ZMYND8 Co-localizes with NuRD on Target Genes and Regulates Poly(ADP-Ribose)-Dependent Recruitment of GATAD2A/NuRD to Sites of DNA Damage

Highlights

- ZMYND8 MYND domain interacts with PPPLΨ motifs in NuRD subunit GATAD2A
- GATAD2A and GATAD2B define mutually exclusive NuRD subcomplexes
- ZMYND8 and NuRD co-localize on active (super)enhancers and promoters
- ZMYND8 recruits GATAD2A/NuRD to DNA breaks in a poly(ADP-ribose)-dependent manner

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In Brief

Spruijt et al. show that the ZMYND8 MYND domain interacts with PPPLΨ motifs in the NuRD subunit GATAD2A. Furthermore, GATAD2A and GATAD2B define mutually exclusive NuRD subcomplexes. The interaction between ZMYND8 and GATAD2A is required for poly(ADP-ribose)-dependent recruitment of NuRD to DNA double-strand breaks.

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ZMYND8 Co-localizes with NuRD on Target Genes and Regulates Poly(ADP-Ribose)-Dependent Recruitment of GATAD2A/NuRD to Sites of DNA Damage

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SUMMARY

NuRD (nucleosome remodeling and histone deacetylase) is a versatile multi-protein complex with roles in transcription regulation and the DNA damage response. Here, we show that ZMYND8 bridges NuRD to a number of putative DNA-binding zinc finger proteins. The MYND domain of ZMYND8 directly interacts with PPPLΦ motifs in the NuRD subunit GATAD2A. Both GATAD2A and GATAD2B exclusively form homodimers and define mutually exclusive NuRD subcomplexes. ZMYND8 and NuRD share a large number of genome-wide binding sites, mostly active promoters and enhancers. Depletion of ZMYND8 does not affect NuRD occupancy genome-wide and only slightly affects expression of NuRD/ZMYND8 target genes. In contrast, the MYND domain in ZMYND8 facilitates the rapid, poly(ADP-ribose)-dependent recruitment of GATAD2A/NuRD to sites of DNA damage to promote repair by homologous recombination. Thus, these results show that a specific substoichiometric interaction with a NuRD subunit paralogue provides unique functionality to distinct NuRD subcomplexes.

INTRODUCTION

The nucleosome remodeling and histone deacetylase (NuRD) complex is a highly conserved chromatin-remodeling complex that is generally associated with transcriptional repression (Allen et al., 2013). Several NuRD complex subunits are rapidly recruited to sites of DNA damage and play roles in DNA-damage-induced transcriptional repression (Chou et al., 2010; Gong et al., 2015; Larsen et al., 2010; Polo et al., 2010; Smeenk et al., 2010). The NuRD complex contains two catalytic activities: the ATP-dependent chromatin-remodeling enzymes CHD3, CHD4, and CHD5 (Allen et al., 2013; Potts et al., 2011) and the histone deacetylases HDAC1 and HDAC2. In addition to these catalytic activities, the NuRD complex contains either methyl-CpG-binding domain (MBD)2 or MBD3; GATAD2A and GATAD2B; MTA1, MTA2, and MTA3; RBBP4 and RBBP7; and CDK2AP1 (Allen et al., 2013; Hendrich and Bird, 1998; Spruijt et al., 2010, 2013). Although Smits et al. (2013) recently determined the average stoichiometry of the complex, questions remain regarding which subunits or paralogues are mutually exclusive and how many functionally distinct NuRD complexes exist.

In previous reports, we and others have identified a number of substoichiometric interactors of NuRD, including ZMYND8 and ZNF687 (Eberl et al., 2013; Kloet et al., 2014; Malovanynaya et al., 2011; Smits et al., 2013). The ZNF687 protein contains ten C2H2-type zinc fingers (ZNFs), which are potential (sequence-specific) DNA-binding domains or protein-protein interaction domains. ZMYND8 has a completely different domain architecture consisting of a PHD finger, BROMO domain, and PWWP domain at its N terminus and a MYND domain located close to its C terminus. This protein is also called protein-kinase-C-binding protein (PKCBP1) or RACK7 (Ansieau and Sergeant, 2003; Fossey et al., 2000). Very recently, the PWWP domain of ZMYND8 has been linked to H3K36me2 and transcriptional regulation of retinoic-acid-induced genes during neuronal differentiation (Adhikary et al., 2016). Furthermore, the BROMO domain of ZMYND8 has been linked to transcriptional repression in response to DNA double-strand breaks (DSBs) (Gong et al., 2015).
Here, we show that ZMYND8 directly interacts with the NuRD subunit GATAD2A through its conserved MYND domain and that GATAD2A and GATAD2B assemble in mutually exclusive NuRD complexes. Although the effects of ZMYND8 depletion on the recruitment to and expression of NuRD target genes are minimal, ZMYND8 plays an important role in the recruitment of GATAD2A-containing NuRD to sites of DNA damage during repair by homologous recombination. Thus, these results show that paralogue-specific substoichiometric interactions further expand the functional diversity of distinct NuRD subcomplexes.

**RESULTS**

**ZMYND8 Connects the NuRD Complex to the Z3 Module via Its MYND Domain**

Recently, ZMYND8 was identified as a novel interactor of the NuRD complex (Eberl et al., 2013; Malovannaya et al., 2011; Smits et al., 2013). To investigate the molecular nature of this interaction, we performed SILAC (stable isotope labeling with amino acid in cell culture)-based GFP affinities purifications for MBDB, MBDB3, and ZMYND8 in HeLa cells (Baymaz et al., 2014). As expected, the MBDB2- and MBDB3-GFP purifications were significantly enriched for NuRD core subunits (Figure 1A; Figure S1A), some ZNF proteins, ZMYND8, and the known NuRD interactor SALL4. Purification of GFP-ZMYND8 resulted in the identification of the BHC complex (consisting of LSD1, RCOR1-3, PHF21A, and HMG20B), subunits of the EMSY complex (Varier et al., 2016), and the NuRD complex (Figure 1B). These results are in agreement with recent data showing that ZMYND8 and three associated ZNF proteins—ZNF532, ZNF592, and ZNF687 (Z3 module)—act as a central hub in a large transcription regulation network (Malovannaya et al., 2011).

To investigate which protein mediates the interaction between the NuRD complex and the Z3 module, we performed label-free quantification (LFQ) purifications of GFP-MBD3 from stable cell lines containing either a scrambled short hairpin RNA (shRNA) or an shRNA targeting ZMYND8, ZNF687, or CDK2AP1 (also called DOC-1) (Figures S1B and S1C) (Cox et al., 2014). We then applied the iBAQ algorithm to estimate the stoichiometry of the core NuRD subunits, ZMYND8 (3%–5%), and ZNF proteins (0%–2%) (Figure 1C, left graph) (Smits et al., 2013). Due to their sequence conservation, the three ZNF proteins share some tryptic peptides, which compromises iBAQ-based stoichiometry estimation for each individual protein. Therefore, we used the intensity of unique peptides that were identified for each ZNF protein instead of iBAQ values to estimate the relative abundance of each protein in the complex (Figure 1C, right graph). Since these values are not normalized for protein size, normalized unique intensities can only be used to compare the abundance of a single protein under different conditions. Knockdown of ZMYND8 (by ~50%) reduced the levels of the ZNF proteins interacting with MBD3 by about 50% (p < 0.05; Figure 1C). In contrast, knockdown by ~60% of ZNF687 significantly reduced the levels of ZNF687 co-purifying with NuRD, whereas the levels of the other ZNF proteins and ZMYND8 were not strongly affected. We corroborated these observations by purifying NuRD in a ZMYND8-knockout (KO) line (Figures 1D and S1D). Thus, these results reveal that ZMYND8 forms a direct link between the Z3 module and the NuRD complex.

Next, we set out to identify the domain in ZMYND8 that is required for the interaction with the NuRD complex and other interactors. ZMYND8 contains three domains that are involved in chromatin binding: a PHD finger, a BROMO domain, and a PWPP domain. In addition to these domains, ZMYND8 has a MYND (MYeloid, Nervy, and Deaf) domain, which is a well-conserved protein-protein interaction domain (Ansieau and Leutz, 2002). We generated GFP-ZMYND8 deletion constructs for all these domains (Figures 1E and S1E), which were then used in label-free-based GFP pull-down experiments. These experiments revealed that deletion of the MYND domain in ZMYND8 abolishes the interaction with the NuRD and BHC complex (Figure 1F; Figures S1F–S1I), which is in agreement with recent data from the Miller lab (Gong et al., 2015). To further substantiate these observations, we performed a SILAC-based GFP affinity purification experiment using a C-terminal fragment of ZMYND8 encompassing the MYND domain (Figure 1E). Both the NuRD and BHC complexes were specifically enriched in this pull-down experiment (Figure 1G). Altogether, these experiments show that ZMYND8 mediates the interaction between NuRD and the Z3 module and that the MYND domain in ZMYND8 is both necessary and sufficient for these interactions.

**ZMYND8 Interacts with NuRD Subunit GATAD2A, which Is Mutually Exclusive with GATAD2B**

Close inspection of the ZMYND8 GFP purifications (Figures 1B, 1F, and 1G) revealed that all known NuRD core subunits co-purify, with the exception of GATAD2B. The NuRD complex...
contains two GATAD2 molecules per complex (Smits et al., 2013), but whether GATAD2A and GATAD2B form heterodimers is not known. Our purifications suggest that ZMYND8 exclusively binds NuRD complexes containing GATAD2A. To further investigate this, we performed label-free GFP purifications for GATAD2A and GATAD2B (Figures 2A and 2B; Figures S2A and S2B). The GATAD2A purification resulted in enrichment of the Z3 module and all NuRD core subunits (Figure 2A).

In contrast, the GATAD2B pull-down resulted in the enrichment of all NuRD subunits except GATAD2A (Figure 2B). A direct comparison of enriched proteins in the GATAD2A and GATAD2B pull-downs further confirms a specific interaction of ZMYND8 and the ZNF proteins with GATAD2A (Figures 2C and S2B). Since ZMYND8 is only enriched in GATAD2A purifications, we hypothesized that ZMYND8 interacts directly with GATAD2A. To test the importance of either GATAD2 protein for the interaction between NuRD and ZMYND8, we made use of stable GATAD2A or GATAD2B shRNA knockdown cell lines containing doxycycline-inducible expression of MBD2-GFP (Figure S2C). We purified MBD2-GFP from nuclear extracts of these cell lines. All purifications enriched for the entire NuRD complex with similar stoichiometries (Figure 2D, top; Figure S2D). The summed stoichiometry of the GATAD2A paralogues in the knockdown lines is remarkably similar to that in the control knockdown line. This implies that, in the absence of either GATAD2, the NuRD complex fully assembles using the other, available GATAD2 parologue. We calculated the abundance of ZMYND8 and each of the ZNF proteins in the Z3 module based on unique peptides. In MBD2-GFP purifications from GATAD2B knockdown cells, ZMYND8, ZNF532, ZNF592, and ZNF687 enriched to similar, or even higher, levels compared to the control knockdown line. In contrast, lower levels of ZMYND8 and proteins of the Z3 module were present in MBD2/NuRD purifications from the GATAD2A knockdown line (Figure 2D, bottom), indicating that GATAD2A is required for the association between ZMYND8 and NuRD.

To establish whether both GATAD2A and GATAD2B form homodimers, we performed co-immunoprecipitation (coIP) experiments with GFP-GATAD2A and mCherry-GATAD2B and analyzed enriched proteins by western blot using antibodies against tagged and endogenous GATAD2A and GATAD2B (Figure 2E). Indeed, purification of GFP-GATAD2A (indicated by G) showed enrichment for endogenous GATAD2A, but not for exogenous or endogenous GATAD2B, thus providing further evidence for the formation of GATAD2A homodimers. Likewise, mCherry-GATAD2B purification (indicated by R) exclusively enriched for endogenous GATAD2B but failed to enrich for endogenous or exogenous GATAD2A.

Altogether, these data revealed that GATAD2A and GATAD2B define mutually exclusive NuRD complexes, similarly to what we have previously shown for MBD2 and MBD3 (Le Guennec et al., 2006). Furthermore, only GATAD2A-containing NuRD complexes interact with the ZMYND8/Z3 module.

**GATAD2A Has Conserved MYND Interaction Motifs**

ZMYND8 exclusively interacts with GATAD2A/NuRD, which suggests that ZMYND8 directly binds to a motif present in GATAD2A that is lacking in GATAD2B. MYND domains are described to recognize different proline-rich motifs, like PxLxP or PPPLxP (Anseieu and Leutz, 2002; Kateb et al., 2013; Liu et al., 2007). Vertebrate GATAD2A contains three consensus PPPLxP motifs, while GATAD2B lacks these motifs (Figures 2F and S2E). To test whether these motifs are required for the direct interaction with ZMYND8, we generated a PPPLxP-deletion mutant of GATAD2A (GATAD2AAPPPLxP). Furthermore, we generated a GFP-fusion protein containing the PPPLxP motifs fused to a nuclear localization sequence (NLS) (GATAD2APPPLxP-NLS). As a control, we included GATAD2APPPLxP, which entails the conserved region that interacts with methyl-binding domain (MBD) proteins (Snanapragasam et al., 2011) (Figure 2F). All of these constructs show a nuclear localization (Figure S2F). We transiently transfected HEK293T cells with these constructs and prepared nuclear extracts. Western blot analyses of GFP purifications from these extracts revealed that wild-type GATAD2A binds ZMYND8, while GATAD2B or GATAD2AAPPPLxP failed to do so (Figure 2G).

In contrast, all proteins, except GFP-GATAD2APPPLxP, interact with the NuRD subunits MBD2 and MBD3. Although the expression level of GFP-GATAD2APPPLxP was low, a specific interaction with ZMYND8 is visible upon a longer exposure of the blot. Similar results were obtained in SILAC-based GFP-affinity purifications (Figure S2G).

To further confirm that the MYND domain of ZMYND8 directly interacts with the PPPLxP motifs in GATAD2A, we expressed GST-ZMYND8MMYND in E. coli and purified the protein using glutathione beads. Then, we incubated the bead-coupled protein with nuclear extract containing GFP, GFP-GATAD2AAPPPLxP, or GFP-GATAD2APPPLxP-NLS. Only the combination of GST-ZMYND8MMYND with GFP-GATAD2APPPLxP resulted in an interaction (Figure 2H).

Since GFP-GATAD2APPPLxP-NLS does not interact with any other NuRD subunit (Figures 2G and S2G), this is a direct interaction.
In conclusion, the MYND domain of ZMYND8 directly interacts with PPPL-b motifs in GATAD2A.

**ZMYND8 and MBD3 Occupy Active Promoters and Enhancers Genome-wide**

To investigate genome-wide binding sites of ZMYND8 and MBD3, we performed chromatin immunoprecipitation sequencing (ChIP-seq) (Figure 3A). Endogenous ZMYND8 ChiP-seq resulted in ~11,000 peaks. These overlap very well with anti-GFP ChiP-seq targeting ZMYND8-GFP (Figure S3A). Furthermore, ChiP-seq for ZMYND8 in ZMYND8-KO cells confirmed that the endogenous ZMYND8 antibody is specific (Figures S3A and S3B). The number of ZMYND8 peaks detected in HeLa cells is lower compared to MCF7 cells, a breast cancer cell line that overexpresses ZMYND8 (Shen et al., 2016).

Based on co-localization with histone marks, the ZMYND8 peaks can be divided into three clusters, one of which mainly contains promoters (Figure 3B). RNA sequencing reveals that most of these promoters are actively transcribed (Figure S3C) and are marked by H3K4me3 and by H3K9, K14, and K27 acetylation (Figure S3D). The two other clusters mainly comprise (super)enhancers (marked by H3K4me1, H3K27Ac, DNase1, and lack of H3K4me3) (Figures 3B and S3D). Strikingly, ZMYND8-bound loci are depleted of DNA methylation (Figure 3C).

Both at promoters and enhancers, ZMYND8 shows a good overlap with MBD3 and RCO1 (Figure 3D). A GFP-tagged version of ZNF687 also occupies ZMYND8 target genes, as determined by ChiP-qPCR (Figure 3E). Given the presence of many putative DNA-binding ZNFs in this module, we hypothesized that this module may serve to recruit the NuRD complex to at least a subset of its genome-wide binding sites. To address this hypothesis, we used a ZMYND8-KO cell line along with a ZMYND8-KO rescue cell line allowing doxycycline-inducible expression of GFP-ZMYND8 WT (Figure 4A). Recruitment of GFP-ZMYND8 WT to laser-induced DSBs (Figure 5D). In conclusion, although ZMYND8 and NuRD share a large number of genome-wide binding sites, the ZMYND8/z3 module does not serve to recruit NuRD to non-methylated target sites in the genome, at least not in steady-state asynchronous cells. Furthermore, genetic deletion of ZMYND8 has only mild effects on gene expression of its target genes.

**ZMYND8 Recruits GATAD2A/NuRD to Sites of DNA Damage**

Since we did not observe a clear role for ZMYND8 in recruiting NuRD to its target genes in steady-state asynchronous cells, we focused on a more dynamic system. Earlier work from us and others revealed that the NuRD complex accumulates at sites of DNA DSBs (Larsen et al., 2010; Polo et al., 2010; Smeenk et al., 2010) and that this process requires the recruitment of ZMYND8 (Gong et al., 2015). Since ZMYND8 exclusively interacts with the GATAD2A-containing NuRD complex, we tested whether ZMYND8 and GATAD2A are recruited to DSBs. Endogenous ZMYND8, as well as endogenous and GFP-tagged GATAD2A, was rapidly (~5 min) recruited to sites of laser-induced DSBs that were marked by phosphorylated H2AX (γH2AX) (Figure 5A; Figure S4A). Knockdown of GATAD2A or genetic deletion of ZMYND8 resulted in the loss of signal in laser tracks, demonstrating the specificity of the used antibodies (Figures S5A and S4B). To address whether ZMYND8 is involved in GATAD2A/B recruitment, we made use of our ZMYND8-KO cells (Figure 4A). Recruitment of GATAD2A to laser-induced DSBs was significantly reduced in ZMYND8-KO cells compared to control cells (Figures 5B and 5C). To monitor GATAD2B recruitment, we induced expression of GFP-GATAD2B in both ZMYND8-KO and control cells. We could detect rapid recruitment of GFP-GATAD2B to laser-induced DSBs (Figure 5D). However, in contrast to GATAD2A, recruitment of GFP-GATAD2B was not affected by the loss of ZMYND8 (Figures 5D and 5E), while DSB levels, as measured by γH2AX formation, were comparable in ZMYND8-KO and control cells. Next, we tested whether the ZMYND8-GATAD2A interaction is required for recruitment of the NuRD complex to DSBs. To this end, we used our inducible MBD2-GFP cell lines with stable shRNA-mediated knockdown of either GATAD2A or GATAD2B.
The rapid recruitment of MBD2-GFP was significantly reduced by knockdown of GATAD2A but was unaffected by depletion of GATAD2B (Figures 5F and 5G). Importantly, DSB induction, as monitored by γH2AX, was comparable in these cells. Assessment of the MBD2-GFP recruitment in the ZMYND8-KO cells confirmed our hypothesis that MBD2 is, at least partially, recruited via the ZMYND8-GATAD2A axis (Figures 5H and 5I). Finally, we sought to address whether the ZNF proteins are involved in the DNA damage response. Local irradiation, indeed, revealed that ZNF687-GFP is rapidly recruited to laser-induced DSBs (Figure 5J). Furthermore, siRNA-mediated knockdown of ZNF687 impaired the recruitment of GFP-CHD4 to DSBs (Figures 5K and 5L). These findings highlight a sequential recruitment model for the MBD2/NuRD complex, which specifically depends on the ZMYND8/ZNF-GATAD2A axis.

The MYND Domain in ZMYND8 Regulates Its Recruitment to Sites of DNA Damage

Previous studies have revealed an important role of poly(ADP-ribose) (PAR) in the accumulation of CHD4 (Chou et al., 2010; Luijsterburg et al., 2012; Polo et al., 2010) and MBD2 (Sun et al., 2016). Although the recruitment of both these proteins is stimulated by ZMYND8 (Figure 5) (Gong et al., 2015), ZMYND8 itself was reported to be recruited to sites of DNA damage through histone acetylation (Gong et al., 2015). To further investigate these findings, we performed functional rescue experiments in our ZMYND8-KO cells. In contrast to control cells, we

Figure 4. ZMYND8 Depletion Does Not Affect NuRD Recruitment and Only Mildly Affects Transcriptional Output of Target Genes

(A) Schematic representation of the cell lines used for the ChIPs in (B). All three cell lines were doxycycline (Dox) treated to correct for any Dox-induced artifacts. (B) Endogenous ChIPs for ZMYND8, GATAD2A, MBD3, CHD4, and RCOR1 in control, ZMYND8-KO, and rescue cell lines. (C) qRTP-PCR analysis of ZMYND8 target genes. (D) Expression analysis of genes with a ZMYND8 peak on their promoter or nearby enhancer. The y axis shows fold change in ZMYND8-KO over control cells. The p values were determined using a two-tailed Student’s t test. Error bars in (B and C) indicate SD. Two-tailed Student’s t test p values: *p < 0.05. See also Figure S3 and Table S2.
found that ZMYND8-KO cells failed to recruit endogenous CHD4 to sites of DNA damage (Figures 6A and 6B). Re-expression of ZMYND8WT and ZMYND8ABROMO fully rescued CHD4 recruitment, while ZMYND8ABRMYND failed to do so (Figures 6A and 6B).

Analysis of the GFP-ZMYND8 mutants also showed that recruitment of ZMYND8 to sites of DNA damage critically depends on its MYND domain (Figures 6A and 6B). These findings show that ZMYND8 recruitment itself, as well as its ability to interact with and recruit the NuRD complex, is fully dependent on its MYND domain, which is in line with our mass spectrometry (MS) data (Figure 1F). To test whether the association of ZMYND8 with the NuRD complex is further induced by DNA damage, we performed LFQ GFP purifications of MBD3 and ZMYND8 in the absence and presence of the DSB-inducing agent phleomycin. This revealed that the stoichiometry of the ZMYND8/NuRD interaction does not change upon induction of DNA damage (p < 0.01; Figures S5C and S5D).

Contrary to our findings, a previous study from the Miller lab reported that ZMYND8 recruitment is mediated through histone acetylation and involves its BRMOM domain (Gong et al., 2015). Based on these results, we first confirmed whether our ZMYND8ABROMO is defective in binding histone acetylation. Indeed, in vitro binding assays confirmed that our ZMYND8ABROMO mutant fails to bind acetylated histone tails, while endogenous and GFP-ZMYND8WT were proficient in doing so (Figures 6C and 6D). In an attempt to resolve this discrepancy, we transiently expressed the ZMYND8ABROMO mutant from the Miller lab and could, indeed, confirm that this protein does not accumulate at sites of DNA damage (Gong et al., 2015) (Figure S4E). Although we did note that this mutant tends to mislocalize in the cytoplasm when expressed at higher levels, we could still detect an interaction with other NuRD subunits in pull-down experiments from nuclear extracts, suggesting at least partial functionality of the mutant. However, the human ZMYND8 gene encodes at least 20 protein-coding transcripts, many of which produce large proteins containing all the functional ZMYND8 domains. We used a different isoform of ZMYND8 (isoform 17, 1,054 amino acids [aa]; ENST00000396281) compared to the one used by Miller (isoform 1, 1,186 aa; ENST00000396281). The discrepancy in the BRMOM-dependent recruitment of ZMYND8 observed in the two studies may be explained by the use of different ZMYND8 isoforms.

Considering the previously reported involvement of PAR chains in NuRD subunit recruitment, we asked whether ZMYND8 recruitment is PAR dependent. This could reconcile our results with previous studies and possibly explain how both PAR and ZMYND8 could stimulate the recruitment of CHD4 and MBD2 (Chou et al., 2010; Luijsterburg et al., 2012; Polo et al., 2010; Sun et al., 2016). Local irradiation experiments revealed that the recruitment of GFP-ZMYND8WT was completely abolished by the treatment of cells with poly(ADP-ribose) polymerase (PARP) inhibitor KU-0058948. Control experiments confirmed the complete loss of PAR chain formation under these conditions (Figures 7A and 7B). Our analysis clearly indicates that the ZMYND8 isoform 17 is functional and able to restore CHD4 recruitment to sites of DNA damage and does not require BROMO-mediated binding to acetylated histones for its recruitment to sites of DNA damage. Instead, we show that recruitment of this ZMYND8 variant fully depends on the MYND domain and requires the formation of DNA damage-induced PAR.

**NuRD Subunits Regulate DSB Repair by Homologous Recombination**

To address whether NuRD subunits are required for DSB repair, we performed several functional assays. Previous studies have reported roles for NuRD components in DSB repair by homologous recombination (HR) (Gong et al., 2015; Pan et al., 2012; Sun et al., 2016). This prompted us to test sensitivity for PARP inhibition, which is a measure of defective HR (McCabe et al., 2006). Knockdown of ZNF687, GATAD2A, or GATAD2B rendered cells highly sensitive to increasing concentrations of PARP inhibitor, suggesting a defect in HR (Figure 7C). We subsequently used the DR (direct repeat)-GFP reporter for HR activity, which measures the HR-dependent repair of a nucleoside-induced DSB, resulting in the restoration of a functional gfp gene (Pierce et al., 1999). Flow-cytometric analysis of GFP fluorescence showed that knockdown of either CHD4 or ZMYND8 significantly reduced HR, although not to the same extent as core HR factor BRCA2 (Figure 7D). To validate these findings, we assessed the formation of RAD51 filaments in laser track experiments, since DNA-bound RAD51 mediates strand invasion and DNA transfer during HR. Analysis of irradiated cells showed that ZMYND8 knockout cells displayed moderately reduced recruitment of RAD51 compared to control cells, explaining the reduced HR efficiency (Figures 7E and 7F).

In summary, these findings suggest overlapping and unique roles of NuRD complex subunits in regulating HR-mediated DSB repair.

**DISCUSSION**

In this study, we have investigated the molecular nature and functional consequences of the interaction between ZMYND8 and the NuRD complex, which was previously reported to be
Figure 6. MYND Domain of ZMYND8 Is Required for Recruitment to Sites of DNA Damage

(A) (Immunofluorescence microscopy of cells 5 min after laser-induced DSBs showing endogenous CHD4 in control and ZMYND8-KO cells. Rescue effect of different GFP-fused ZMYND8 mutants is assessed.

(B) Quantification of (A). Error bars indicate SEM. Two-tailed Student’s t test p values: *p < 0.05; **p < 0.005.

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sub-stoichiometric (Smits et al., 2013). Our work revealed that ZMYND8 bridges the GATAD2A/NuRD complex to three ZNF proteins, ZNF532, ZNF592, and ZNF687, each of which contains a large number of putative DNA-binding ZNFs. Therefore, we hypothesized that this so-called Z3 module may serve to recruit GATAD2A/NuRD complexes to non-methylated target sites in the genome. Indeed, ChIP-seq for ZMYND8 and MBD3 revealed a substantial overlap of genome-wide binding sites, mostly active enhancers and promoters. However, ChIP-seq and ChIP-qPCR experiments in ZMYND8 knockout and rescue cell lines revealed that loss of ZMYND8 does not result in a loss of NuRD from its target genes. Based on these results, we hypothesized that the ZMYND8-GATAD2A/NuRD interaction is particularly important for an immediate recruitment to target genes that become induced or repressed following a stimulus. Future studies should shed further light on the kinetics and the importance, if any, of the ZMYND8-GATAD2A/NuRD interaction for NuRD-mediated transcription regulation in different cellular contexts. Support for the dynamic function of ZMYND8 is provided by its rapid poly(ADP-ribose)-dependent recruitment to DSBs. Once recruited, ZMYND8 contributes to the efficient recruitment of GATAD2A and CHD4, without an appreciable impact on GATAD2B. Efficient recruitment of MBD2 was also dependent on ZMYND8-GATAD2A/NuRD, but not GATAD2B, suggesting a sequential recruitment model for these NuRD subunits through the ZMYND8-GATAD2A interaction. The genetic interdependencies of NuRD subunit recruitment to DSBs was often partial, arguing that several mechanistically distinct NuRD recruitment mechanisms contribute to its roles in the DSB response (Luijsterburg et al., 2012; Smeenek et al., 2010). We consistently observed defects in DSB repair by HR upon the loss of several distinct NuRD subunits, suggesting an important role for the NuRD complex in this repair pathway. Additional mechanistic studies will be required to unravel the unique and overlapping roles of individual NuRD subunits in DSB repair.

ChIP-seq experiments revealed a striking genome-wide correlation between ZMYND8/NuRD and active promoters and enhancers marked with H3K4me3 or H3K4me1 and histone acetylation marks (Figure 3B). ChIP-seq provides correlative data, and observed signals are derived from thousands of asynchronously dividing cells. However, expression analysis of ZMYND8/NuRD target genes revealed that NuRD bound genes tend to be highly expressed (Figure S3C). This positive correlation between NuRD and gene expression has also been observed by others (Shimbo et al., 2013; Whyte et al., 2012). Nevertheless, RNA-seq in ZMYND8 KO cells revealed a slight increase in expression of genes that are regulated by a nearby ZMYND8-bound (super) enhancer. This indicates that ZMYND8/NuRD mildly represses and, thus, fine-tunes expression of these genes, which is in agreement with two recent publications (Li et al., 2016; Shen et al., 2016). In any case, these observations force us to reconsider the textbook description of the NuRD complex as an exclusive transcriptionally repressive complex (Baymaz et al., 2015; Reynolds et al., 2013; Whyte et al., 2012). To investigate the exact function of the NuRD complex in regulating gene expression, time course experiments in which NuRD recruitment to target genes can be induced need to be designed. Such experiments will allow deciphering of the exact order of events and the transcriptional output following NuRD recruitment.

Finally, the mammalian NuRD complex contains a striking number of protein paralogues such as MBD2/3, MTA1/2/3, HDAC1/2, CHD3/4, and GATAD2A/B. These paralogues are vertebrate specific, as Drosophila NuRD only contains one GATAD2, CHD, MTA, and HDAC protein, for example. Apparently, during evolution, multiple NuRD subunit paralogues with distinct biological functions evolved. In this study, we have shown that the GATAD2A and GATAD2B paralogues define mutually exclusive NuRD subcomplexes. Furthermore, only GATAD2A interacts with the ZMYND8/Z3 module. Currently, it is not clear yet whether and which of the other NuRD subunit paralogues form hetero- or homomers and, thus, how many different functional compositions of the NuRD complex exist. In the future, paralogue-specific affinity purifications will surely shed more light on the biochemical and functional diversity of NuRD, which should be classified as a family of closely related protein complexes rather than a single biochemical entity.

**EXPERIMENTAL PROCEDURES**

**Cloning**

ZMYND8 isoform 17 was cloned into pEGFP-C3 (Clontech). This is a refer to as ZMYND8WT throughout this study (Supplemental Information). GFP-ZMYND8WT and GFP-ZMYND8TRICOMO mutant (isoform 1) constructs were kindly provided by Kyle Miller. GFP-GATAD2A was PCR amplified from a construct kindly provided by Dr. Imre Berger and cloned into the pEGFP-C3 vector. pDNA5-FRT-TO-GFP constructs were created by Gateway cloning (Thermo Fisher Scientific). pCMV-mCHERRY-p66B was generously provided by Dr. Rainer Renkawitz and formed the template for PCR of GATAD2B.

The ZMYND8 MYND domain was GST tagged in vector pRPN256NB.

**Cell Culture and Generation of Stable Cell Lines**

Hek293T, U2OS, HeLa Kyoto, or HeLa FRT-TO cells were grown according to standard protocols. Generation of stable inducible lines, shRNA-mediated knockdown lines, and CRISPR (clustered regularly interspaced short palindromic repeat) knockdown cells, as well as details about SILAC culturing and siRNA transfections, can be found in the Supplemental Experimental Procedures. U2OS cells stably expressing GFP-CHD4 were previously described (Smeenek et al., 2010).

**Nuclear Extract Preparation**

Nuclear extracts (NEs) were prepared essentially as described previously (Dignam et al., 1983).

**GFP Affinity Purification**

GFP purifications were performed essentially as described previously (Baymaz et al., 2014).

**MS**

Liquid chromatography-tandem MS (LC-MS/MS) measurements were performed as described previously (Kloet et al., 2014).
Figure 7. ZMYND8 and NuRD Function in a PARP-Dependent DNA Damage Pathway

(A) Visualization of GFP-ZMYND8 recruitment in DMSO- or PARPi-treated cells.

(B) Quantification of (A).

(C) Survival assays of GATAD2A and GATAD2B knockdown HeLa cells and siZNF687-treated U2OS cells. siBRCA2 is a positive control.

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MS Data Analysis
Raw data were analyzed using the MaxQuant software package, Perseus, and R (Cox et al., 2014; Cox and Mann, 2008; Tyanova et al., 2016). Details about data analyses are described in the Supplemental Information. Processed MS data can be found in Table S1.

ChIP-Seq
Details about chromatin preparation and ChIP, such as antibodies, can be found in Supplemental Experimental Procedures. X-linking of cells was performed using disuccinimidyl glutarate (DSG) and formaldehyde. Library preparation was performed using the KAPA Kit and Nextflex adaptors. Purified DNA was analyzed by deep sequencing on the Illumina GAIIx or Illumina HiSeq2000 genome analyzers.

Library Sequencing and Data Analysis
Reads were mapped to hg19 (NCBI build 37) using BWA, allowing one mismatch. Peak-calling was performed with the MACS 2.0 tool. ZMYND8 peaks and their assigned target genes are listed in Table S2.

The Python package used for K-means clustering and generation of heatmaps and bandplots is available at http://smonvh.github.io/fluff/ (Georgiou and van Heeringen, 2016). R was used to generate bandplots.

Available RCOR1 ChIP-seq data in HeLa S3 (GSM1104353) were used to generate heatmaps (Zhang et al., 2013). DNA methylation was analyzed using ENCODE (Encyclopedia of DNA Elements) dataset GSM999337. The ChIP-seq datasets for H3K4me1, H3k27ac, DNase I, and P300 were retrieved from the ENCODE data repository site (http://genome.ucsc.edu/ENCODE/).

RNA-Seq
RNA was purified using the QIAGEN RNeasy Mini Kit. rRNA was removed using ENCODE (Encyclopedia of DNA Elements) dataset GSM999337. The ChIP-seq datasets for H3K4me1, H3k27ac, DNase I, and P300 were retrieved from the ENCODE data repository site (http://genome.ucsc.edu/ENCODE/).

Library Sequencing and Data Analysis
Reads were mapped to hg19 (NCBI build 37) using BWA, allowing one mismatch. Peak-calling was performed with the MACS 2.0 tool. ZMYND8 peaks and their assigned target genes are listed in Table S2.

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UV-A Laser Micro-irradiation
Cells were grown on coverslips and sensitized with bromodeoxyuridine (BrdU). When required, cells were simultaneously induced with doxycycline. The cells were grown on coverslips and sensitized with bromodeoxyuridine (BrdU).

U2OS cells containing the DR-GFP reporter were used to measure the repair of I-SceI-induced DSBs by HR as described previously (Pierce et al., 1999). For more details, see Supplemental Experimental Procedures.

PARP Inhibitor Survival
U2OS cells were transfected with small interfering RNA (siRNA) and, after 48 hr, trypsinized, seeded, and exposed to PARP inhibitor KU-0058948 (AstraZeneca) at the indicated concentration. After 7 days, the cells were stained with methylene blue. Colonies containing more than 20 cells were scored.

Statistical Methods
The p values in SILAC-based GFP affinity purifications were calculated using significance B in Perseus. Specific interactors in LFO-based GFP affinity purifications were determined using an adapted two-tailed Student’s t test in the Perseus software (permutation-based false discovery rate [FDR] with 250 randomizations); s0 and FDR are indicated in the graphs. For ChIP-qPCR and qRT-PCR, p values were determined using two-tailed Student’s t tests on technical triplicates of one of the representative experiments. For micro-irradiation experiments, mean and SEM were determined based on more than 40 cells per condition divided over multiple experiments. The p values were calculated using two-tailed Student’s t test.

ACCESSION NUMBERS
The accession number for raw and processed MS data reported in this paper is PXD: PXD003856. These data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaino et al., 2016). The accession number for all high-throughput sequencing data reported in this paper is GEO: GSE79836.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.037.

AUTHOR CONTRIBUTIONS
C.G.S. and M.V. conceived the project and wrote the manuscript, with input from all other authors. C.G.S. performed all MS and cloning experiments, as well as most ChIP and qRT-PCR experiments. M.S.L., W.W.W., and H.V.A. performed DNA damage experiments. R.M. and R.G.H.L. performed analysis of ChIP-seq and RNA-seq data, respectively. P.W.T.C.J. and H.R.V. assisted the MS experiments. R.R.E. and M.P.B. made CRISPR ZMYND8 knockout cell lines. F.M. performed the ZMYND8-GFP ChIP-seq, I.P., M.C.V.-A. and A.M. made stable cell lines. H.G.S., H.V.A., and M.V. supervised the project.

(D) Bar graph showing HR efficiency in siRNA-mediated knockdown cells as measured by a DR-GFP assay. The reporter cell line contains two dysfunctional gfp genes. Expression of I-SceI induces a DNA double-strand break, and repair of this break by homologous recombination leads to a functional gfp gene.

(E) Formation of RAD51 foci in control or ZMYND8-KO cells.

(F) Quantification of (E).

Error bars indicate SEM. Two-tailed Student’s t test p values: *p < 0.05; **p < 0.005; ***p < 5.10^{-6}. See also Figure S4.

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.037.
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