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# Identification of SUMO-Dependent Chromatin-Associated Transcriptional Repression Components by a Genome-wide RNAi Screen

Bastian Stielow,[1,3] Alexandra Sapetschnig,[1,3,4] Imme Krüger,[1] Natascha Kunert,[1] Alexander Brehm,[1] Michael Boutros,[2] and Guntram Suske[1,*]
[1]Institute of Molecular Biology and Tumor Research, Philipps-University of Marburg, Emil-Mannkopff-Strasse 2, D-35032 Marburg, Germany
[2]German Cancer Research Center, Boveri-Group Signaling and Functional Genomics, Im Neuenheimer Feld 580, D-69120 Heidelberg, Germany
[3]These authors contributed equally to this work.
[4]Present address: Wellcome Trust/Cancer Research UK Gurdon Institute, University of Cambridge, The Henry Wellcome Building of Cancer and Developmental Biology, Tennis Court Road, Cambridge CB2 1QN, UK.
*Correspondence: suske@imt.uni-marburg.de
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## SUMMARY

**SUMO modification of many transcription factors is linked to transcriptional repression. The molecular mechanisms by which SUMO attachment represses transcription are largely unknown. Here we report a genome-wide RNA interference screen in *Drosophila melanogaster* cells for components regulating and mediating SUMO-dependent transcriptional repression. Analysis of >21,000 double-stranded RNAs (dsRNAs) identified 120 genes whose dsRNA-mediated knockdowns impaired SUMO-dependent transcriptional repression. Several of these genes encode chromatin-associated proteins, including the ATP-dependent chromatin remodeler Mi-2, the *D. melanogaster* ortholog of the *C. elegans* protein MEP-1, and the polycomb protein Sfmbt. Knockdown of these proteins did not impair SUMO conjugation, demonstrating that they act downstream of SUMO attachment. Biochemical analyses revealed that MEP-1, Mi-2, and Sfmbt interact with each other, bind to SUMO, and are recruited to promoters in a SUMOylation-dependent manner. Our results suggest that MEP-1, Mi-2, and Sfmbt are part of a common repression complex established by DNA-bound SUMO-modified transcription factors.**

## INTRODUCTION

The small ubiquitin-related modifier (SUMO) is reversibly attached to lysine residues of a still-growing number of proteins (Gill, 2004; Hay, 2005; Watts, 2004). SUMO conjugation to target proteins involves a cascade of enzymatic activities composed of the heterodimeric SUMO-activating E1 enzyme (Aos1/Uba2), the SUMO-conjugating E2 enzyme (Ubc9), and a SUMO E3 ligase that increases the efficiency of SUMO conjugation (Johnson, 2004). Modification of proteins by SUMO is reversible, and SUMO-specific isopeptidases catalyze removal of SUMO conjugates from their substrates (Gong and Yeh, 2006; Melchior et al., 2003).

SUMO and the conjugation pathway are highly conserved in eukaryotes including budding yeast (Johnson and Gupta, 2001; Shih et al., 2002), nematodes (Jones et al., 2002), flies (Bhaskar et al., 2002; Bhaskar et al., 2000; Shih et al., 2002), and vertebrates. Four different SUMO proteins have been identified in mammals. In contrast, invertebrates have a less-complex SUMOylation system with a single SUMO species.

The majority of SUMO-modified proteins are transcriptional regulators, including promoter-specific transcription factors, cofactors, and chromatin-modifying enzymes (reviewed in Gill, 2005; Hay, 2005; Seeler and Dejean, 2003). In most cases, post-translational modification by SUMO is linked to transcriptional repression, since removal of the SUMO moiety by mutation of the SUMO acceptor lysine residue increases the transcription factor activity. Examples are steroid hormone receptors (Holmstrom et al., 2003; Nishida and Yasuda, 2002), Lef1 (Sachdev et al., 2001), C/EBPs (Kim et al., 2002; Subramanian et al., 2003), Elk-1 (Yang et al., 2003), and Sp3 (Ross et al., 2002; Sapetschnig et al., 2002, 2004).

The molecular mechanisms by which SUMO attachment regulates transcription are largely unknown. In some cases, it has been suggested that SUMOylation alters the subnuclear localization of the SUMO target protein. For instance, accumulation in PML nuclear bodies has been reported for a number of SUMO-modified transcription factors, including Lef1 (Sachdev et al., 2001). Another model proposes that SUMO-modified transcriptional regulators recruit corepressor molecules to promoters that in turn induce changes in chromatin structure leading to transcriptional repression (Hay, 2005). In line with such a model, the recruitment of histone deacetylases (Girdwood et al., 2003; Yang and Sharrocks, 2004) and the corepressor Daxx (Chang et al., 2005; Kuo et al., 2005; Lin et al., 2004) to SUMO-modified transcription factors has been reported. However, other proteins might contribute to SUMO-dependent inhibition of transcription.

Advances in getting mechanistic insights into SUMO-mediated transcriptional repression are mainly hampered by several limitations. (1) Usually only a very small portion of a given

transcription factor is SUMOylated, while the mutation of the SUMO acceptor site often has a dramatic effect on transcription factor activity. This suggests a transient SUMOylation event that ultimately leads to alterations at the chromatin level, which are maintained by other factors. (2) Due to the absence of specific SUMO protease inhibitors, unbiased biochemical approaches to identify proteins associated with SUMOylated transcription factors have failed. Unspecific protease inhibitors such as NEM modify cysteine residues also in all other proteins, thereby disturbing the structure and very likely also the molecular and biological function of a corepressor. (3) Yeast two-hybrid screens for SUMO-interacting proteins would be biased toward identifying strong direct interactors, which would not necessarily represent the putative situation in the living cell.

To circumvent these limitations, we have performed a genome-wide RNA interference screen in cultured *Drosophila* cells for components mediating SUMO-dependent transcriptional repression. Using the transcription factor Sp3 as a paradigm, we identified a broad range of genes involved in SUMO conjugation and SUMO-mediated transcriptional repression. Several of these genes encode proteins that are linked to transcriptional repression, including MEP-1, the chromatin remodeler Mi-2, and the polycomb protein Sfmbt. All three proteins interact with each other as well as with SUMO in vitro. Coimmunoprecipitation experiments also revealed robust association of endogenous MEP-1 and Mi-2 in vivo. Furthermore, we demonstrate SUMOylation-dependent recruitment of MEP-1, Mi-2, and Sfmbt to a stably integrated transgene. Finally, mammalian orthologs of *Drosophila* Mi-2 and Sfmbt, namely mouse Mi-2 and L3mbtl2, were found to be present at the endogenous *Dhfr* promoter in wild-type MEFs, but not in $Sp3^{-/-}$ MEFs. Our studies strongly suggest that *Drosophila* MEP-1, Mi-2, and Sfmbt, as well as their mammalian orthologs, directly mediate SUMOylation-dependent transcriptional repression, likely by promoting the formation of repressive chromatin structures.

## RESULTS

### A Genome-wide RNA Interference Approach to Identify SUMO-Dependent Transcriptional Repression Components

In order to identify SUMO-dependent corepressor components, we designed a genome-wide RNA interference screen in *Drosophila melanogaster* cells using the transcription factor Sp3 as a paradigm (Figure 1A), whose activity is strongly controlled by SUMO modification. In mammalian as well as in insect cells, SUMO-modified wild-type Sp3 is almost inactive, whereas SUMOylation-deficient Sp3 mutants act as strong activators (see the Supplemental Data, Figure S1, available online) (Dennig et al., 1996; Sapetschnig et al., 2002, 2004). We hypothesized that transfection of wild-type Sp3 along with a reporter construct does not lead to activation, because a corepressor complex is recruited to SUMO-modified Sp3. By RNA interference, one could target proteins involved in SUMO conjugation and SUMO-dependent transcriptional repression leading to reporter gene activation (Figure 1A).

A sensitive SUMOylation-dependent reporter system was generated by fusing two Sp3-binding sites and a TATA box to the firefly luciferase gene, hereinafter referred to as (GC)2-FLuc. Upon transfection of *Drosophila* cells with (GC)2-FLuc and an expression plasmid for Sp3, no induction of the reporter gene is observed. Repression is due to posttranslational modification of Sp3 by *D. melanogaster* SUMO (also designated Smt3) at lysine 551 since the Sp3 K551R mutant strongly induces the reporter activity in a situation in which wild-type Sp3 and the SUMOylation-deficient K551R mutant are expressed at a similar level (Sapetschnig et al., 2002, 2004).

As proof of principle, we examined whether the knockdown of *D. melanogaster* SUMO would induce activation by wild-type Sp3. Incubation of $Kc_{167}$ cells with a dsRNA targeting SUMO resulted in strongly increased wild-type Sp3 activity, whereas the activity of the SUMOylation-deficient Sp3 K551R mutant was barely affected (Figure 1B). dsRNAs targeting GFP or *D. melanogaster* ubiquitin (*Ubi63p*) did not relieve repression. Thus, the firefly reporter activity reflects impaired SUMO modification of wild-type Sp3.

### Genome-wide RNAi Screen and Data Analysis

We performed a genome-wide RNAi screen using a dsRNA library in 384-well plates consisting of more than 21,000 dsRNAs targeting approximately 91% of the annotated *D. melanogaster* transcripts (Figure 1C). Normalized firefly/*Renilla* ratios were calculated, and potential candidate genes were assigned based on their deviation from the plate median for each given plate. The original screening results are available online at http://www.imt.uni-marburg.de/suske/SUMO_data.xls. Plots showing the experimental variation of the screen are depicted in Figures 1D and 1E. As a threshold for primary candidate selection, we used the lowest normalized average firefly value obtained with a dsRNA probe targeting SUMO and applied additional stringent selection criteria (see the Experimental Procedures for details) yielding 265 dsRNAs. A scatter plot of these dsRNAs is depicted in Figure 1F.

To further minimize potential false positives, several rescreens were performed (Figure 2A), including a rescreen with another reporter construct containing five Sp3 binding sites and an expression vector for the small isoform of Sp3 (Sp3si) lacking the N-terminal transactivation domain (Sapetschnig et al., 2004). In addition, candidate genes were also assayed with the SUMOylation-deficient Sp3si K551D mutant to exclude those proteins that act negatively on Sp3 independently of SUMO modification. These additional control experiments resulted in 120 strong candidates for regulating the SUMO conjugation pathway or conferring SUMO-dependent transcriptional repression (Supplemental Data, Table S1).

Based on predicted molecular and biological functions, protein domains, and reports from the literature, we assigned proteins to functional groups (Figure 2B and Table S1), such as signaling pathway components (34), transcriptional regulators (23), translational regulators (5), metabolic enzymes (18), structural proteins (2), and transporter molecules (8). One fourth of the proteins (30) could not be assigned into any of these categories due to the lack of biochemical and biological information.

Among the genes identified in the screen, *lesswright* encoding the *D. melanogaster* SUMO-conjugating E2 enzyme Ubc9 is present, confirming the effectiveness of the screen. Yet another

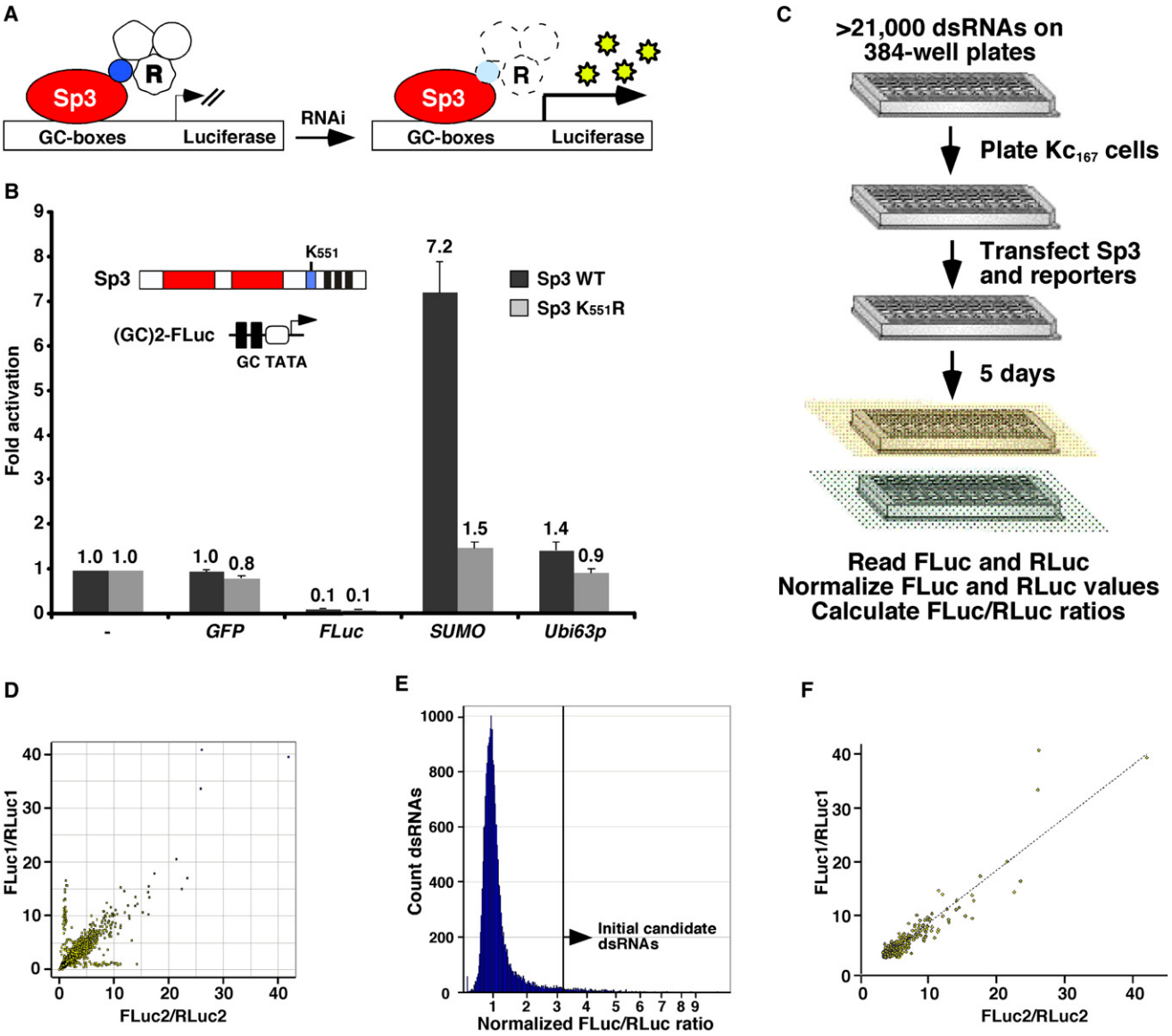



**Figure 1. Genome-wide RNAi Screen for SUMO Conjugation and SUMO-Dependent Repression Components**

(A) Design of the RNAi screen.

(B) Proof of principle: dsRNA targeting SUMO relieves repression. *D. melanogaster* $Kc_{167}$ cells were incubated with the indicated dsRNAs and subsequently transfected with the (GC)2-FLuc reporter along with expression constructs for wild-type Sp3 or the SUMOylation-deficient Sp3 K551R mutant and a *Renilla* luciferase control reporter. Data are represented as mean ±SD.

(C) Outline of the genome-wide RNAi screen. A total of more than 21,000 dsRNAs on 384-well plates were incubated with $Kc_{167}$ cells. After 24 hr cells were transfected with an expression plasmid for wild-type Sp3, the (GC)2-FLuc, and an actin promoter-driven *Renilla* luciferase construct. Five days posttransfection, cells were lysed and luciferase activities determined and subjected to computational analyses.

(D) Scatter plot showing the experimental variation of the screen. Normalized firefly luciferase/*Renilla* luciferase (FLuc/RLuc) ratios of all 384-well plates (2 × 57) were plotted against each other.

(E) Histogram of normalized firefly/*Renilla* luciferase ratios of all 21,000 dsRNAs.

(F) Scatter plot of selected 265 candidate genes.

isolated gene linked to SUMO conjugation is *Su(var)2-10* coding for *Drosophila* PIAS (also designated Zimp). *D. melanogaster* PIAS is the ortholog of mammalian PIAS1 that acts as a SUMO E3 ligase on Sp3 SUMOylation in vitro (Sapetschnig et al., 2002).

## Identification of Proteins that Act Downstream of SUMO Conjugation

The design of the screen did not allow us to distinguish between proteins that modulate SUMOylation and those that act downstream of SUMO conjugation by regulating or mediating

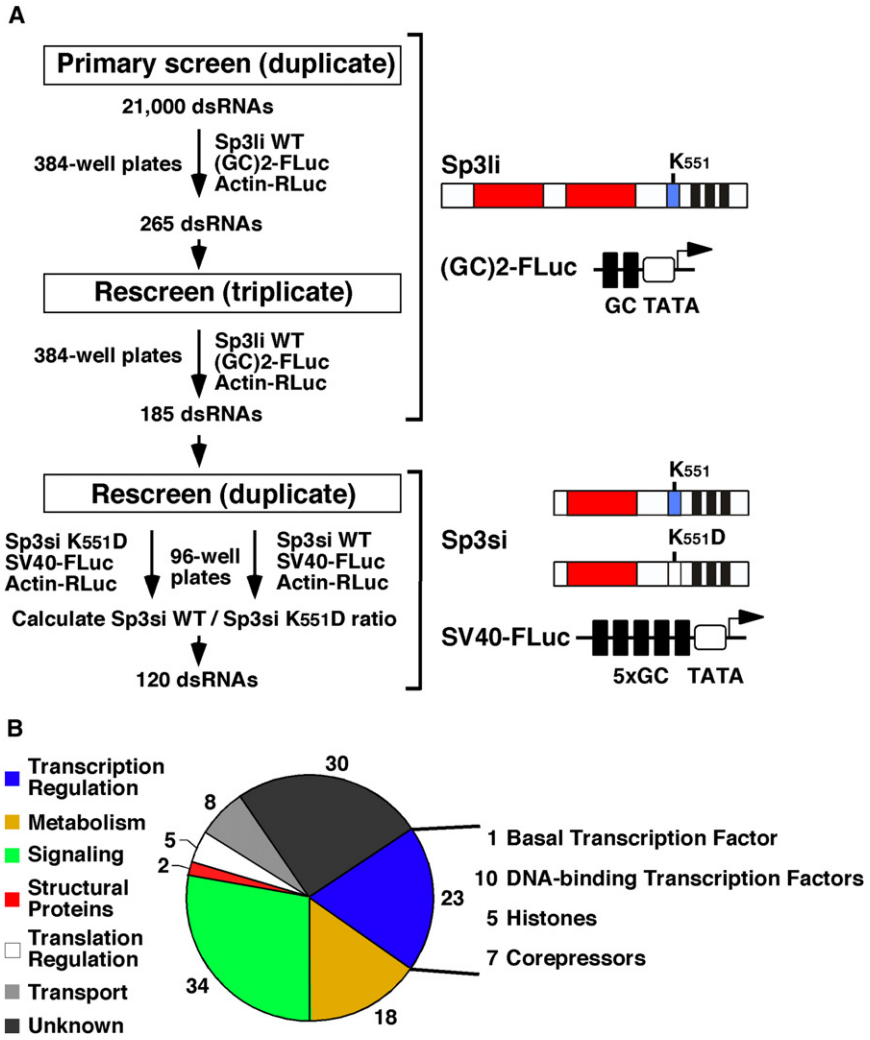



**Figure 2. Schematic Outline of Secondary Screens and Results**

(A) Schematic outline of screening procedures.

(B) Functional classification of the 120 genes obtained after the multiple rescreening procedures.

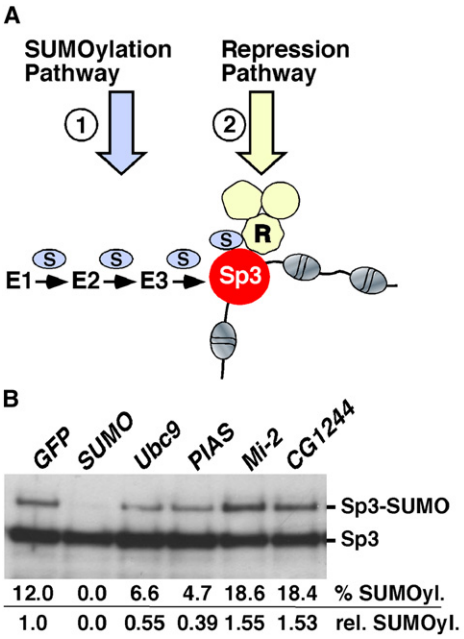
Sp3-SUMO-dependent transcriptional repression. RNAi against candidates that act on the SUMOylation pathway reduces SUMO modification of Sp3. In contrast, proteins that act downstream of SUMO conjugation are not expected to decrease the amount of SUMOylated Sp3 (Figure 3A). SUMO modification of Sp3 was monitored by quantitative western blot analyses in the presence of dsRNAs using fluorescence-labeled secondary antibodies (Figure 3). Because the limited accuracy of quantitative western blots does not allow for the analyses of weakly active dsRNAs that potentially affect Sp3 SUMOylation levels only slightly, we focused on a subset of dsRNAs that strongly relieved repression. Depletion of SUMO, which was very efficient in these experiments, leading to more than 40-fold Sp3-dependent reporter gene activation, resulted in complete loss of Sp3 SUMOylation (Figures 3B and 3C). Similarly, knockdown of Ubc9 but also knockdown of PIAS significantly reduced SUMO modification of Sp3 (Figures 3B and 3C). This result is consistent with the function of Ubc9 as SUMO-conjugating E2 enzyme and strongly suggests that *D. melanogaster* PIAS acts as a SUMO E3 ligase for Sp3 SUMO modification in insect cells. In addition to dsRNAs targeting SUMO, Ubc9, and PIAS, only two other dsRNA probes reduced SUMO modification (Figure 3C). Knockdown of an additional 15 proteins that were analyzed by western blotting did not reduce SUMO modification (Figures 3B and 3C), suggesting that they act downstream of SUMO attachment.

## Potential SUMO-Dependent Corepressors

Proteins that may directly act as SUMOylation-dependent corepressors must (1) act downstream of SUMO conjugation and (2) be nuclear proteins that can be linked to transcriptional control but (3) are most likely not DNA-binding transcription factors. Proteins that fulfill these criteria include Mi-2 (CG8103), Chd3 (CG9594), CG1244, Sfmbt (CG16975), and Sbb (CG5580, also designated Brakeless/Bks or MTV) (Figure 4A). Mi-2 is an ATP-dependent chromatin remodeler that associates with other proteins to form multisubunit complexes termed nucleosome-remodeling histone deacetylase (NuRD) (Bouazoune and Brehm, 2006; Bowen et al., 2004). Chd3 is a yet-uncharacterized paralog of Mi-2. CG1244 (hereinafter referred to as MEP-1) is a seven-zinc finger protein, orthologous to *C. elegans* MEP-1, that has been shown to be associated with the nematode Mi-2 (LET-418/CHD4) (Unhavaithaya et al., 2002). Sfmbt is a 4 mbt domain containing protein that binds to mono- and dimethylated histone H3-K9 and H4-K20 residues (Klymenko et al., 2006). Scribbler (Sbb) is listed as "transcription factor" in FlyBase due to the presence of a zinc finger. Recent reports link Sbb to transcriptional repression (Haecker et al., 2007; Wehn and Campbell, 2006).

## Redundancy and Rescue Experiments Confirmed a Role of Corepressors in SUMO-Mediated Repression

To validate the RNAi phenotype of dsRNAs targeting Mi-2, Chd3, MEP-1, Sfmbt, and Sbb, we targeted these proteins in another cell line (Supplemental Data, Figure S2) and analyzed several additional independent RNAi probes (Figure 4B). In the case of Mi-2, MEP-1, Sfmbt, and Sbb, we also monitored the knockdown efficiencies of the endogenous proteins by western blotting (Figure 4C). All alternative dsRNAs except one specifically enhanced the activity of wild-type Sp3, but not of the Sp3 K551D mutant. Immunoblot analysis demonstrated that the inefficient *MEP-1(5U)* probe did not result in significant MEP-1 protein depletion (Figure 4C).

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| dsRNA | Relative Sp3 SUMOylation |
|---|---|
| *GFP* | 1.00 |
| *SUMO* (5) | 0.00 |
| *Ubc9* (2) | 0.55 +/- 0.09 |
| *PIAS (2)* | 0.39 +/- 0.10 |
| *CG13088* (5) | 0.74 +/- 0.11 |
| *Jra* (5) | 0.76 +/- 0.16 |
| *Mi-2* (3) | 1.55 +/- 0.54 |
| *CG1244* (3) | 1.53 +/- 0.44 |
| *Sfmbt* (2) | 1.27 +/- 0.09 |
| *Sbb* (5) | 1.08 +/- 0.17 |
| *Kay* (5) | 1.29 +/- 0.17 |
| *Pcp* (2) | 1.21 +/- 0.17 |
| *CG14972* (2) | 1.27 +/- 0.36 |
| *CG10616* (5) | 0.93 +/- 0.14 |
| *Hoe1* (2) | 1.26 +/- 0.16 |
| *CG9432* (2) | 1.51 +/- 0.35 |
| *Map205* (2) | 1.29 +/- 0.33 |
| *CG9067* (5) | 0.96 +/- 0.08 |
| *CG3964* (2) | 1.21 +/- 0.01 |
| *eIF4AIII* (2) | 0.96 +/- 0.07 |
| *Histon 4R* (2) | 1.99 +/- 0.74 |

**Figure 3. Analyses of SUMO Conjugation and Repression Modulators**

(A) Schematic presentation of the SUMOylation and repression pathways.

(B) Example western blot to distinguish between genes involved in SUMOylation or SUMO-dependent repression. $Kc_{167}$ cells were plated with the indicated dsRNAs, transfected with an expression construct for wild-type Sp3, and analyzed by immunoblotting using anti-Sp3 antibodies. The values (percent SUMOylation and relative SUMOylation) were derived from immunoblots with fluorescence-labeled secondary antibodies.

(C) Summary of quantitative western blot analyses. The values for relative Sp3 SUMOylation are means ±SD. Numbers in parentheses refer to the number of independent experiments.

All together, these control experiments revealed a very tight correlation between activation and knockdown efficiencies. In this context, it should be mentioned that the alternative dsRNA probe *MRC009_F11* targeting *Chd3* depletes specifically Chd3 and does not affect Mi-2 protein level (data not shown), suggesting that both highly homologous proteins are involved in SUMOylation-mediated repression. Similar redundancy control experiments with new, if possible nonoverlapping dsRNAs targeting a different region of the corresponding mRNAs were performed for additional 35 top candidates (see the Supplemental Data, Figure S3).

As an additional proof for the involvement of Mi-2, MEP-1, and Sfmbt in the Sp3-SUMO-mediated repression mechanism, we performed rescue experiments. Endogenous Mi-2, MEP-1, and Sfmbt were targeted with dsRNA probes against the ORFs or the 3′UTRs, respectively, of the corresponding mRNAs (Figures 4D–4F). Expression of epitope-tagged versions of Mi-2, MEP-1, and Sfmbt using expression constructs containing the ORFs only (lacking the 3′UTR) rescued repression slightly when dsRNAs against the ORFs were used for targeting. A complete rescue of the loss-of-function phenotype occurred when the 3′UTR of the endogenous mRNAs was targeted (Figures 4D–4F). Western blot control experiments confirmed that the transfected proteins were expressed properly (Figure 4G). In the case of Mi-2, we expressed in addition a mutant carrying a deletion in the catalytic ATPase domain (Bouazoune and Brehm, 2005). This mutant is expressed at a similar level as the wild-type protein but did not rescue the RNAi phenotype (Figure 4D), indicating that the ATPase domain of Mi-2 is essential for SUMO-mediated repression.

To address the question of whether proteins that mediate repression by Sp3-SUMO are also involved in the repression mechanism of other SUMO-modified transcription factors, we have performed RNAi experiments with the *Drosophila* transcription factor Dorsal. Dorsal is a target for SUMOylation in *D. melanogaster* cells, and the SUMO-deficient Dorsal mutant (Dorsal K382R) is an up to 10-fold stronger activator than wild-type Dorsal (Supplemental Data, Figure S4A) (Bhaskar et al., 2002). Knockdown of Mi-2, MEP-1, and Sfmbt, but also knockdown of Chd3 and Sbb, activated specifically wild-type Dorsal (Supplemental Data, Figure S4B), demonstrating that these factors can act beyond Sp3 on other SUMOylated transcription factors.

## The Deacetylase of the NuRD Complex Is Not Required for Transcriptional Repression

Several studies imply that *D. melanogaster* Mi-2 exists in a multisubunit complex, similar to vertebrate NuRD complexes, where it is associated with the histone deacetylase RPD3 (HDAC1/2) and a number of additional components (Figure 5A) (Bouazoune and Brehm, 2006). It is generally believed that it is predominantly the HDAC activity of the NuRD complex that mediates transcriptional repression. However, targeting of RPD3 with two different dsRNA probes did not result in transcriptional activation of Sp3 (Figure 5B), although control immunoblots revealed almost complete depletion of the RPD3 protein (Figure 5C). We also employed the HDAC inhibitors trichostatin A (TSA) and nicotine amid (NAM). Both drugs did not activate wild-type Sp3 (Figure 5D), further confirming that histone deacetylase activities are not essential for transcriptional repression by Sp3-SUMO.

Initial results suggested that MTA1-like might be involved in SUMO-mediated repression, since knockdown of MTA1-like by the *HFA12382* dsRNA probe activated wild-type Sp3 slightly. However, analyses of the *HFA12382* sequence revealed 121 > 18 nt potential off-targets as well as CAR repeats that might cause off-target effects (Ma et al., 2006). In addition, an alternative dsRNA probe for MTA1-like did not relieve repression. Thus, we consider it rather unlikely that MTA1-like is involved in SUMO-mediated repression. dsRNAs against all other subunits of the NuRD complex, namely p55, p66/68, and MBD2/3, were also present in the RNAi library used for initial screening, but transcriptional activation of Sp3 occurred in no case. We conclude that the action of Mi-2 is independent of the HDAC RPD3 and of other subunits of the NuRD complex.

### MEP-1, Mi-2, and Sfmbt Bind to SUMO and Are Recruited to Promoters in a SUMOylation-Dependent Manner

We investigated whether MEP1, Mi-2, and Sfmbt interact directly with SUMO using GST-SUMO fusions and in vitro-translated Mi-2, MEP-1, and Sfmbt. All three proteins interacted with *Drosophila* SUMO as well as with human SUMO-1, whereby the strongest interaction was observed with MEP-1 (Figure 6A). We also analyzed whether Mi-2, MEP-1, or Sfmbt interacts with Sp3 or Sp3-SUMO (Figure 6B). Mi-2, MEP-1, and predominantly Sfmbt interact with unmodified Sp3. However, quantification of the relative binding to SUMO-modified and unmodified Sp3 revealed that Mi-2, Sfmbt, and particularly MEP-1 bind preferentially to SUMO-conjugated Sp3 (Figure 6B). This result is consistent with the finding that predominantly MEP-1 strongly interacts with SUMO on its own.

To determine whether Mi-2, MEP-1, and Sfmbt are recruited to the (GC)2-promoter by Sp3 in a SUMOylation-dependent manner in vivo, we performed chromatin immunoprecipitation experiments with SL2 cell lines stably transfected with the (GC)2-FLuc reporter, the actin-driven RLuc coreporter and a $Cu^{2+}$-inducible expression vector for wild-type Sp3 or a SUMOylation-deficient Sp3 mutant (Sp3 SD), respectively (Supplemental Data, Figure S5). Both wild-type Sp3 and mutant Sp3 are recruited to the (GC)2-promoter upon induction (Figure 6C). MEP-1 and Sfmbt are associated with the (GC)2-promoter in the presence of wild-type Sp3 but neither in the absence of Sp3 nor in the presence of the SUMOylation-deficient Sp3 SD mutant (Figure 6C). Mi-2 that is to some extent already present at the promoter in the absence of Sp3 or in the presence of the SUMOylation-deficient Sp3 mutant becomes highly enriched in the presence of SUMOylated wild-type Sp3. The recruitment of Mi-2, MEP-1, and Sfmbt by Sp3-SUMO demonstrates that all three proteins are directly involved in mediating SUMO-dependent repression.

We analyzed whether MEP-1, Mi-2, and Sfmbt can interact with each other. All three proteins bound to each other in vitro, whereby the strongest interactions were observed between MEP-1 and Mi-2 and between MEP-1 and Sfmbt (Figure 6D). Coimmunoprecipitation experiments revealed also strong association of endogenous MEP-1 with Mi-2 in vivo (Figure 6E). An in vivo association between Sfmbt and Mi-2 or MEP-1 was not detectable under our experimental conditions. Thus, it remains an open question whether Sfmbt is only loosely or transiently associated with MEP-1 and Mi-2 or whether promoter recruitment of Sfmbt by SUMOylated Sp3 occurs independently of Mi-2 and MEP-1. Taken together, our protein-protein interaction and promoter recruitment studies strongly suggest that MEP-1, Mi-2, and Sfmbt are part of a protein complex established by Sp3-SUMO bound to the (GC)2-promoter (Figure 6F).

### Mammalian Orthologs of *Drosophila* Mi-2 and Sfmbt Occupy the Mouse *Dhfr* Promoter in the Presence, but Not in the Absence, of Sp3

We asked whether mammalian orthologs of the identified SUMO-dependent corepressors are operative at endogenous Sp3-regulated target genes. Sp3 has been implicated in the repression of the *Dhfr* promoter that contains four Sp1/Sp3 binding sites (Birnbaum et al., 1995; Kennett et al., 1997) (Figure 7A). Consistently, *Dhfr* expression is increased in $Sp3^{-/-}$ MEFs (Figure 7B) derived form Sp3 knockout mice (Bouwman et al., 2000). ChIP experiments revealed that Sp3, as well as the paralogous transcription factor Sp1, is bound to the endogenous *Dhfr* promoter in wild-type MEFs, whereas in $Sp3^{-/-}$ MEFs only Sp1 is present at the *Dhfr* promoter (Figure 7C).

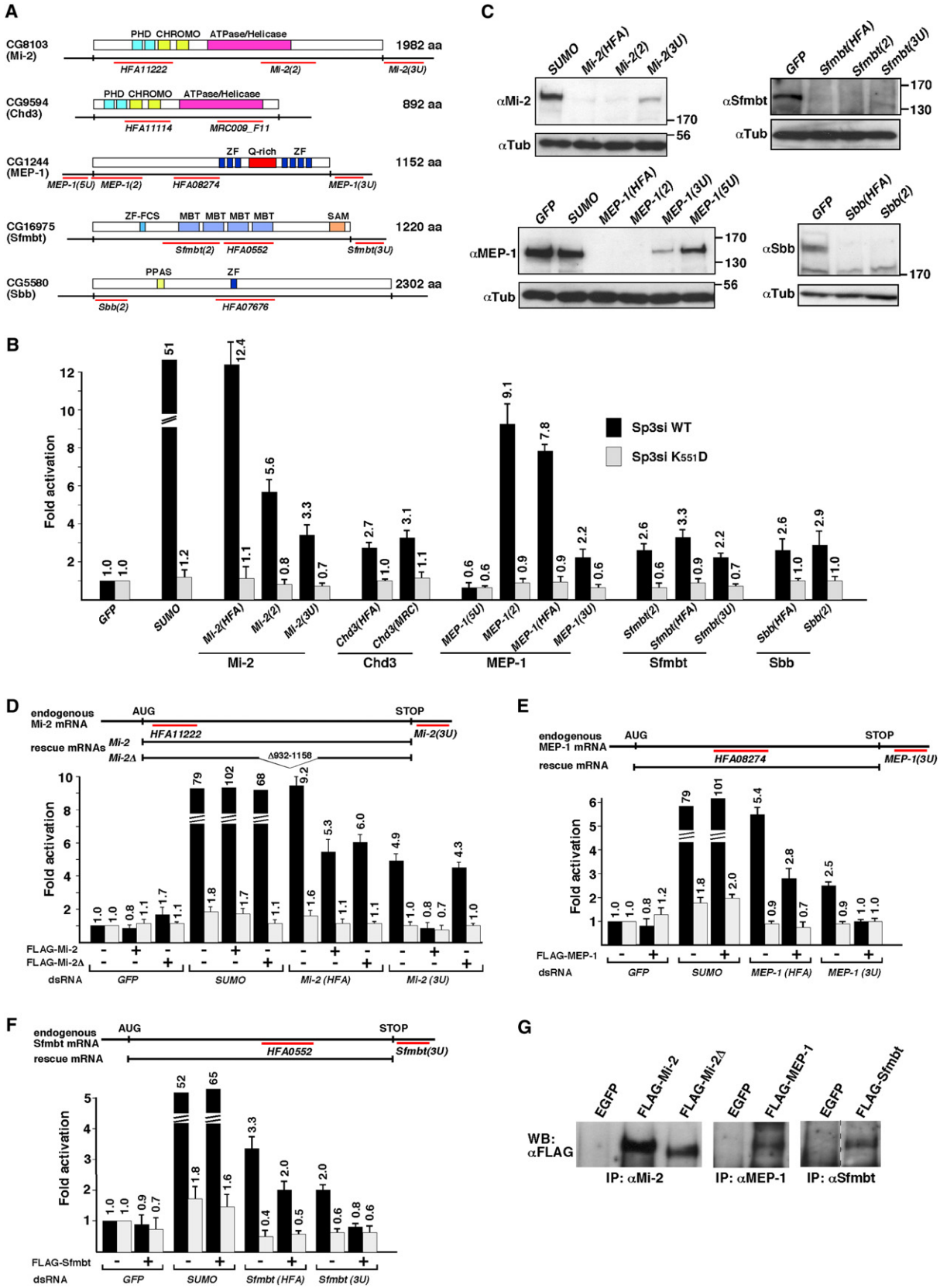
We analyzed occupancy of the *Dhfr* promoter by Mi-2 and L3mbtl2, which are the most closely related mammalian orthologs of *Drosophila* Mi-2 and Sfmbt, respectively. MEP1 could not be included in these studies, since a mammalian ortholog of this protein is currently unknown. Mi-2 and L3mbtl2 are present at the *Dhfr* promoter in wild-type MEFs, but not in Sp3-deficient MEFs (Figure 7C). Control immunoblots revealed that both proteins are expressed in wild-type and $Sp3^{-/-}$ MEFs at similar levels (Figure 7D). Sp3 and Sp1, but also Mi-2 and L3mbtl2, were not detectable approximately 1 kb upstream of the *Dhfr* promoter, further showing specificity of the assay (data not shown). These findings demonstrate local recruitment of Mi-2 and L3mbtl2 to the *Dhfr* promoter that is dependent on the presence of Sp3. In connection with our results in *Drosophila* cells, this strongly suggests that it is the SUMO modification of Sp3 that mediates recruitment of Mi-2 and L3mbtl2.

## DISCUSSION

Numerous transcription factors are targets for SUMO modification that confers reduced transcriptional activation. In most situations, the SUMO-modified transcription factor fraction at the steady state is very small in relation to the total pool of the transcription factor. Nevertheless, the transcription factor appears to be maximally repressed, since mutation of the SUMO acceptor site relieves repression. This enigma led to the hypothesis that a transiently conjugated transcription factor recruits a repressive complex that would be retained even after removal of SUMO (Hay, 2005). In this scenario, SUMO is required to initiate repression but is not necessary for maintenance of repression. Transient and possibly weak interactions between SUMO-modified transcription factors and potential corepressors present severe limitations to biochemical approaches for the identification of SUMO-dependent repression components. Our genome-wide screening approach provided a resort to this dilemma and led to the identification of SUMO-dependent repression components.

### MEP-1, Mi-2, and Sfmbt Are SUMO-Dependent Corepressors

MEP-1, Mi-2, and Sfmbt are directly involved in mediating SUMO-dependent repression. This conclusion is based on the following observations: (1) knockdown of MEP-1, Mi-2, and Sfmbt by RNAi relieved repression by the SUMO-modified transcription factor Sp3 but (2) did not impair SUMO modification demonstrating that they act downstream of SUMO attachment; (3) Mi-2, Sfmbt, and particularly MEP-1 interact with SUMO and with SUMO-modified Sp3; (4) MEP-1, Mi-2, and Sfmbt are recruited to the (GC)2-promoter in the presence of SUMO-modified wild-type Sp3 but neither in its absence nor in the presence of the SUMOylation-deficient Sp3 mutant. (5) Finally, mammalian orthologs of *Drosophila* Mi-2 and Sfmbt (mouse


A
CG8103 (Mi-2)
PHD CHROMO ATPase/Helicase
1982 aa
HFA11222
Mi-2(2)
Mi-2(3U)
CG9594 (Chd3)
PHD CHROMO ATPase/Helicase
892 aa
HFA11114
MRC009_F11
CG1244 (MEP-1)
ZF Q-rich ZF
1152 aa
MEP-1(5U) MEP-1(2)
HFA08274
MEP-1(3U)
CG16975 (Sfmbt)
ZF-FCS MBT MBT MBT MBT SAM
1220 aa
Sfmbt(2)
HFA0552
Sfmbt(3U)
CG5580 (Sbb)
PPAS ZF
2302 aa
Sbb(2)
HFA07676
C
SUMO Mi-2(HFA) Mi-2(2) Mi-2(3U)
αMi-2
αTub
170
56
GFP Sfmbt(HFA) Sfmbt(2) Sfmbt(3U)
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170
130
GFP SUMO MEP-1(HFA) MEP-1(2) MEP-1(3U) MEP-1(5U)
αMEP-1
αTub
170
130
56
GFP Sbb(HFA) Sbb(2)
αSbb
αTub
170
B
Fold activation
Sp3si WT
Sp3si K551D
GFP SUMO Mi-2(HFA) Mi-2(2) Mi-2(3U) Chd3(HFA) Chd3(MRC) MEP-1(5U) MEP-1(2) MEP-1(HFA) MEP-1(3U) Sfmbt(2) Sfmbt(HFA) Sfmbt(3U) Sbb(HFA) Sbb(2)
Mi-2
Chd3
MEP-1
Sfmbt
Sbb
D
endogenous Mi-2 mRNA
AUG STOP
HFA11222
Mi-2(3U)
rescue mRNAs
Mi-2
Mi-2Δ
Δ932-1158
Fold activation
FLAG-Mi-2
FLAG-Mi-2Δ
dsRNA
GFP SUMO Mi-2 (HFA) Mi-2 (3U)
E
endogenous MEP-1 mRNA
AUG STOP
HFA08274
MEP-1(3U)
rescue mRNA
Fold activation
FLAG-MEP-1
dsRNA
GFP SUMO MEP-1 (HFA) MEP-1 (3U)
F
endogenous Sfmbt mRNA
AUG STOP
HFA0552
Sfmbt(3U)
rescue mRNA
Fold activation
FLAG-Sfmbt
dsRNA
GFP SUMO Sfmbt (HFA) Sfmbt (3U)
G
EGFP FLAG-Mi-2 FLAG-Mi-2Δ EGFP FLAG-MEP-1 EGFP FLAG-Sfmbt
WB: αFLAG
IP: αMi-2
IP: αMEP-1
IP: αSfmbt

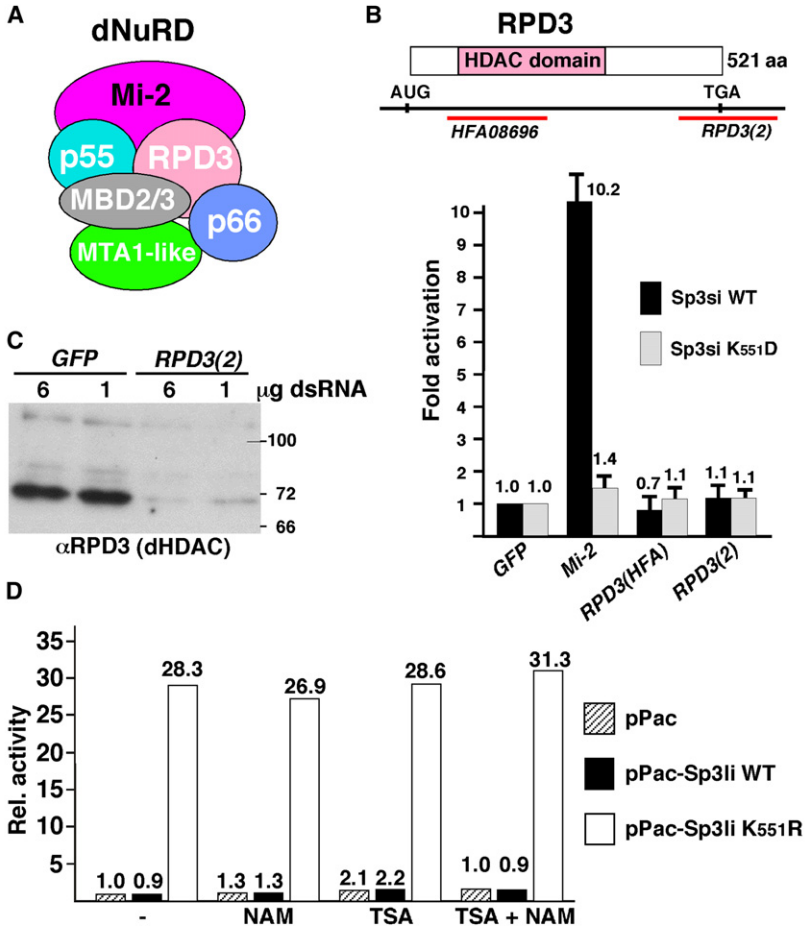


**Figure 5. HDACs Are Not Involved in Sp3-SUMO-Mediated Repression**

(A) Schematic presentation of the NuRD complex (adapted from Bouazoune and Brehm [2006]).

(B) DsRNAs targeting RPD3 do not relieve repression. *D. melanogaster* $KC_{167}$ cells were incubated with dsRNAs directed against GFP, Mi-2, and two different regions of the RPD3 mRNA as indicated and subsequently transfected with reporters and Sp3 variants as described in the legend to Figure 4B. Data are expressed as mean ± SD.

(C) Control western blot showing efficient depletion of RPD3.

(D) HDAC inhibitors do not enhance transcriptional activity of Sp3. Insect cells were transfected with an expression plasmid for wild-type Sp3li or the Sp3li K551R mutant and corresponding reporter plasmids. Twenty-four hours posttransfection, cells were treated with 5 mM NAM, 1 μM TSA, or both for additional 24 hr. Control cells (–) were incubated with the solvents. Values are means of two independent experiments.

Mi-2 and L3mbtL2) are present at the endogenous *Dhfr* promoter in wild-type MEFs, but not in *Sp3*$^{-/-}$ MEFs.

Several studies imply that *D. melanogaster* Mi-2 associates with the histone deacetylase RPD3 and several other proteins in a complex similar to the Mi-2-containing NuRD complex in vertebrates (Bouazoune and Brehm, 2006). However, our results demonstrate that for SUMO-mediated transcriptional repression, other subunits of the NuRD complex are not essential, which is consistent with the finding that HDAC inhibitors do not activate Sp3. Negative regulation of the DNA replication-related element factor (dDREF) by Mi-2 is also HDAC independent (Hirose et al., 2002). It remains to be established whether there exists a Mi-2 fraction in the cell that is not associated with HDACs or other NuRD subunits.

Our coimmunoprecipitation experiments demonstrate that Mi-2 is associated with MEP-1 in vivo. Association of MEP-1 with Mi-2 in vivo was also reported in *C. elegans*, where both proteins are required to prevent germline development in the soma (Unhavaithaya et al., 2002). A previous report also linked SUMO-mediated transcriptional repression to *C. elegans* MEP-1. SUMOylation of the *C. elegans* ETS transcription factor LIN-1 promoted transcriptional repression and mediated an interaction with *C. elegans* MEP-1 in a yeast two-hybrid assay (Leight et al., 2005). Inspection of the *Drosophila* MEP-1 amino acid sequence revealed several putative strong SUMO-interaction sites (data not shown). However, these SUMO-interacting motifs are not conserved in the *C. elegans* ortholog, and it is unclear at this stage whether the *C. elegans* protein can directly interact with SUMO as shown here for *Drosophila* MEP-1.

Thus far, the polycomb protein Sfmbt has been linked neither to SUMO-dependent repression nor to MEP-1 or Mi-2. Sfmbt is required for repression of *Drosophila* E2F activity (Lu et al., 2007), and the human ortholog L3MBTL2 was

**Figure 4. Verification of Candidate Corepressors by Targeting Different Regions and Rescue Experiments**

(A) Structural features of candidate corepressors. Black lines indicate target gene mRNAs, red lines size and position of dsRNA probes. PHD, plant homeodomain finger; CHROMO, chromatin organization modifier domain; ATPase/helicase, a DEAD-like helicase domain; ZF, C2H2 type zinc finger; Q-rich, glutamine-rich domain; ZF-FCS, FCS-type zinc finger; MBT, malignant brain tumor domain; SAM, sterile alpha motif; PPAS, phosphopantetheine binding domain.

(B) Relief of repression by alternative dsRNA probes. $Kc_{167}$ cells were incubated with dsRNAs targeting different mRNA regions (indicated in [A]) of Mi-2, Chd3, MEP-1, Sfmbt, and Sbb, subsequently transfected with the SV40-FLuc reporter, the *Renilla* luciferase coreporter, and an expression plasmid for Sp3si WT or the Sp3si K551D mutant, respectively. Normalized data are expressed as the mean ± SD of at least two independent experiments performed in duplicate.

(C) Immunoblot analysis of Mi-2, MEP-1, Sfmbt, and Sbb showing the knockdown effectiveness of individual dsRNAs.

(D–F) Rescue of RNAi phenotypes. FLAG-tagged Mi-2 (D), MEP-1 (E), and Sfmbt (F), but not a Mi-2 mutant lacking the ATPase domain (Mi-2Δ) (D), rescued the RNAi phenotype caused by the 3′UTR dsRNA. Data are expressed as mean ± SD.

(G) Control western blot experiments showing expression of FLAG-tagged Mi-2, Mi-2Δ, MEP-1, and Sfmbt.

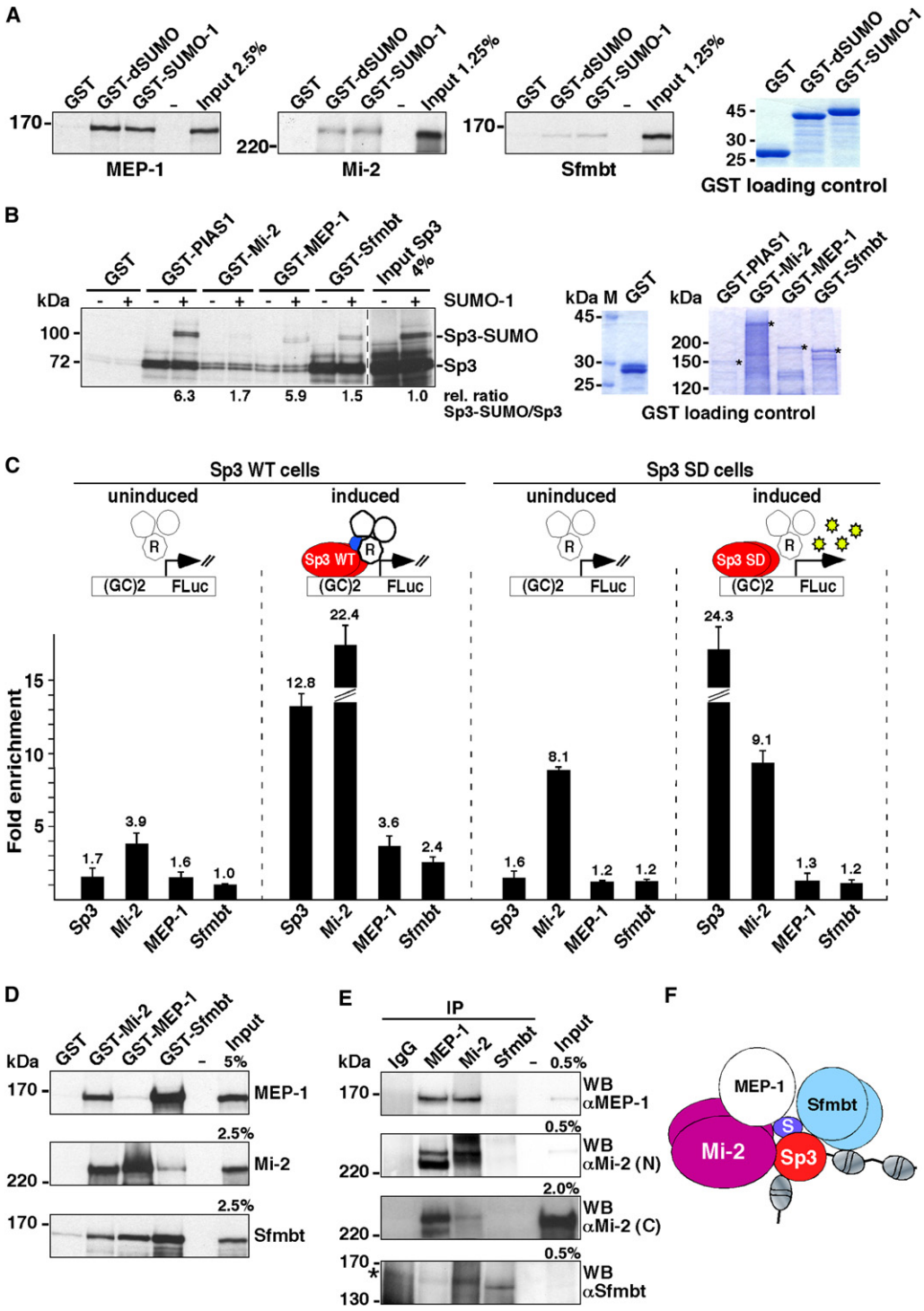


**Figure 6. SUMOylation-Dependent Recruitment of MEP-1, Mi-2, and Sfmbt**

(A) MEP-1, Mi-2, and Sfmbt interact with SUMO in vitro. [$^{35}$S]-labeled MEP-1, Mi-2, and Sfmbt were incubated with equal amounts of GST, GST-dSUMO, and GST-SUMO-1 as indicated. Bound proteins were resolved by SDS-PAGE and visualized by fluorography.

(B) MEP-1, Mi-2, and Sfmbt interact preferentially with SUMO-modified Sp3 in vitro. Partially SUMO-modified [$^{35}$S]-labeled Sp3 was incubated with similar amounts of immobilized GST, GST-PIAS1, GST-Mi-2, GST-MEP-1, and GST-Sfmbt. Bound Sp3 proteins were resolved by SDS-PAGE and visualized by fluorography. Quantification of relative binding to Sp3-SUMO was by phosphoimaging.

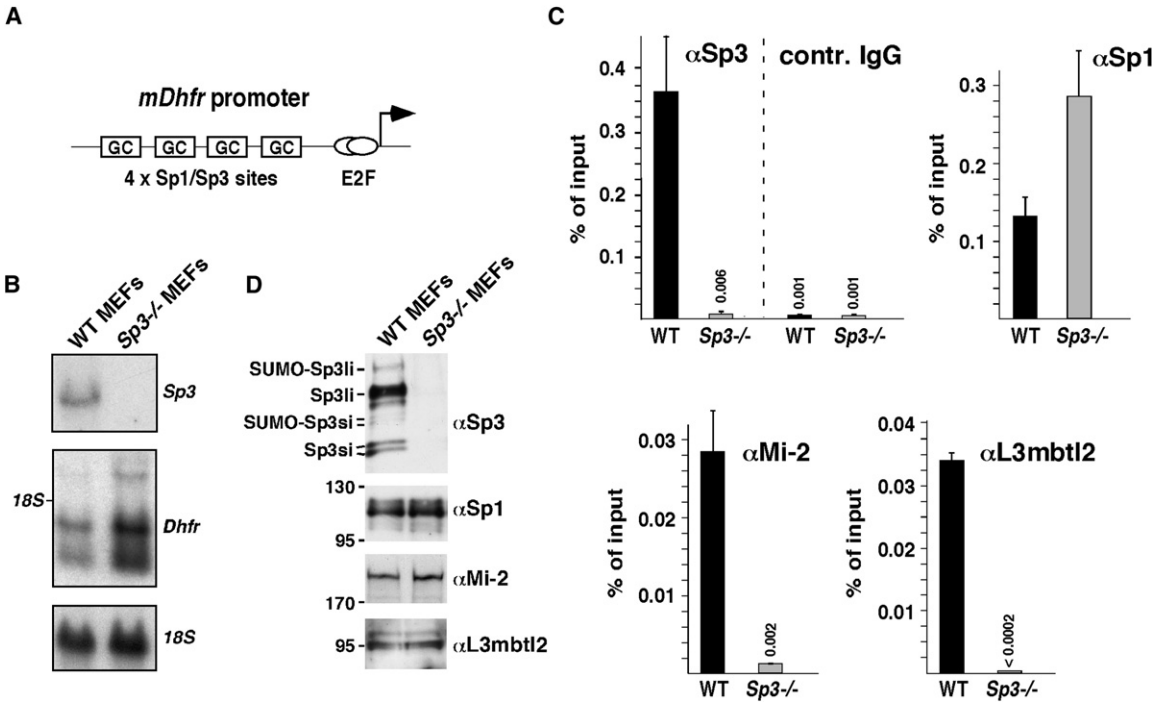


**Figure 7. Recruitment of Mi-2 and L3mbtl2 to the Endogenous Mouse *Dhfr* Promoter in Wild-Type and *Sp3*$^{-/-}$ MEFs**

(A) Schematic drawing of the mouse *Dhfr* promoter.

(B) Northern blot analysis of *Dhfr* expression in WT and *Sp3*$^{-/-}$ MEFs.

(C) ChIP assays. Immunoprecipitated DNA from WT and *Sp3*$^{-/-}$ MEFs was amplified by qPCR with primers specific for the *Dhfr* promoter. DNA recoveries are expressed as percentage of input (mean ± SD).

(D) Immunoblot analysis of Sp3, Sp1, Mi-2, and L3mbtl2 in WT and *Sp3*$^{-/-}$ MEFs.

previously identified as a member of the E2F6 repression complex (Ogawa et al., 2002). The MBT domains of L3MBTL1, a close homolog of L3MBTL2, can compact nucleosomal arrays dependent on mono- and dimethylation of histone H4K20 and of histone H1bK26. Moreover, L3MBTL1 and L3MBTL2 are both associated with HP1γ linking transcriptional repression by MBT domain proteins to chromatin compaction (Trojer et al., 2007).

Orthologs of *Drosophila* Mi-2, MEP-1, and Sfmbt as well as SUMO and the SUMOylation conjugation enzymes are synMuv (synthetic multivulval) genes in *C. elegans* that antagonize Ras signaling to prevent erroneous vulval fate but are also involved in gene repression outside the vulva (Poulin et al., 2005). We hypothesize that similar synMuv-like interactions may be also relevant for developmental decisions in *D. melanogaster* and vertebrates. SUMOylation might be a general mechanism that contributes to the recruitment of chromatin-associated proteins, thereby promoting target gene silencing.

Three other proteins identified in our screen that may be also directly involved in SUMO-mediated repression are Chd3 and Sbb (see Results) as well as CG15636 (HP6) encoding a heterochromatic 106 amino acid protein containing a single chromoshadow domain (Greil et al., 2007). In addition, identified proteins of unknown function might also act as SUMO-dependent corepressors. Future investigations will also address the role of these proteins in SUMO-mediated repression.

While the transcription factor Sp3 was used as a paradigm, it is very likely that the identified corepressors are generally important, and we suggest that they also confer transcriptional repression by other SUMO-modified transcription factors. For the *D. melanogaster* transcription factor Dorsal, we have explicitly shown this to be the case (Supplemental Data, Figure S4).

(C) Promoter recruitment of Mi-2, MEP-1, and Sfmbt by SUMO-modified wild-type Sp3. Crosslinked chromatin was isolated from untreated and $Cu^{2+}$-induced SL2 cells expressing either wild-type Sp3 or the SUMOylation-deficient Sp3 SD mutant (see Supplemental Data, Figure S5) and incubated with antibodies against the indicated proteins. Precipitated material was used as template for qPCR.

(D) MEP-1, Mi-2, and Sfmbt interact with each other in vitro. [$^{35}$S]-labeled Mi-2, MEP-1 and Sfmbt were incubated with GST-fusions as indicated and analyzed as in (A).

(E) MEP-1 and Mi-2 are associated in vivo. Mi-2, MEP-1, and Sfmbt were immunoprecipitated and subsequently analyzed by western blotting. Two different antibodies were used to detect Mi-2 (αMi-2[N] and αMi-2[C]). The star at the Sfmbt blot denotes a polypeptide that is likely to be unspecific because it is also detected in the IgG control.

(F) Model depicting the recruitment of Mi-2, MEP-1, and Sfmbt by SUMO-modified Sp3.

### Other Components Identified in the Screen

Our RNAi screen has uncovered a wide range of components in addition to the SUMO-dependent corepressors. One of the genes identified, *Su(var)2-10*, encodes *Drosophila* PIAS. Although not formally proven, it is very likely that *Drosophila* PIAS acts also as SUMO E3 ligase, since its dsRNA-mediated knockdown reduced SUMO modification of Sp3 as well as SUMO modification in general (data not shown). Other genes may be involved in activating or regulating the SUMO-dependent corepressors or the enzymes of the SUMOylation machinery. Some of the identified genes were also isolated in other genome-wide RNAi studies (see Table S1), including a genome-wide screen for MAPK (Friedman and Perrimon, 2006) and Wnt-Wingless (DasGupta et al., 2005) signaling regulators. This is consistent with reports on the interplay of the SUMO and the MAP kinase pathways (Yang et al., 2003) and on the involvement of SUMOylation in regulating Tcf/Lef proteins (Sachdev et al., 2001; Yamamoto et al., 2003) that function as nuclear effectors of the Wnt signaling pathway.

Clearly, the results from the screen presented here open many new avenues for investigations of SUMO signaling and SUMO-mediated transcriptional repression events. In particular, the interplay of all the proteins involved in regulating and conferring SUMOylation-dependent repression may now be addressed.

## EXPERIMENTAL PROCEDURES

### Genome-wide High-Throughput RNA Interference Screen

A library of >21,000 dsRNAs on polystyrene 384-well plates representing the *D. melanogaster* genome was used as starting material (Boutros et al., 2004; Müller et al., 2005). As internal negative and positive controls, each 384-well plate contained wells with dsRNA targeting SUMO, GFP, and FLuc. $Kc_{167}$ cells were dispensed at a density of 13,500 cells per well in 15 μl serum-free medium using an automated liquid dispenser (MultiDrop, Thermo Electron Corporation) and incubated for 60 min at 25°C. Subsequently, 20 μl serum-containing medium was added, and cells were cultured for 24 hr at 25°C. Transfection was performed with an automated liquid dispenser (MultiDrop) using the Effectene transfection reagent (QIAGEN). Each well obtained a total of 44 ng DNA (2 ng pPacUSp3, 40 ng [GC]2-FLuc, and 2 ng pPac-RLuc) in a volume of 20 μl. Five days posttransfection, firefly and *Renilla* luciferase activities were determined as described (Müller et al., 2005).

Firefly and *Renilla* luciferase values of individual wells were normalized to the median of the FLuc or RLuc values, respectively, of each plate. Then the ratio of each normalized FLuc and RLuc value, as well as the average of the duplicates, was calculated. Normalized FLuc/RLuc values higher than the lowest value obtained with dsRNA targeting *D. melanogaster* SUMO were considered as candidate dsRNAs leading to the initial selection of 585 candidate dsRNAs. Additional stringent selection criteria were applied to reduce the number of genes for further analyses. dsRNAs with the following features were not considered in secondary screens: (1) dsRNAs exhibiting an extreme viability phenotype leading to normalized FLuc values lower than 0.5, (2) dsRNAs that enhanced actin promoter-driven *Renilla* luciferase by more than 1.25-fold, and (3) dsRNAs with a specificity less than 70% or more than six potential 21 nt off-targets.

### Antibodies

For immunodetection and ChIPs, the following antibodies were used. For commercial antibodies, anti-Sp3 D20 (Santa Cruz), 1:3000 for chemoluminescence and 1:1000 for fluorescence blots; anti-FLAG M2 (Sigma), 1:1000; anti-β-tubulin (Chemicon International), 1:15,000; anti-Mi-2α/β H242 (Santa Cruz), 1:1000; and anti-L3MBTL2 (Lake Placid Biologicals), 1:2000; secondary HRP-coupled antibodies (Amersham Biosciences), 1:20,000 were used. For fluorescence western blot imaging, the IRDye 680-labeled anti-rabbit secondary antibody (1:5000) was used (LI-COR Biosciences). For noncommercial antibodies, anti-Sp1 (Hagen et al., 1994) 1:2000; anti-dSfmbt (Klymenko et al., 2006), 1:1000 ; anti-dMi-2(N) and anti-dMi-2(C) (Kehle et al., 1998),1:10.000; anti-RPD3 (Brehm et al., 2000), 1:1000; anti-dMEP-1 (N.K. and A.B., unpublished data, polyclonal rabbit serum), 1:5000; and anti-Bks/Sbb (Senti et al., 2000), 1:1000 were used.

### Chromatin Immunoprecipitation

ChIPs, reversal of crosslinks, and DNA purifications were performed according to Upstate Biotechnology's protocol. Immunoprecipitated samples were subjected to quantitative real-time PCR using the following primers: (GC)2-FLuc promoter region, 5′-ACTATGCGGCATCAGAGCAG-3′ and 5′-CGAGATCTGCGGATCCTAAG-3′; mouse *Dhfr* promoter, 5′-CACGCCTCAACCTGTGGGGGA-3′ and 5′-GCGGGGATAAAATCCTACCAGCC-3′; and mouse *Dhfr* −1 kb upstream sequences, 5′-CTTAAACTGATTTGCAACTGCAG-3′ and 5′-CGTTTTACTGTACAGATTTCCAG-3′. Results are presented as the percentage of input or as fold enrichment calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the input sample as reference and the sample with unrelated antibodies (Sp3 preimmune serum and anti-Gal4 antibodies) as calibrator.

Further details on the establishment of the screen, as well as information on plasmids, cell lines, and other experimental procedures, are provided in the Supplemental Data.

## SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two tables, six figures, and Supplemental References and can be found with this article online at http://www.molecule.org/cgi/content/full/29/6/742/DC1/.

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