for mapping, co-immunoprecipitation, and mammalian two-hybrid experiments in transfected cells showed good affinity between c-Myb and Mi-2α. We also employed confocal imaging to try to visualize the co-localization between c-Myb and Mi-2α. However, the uniform distribution of Mi-2α observed in the nucleoplasm, as opposed to
Myb, which stains both punctual and uniformly (27), precluded any conclusions to be drawn from these experiments (results not shown).

What could be the functional implication of c-Myb interacting with Mi-2α? Our first obvious hypothesis was recruitment of the NuRD complex by c-Myb to repress selected target genes. The NuRD complex possesses both chromatin remodeling and histone deacetylase activity and has been suggested to be involved in gene repression, acting both globally and on specific genes (17). A role for NuRD in specific gene regulation comes from reports on NuRD recruitment by specific factors, such as p53, Hunchback, KAP-1, and FOG-1 (reviewed in Ref. 51) (20). All these examples are associated with gene repression. A more promiscuous recruitment of the NuRD complex to chromatin is governed by the MBDs, where MBD2 was reported to direct MBD2/NuRD to CpG islands in a methylation-sensitive way, promoting methylation of arginines in histone tails through co-recruitment of PRMT5 (22).

We addressed the interaction between c-Myb and other components of the NuRD complex by performing various co-immunoprecipitation experiments both in transfected cells and with endogenous factors in c-Myb expressing, human hematopoietic Jurkat and K562 cells, using antibodies to detect NuRD subunits such as HDAC1, RbAp46, and MTA2. However, we could only detect very weak interactions (results not shown). This suggests that recruitment of Mi-2α by c-Myb does not necessarily imply recruitment of other NuRD subunits and implies a possible NuRD-independent function of Mi-2α.

A key observation was that Mi-2α enhanced c-Myb-dependent activation in all functional analyses we performed (Figs. 4–6). It therefore appears that c-Myb does not interact with Mi-2α in order to recruit a repressive complex, but rather cooperate with Mi-2α as a co-activator. This conclusion required that other more trivial explanations were excluded, one of them...
being the possibility of Mi-2α stabilizing c-Myb, thereby causing enhanced reporter activation. However, co-transfection of c-Myb with Mi-2α did not seem to have any influence on the c-Myb protein level (Fig. 9B). We also excluded that the co-activation observed was restricted to transiently transfected reporters, which may not be fully chromatinized and hence not suited to monitor the action of a chromatin-remodeling factor. Use of an endogenous, chromatinized c-Myb target (Fig. 6) showed the same co-activation with c-Myb and Mi-2α as observed with transfected plasmid reporters (Fig. 4). Because Mi-2α had no influence on the basal expression level in either system, a trivial global enhancement of transcription, caused by the chromatin remodeling activity of Mi-2α could also be excluded. Finally, specific knock-down of Mi-2α in c-Myb-expressing K562 cells, resulted in down-regulation of the bona fide c-Myb target genes Neuromedin U (42) and ADA (44) (Fig. 8), consistent with Mi-2α functioning as a co-activator at the endogenous level, enhancing the transcription of specific c-Myb target genes.

An evident possibility considered was that the chromatin remodeling activity of Mi-2α is exploited by c-Myb to open promoters leading to more efficient reporter activation. In principle, increased fluidity of chromatin may help both activating and repressive processes. A similar mechanism has been suggested for the Ikaros-NuRD complex, which is able to potentiate gene expression (52, 53). However, when comparing wild-type and helicase-dead Mi-2α, the Myb-Mi-2α co-activation seemed to be independent of Mi-2α helicase activity (Figs. 5 and 6). This suggests a different mechanism dependent on other properties of Mi-2α than its helicase activity. Whether the Ikaros-NuRD co-activation requires an active helicase, is not known.

A key experiment in our mechanistic analysis was the Gal4 tethering assays showing that Mi-2α wild-type, when overexpressed and tethered to two different promoters, is able to repress the reporter transcription and that this repression is highly helicase-dependent (Fig. 7). These observations directly suggest a dual function of the Mi-2α protein, being able to repress in a helicase-dependent fashion and to activate in a helicase-independent fashion. The first activity is what is expected from Mi-2α being associated with NuRD function. The second activational function is novel and implicates an intrinsic transactivation function in the protein, which might be able to recruit other co-activators. Interestingly, NuRD components have been found associated with co-activator protein complex subunits (54, 55), suggesting involvement in a switch-mechanism between transcriptional activation and repression. Whether Mi-2α plays a role in gene activation has to date been unknown. However, several reports have shown that the parologue Mi-2β has both transcriptional activating as well as repressive activity (40, 41, 56). These observations as well as evidence for NuRD complexes of variable composition (21), including conflicting evidence regarding the exact Mi-2 composition of NuRD complexes (17), make us believe that the concept of Mi-2 proteins as being only the helicase components of a single NuRD complex with repressive function is too simplistic.

One interesting finding in this context is the identification of an intrinsic transactivation domain in Mi-2β. Shimono and co-workers (40) have shown that when the N-terminal part of Mi-2β (N-term-PHD: amino acid residues 1–360) was tethered to a Gal4-responsive promoter, this domain activated more than 10-fold, while all other Mi-2β domains as well as the protein as a whole repressed the reporter. We therefore performed a similar Gal4 tethering experiment with Mi-2α and concluded that also this family member harbors an activation function located in the N-terminal domain (Fig. 7B; amino acid residues 1–315). This is an obvious candidate for the transactivation function disclosed in the helicase mutant. Shimono and co-workers also showed that BRG1, a SWI/SNF component, interacts with this domain in a co-activating fashion. Noteworthy, it is the same domain in Mi-2α that shows the highest affinity for c-Myb (Fig. 2C; Mi-2α amino acid residues 1–315). In fact, this domain alone was enough to enhance the c-Myb-dependent reporter activation to a level comparable to that of full-length Mi-2α (supplemental Fig. S1 and Fig. 4), indicating that the N-terminal part of both Mi-2α and β might be responsible for their helicase-independent activation function. Curiously, there is a putative HMG-box within this common transactivating domain of the Mi-2s (amino acid residues 165–221 in Mi-2α; Fig. 2A), which might stabilize c-Myb binding to DNA in vivo.

A functional role for Mi-2α in transcriptional activation is also consistent with recent data suggesting Mi-2α to have properties as a histone code reader because of its PHD finger showing preferential binding to trimethylated histone H3K36 (57). Methylated H3K36 is found enriched in active chromatin, where H3K36me3 generally accumulates toward the 3′-region of transcribed genes (58). Particularly intriguing is the fact that the DBD of c-Myb associates with exactly the same tail region of histone H3 (amino acid residues 27–42) (59). Future work will show whether the two regulators meet in this tail region and how they mechanistically co-operate.

Given the dual function of Mi-2α there must be mechanisms determining whether repression or activation should dominate. It would be interesting to assay the helicase activity of Mi-2α in the presence and absence of c-Myb, to monitor whether this activity is inhibited in the complex. Another possibility is that the c-Myb-Mi-2α complex, through a composite interaction surface, recruits co-activators leading to the domination of the activation function.

Closely related to this, is the finding of the tripartite complex HEB:Mi-2β:p300 that is able to bind to the CD4 enhancer region in T-cells, increasing histone H3 acetylation and enhancing CD4 gene transcription (41). p300/CBP is a well established c-Myb co-activator, which is required for a functional hematopoiesis (48–50). We show in this paper that p300 potentiates the Myb-Mi-2α transactivational co-operation. However, we were not able to detect any physical interaction between p300 and Mi-2α or c-Myb:Mi-2α (data not shown). It is possible that p300 and Mi-2α cooperate on c-Myb responsive promoters without physically interacting, or there might be other co-activator(s) in the Mi-2α protein interaction repertoire that are recruited to c-Myb via Mi-2α.

Finally, we addressed whether we could see a link between SUMO-conjugation of c-Myb and the action of Mi-2α, particularly because co-repressor recruitment is a main concept in
current thinking on the negative SUMO-function in transcription (46). No SUMO binding was found for Mi-2α. In functional assays, we observed the strongest co-activation of Mi-2α in the absence of c-Myb SUMO-conjugation, which indirectly supports our conclusion that Mi-2α in association with c-Myb rather operates as a co-activator.

It is noteworthy that Mi-2α has been reported to interact with two master transcriptional regulators of the mammalian hematopoietic system, Ikaros and Aiolos (60). The finding that c-Myb, another transcription factor important for gene regulation in hematopoietic cells, also associates with Mi-2α, suggests a central role for this helicase in hematopoietic gene regulation. Having found this novel link between c-Myb and Mi-2α, further in-depth mechanistic studies are needed to shed light on Mi-2α co-activation properties and their role in assisting c-Myb in its hematopoietic functions.

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