Supplemental Figures

Figure S1

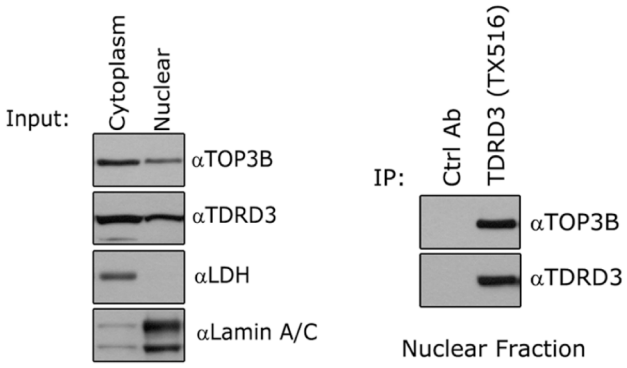


Figure S2

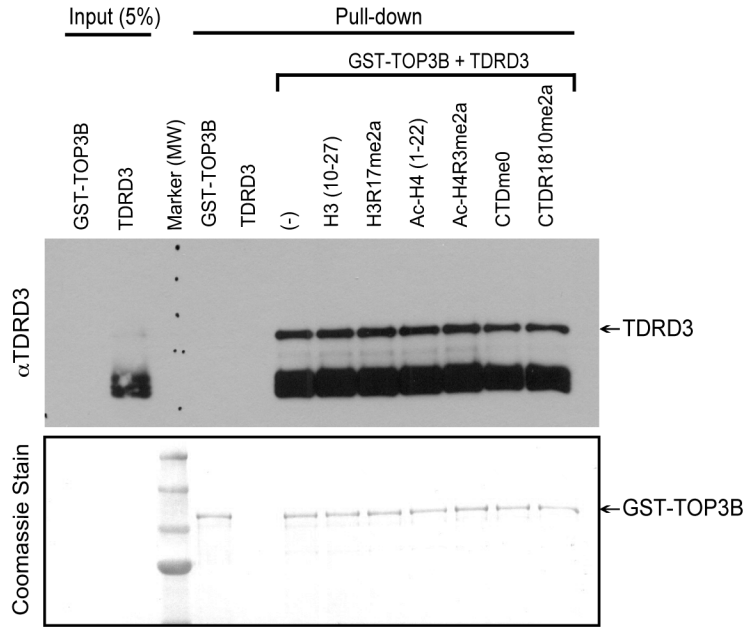
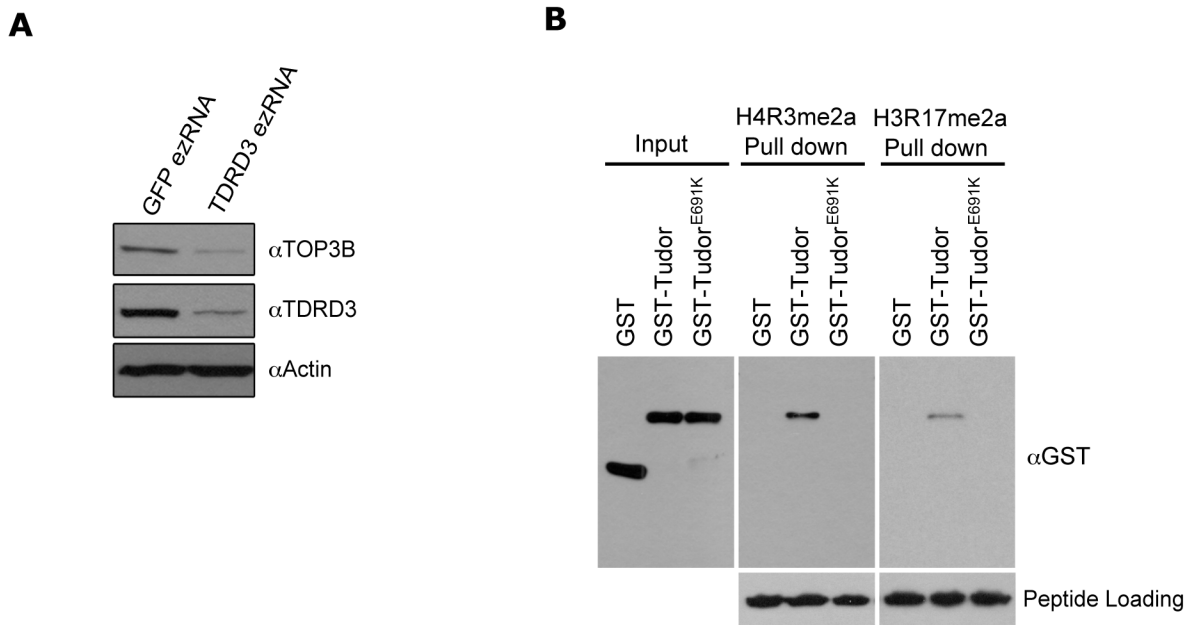

Input (5%)
Pull-down
GST-TOP3B + TDRD3
GST-TOP3B
TDRD3
Marker (MW)
GST-TOP3B
TDRD3
(-)
H3 (10-27)
H3R17me2a
Ac-H4 (1-22)
Ac-H4R3me2a
CTDme0
CTDR1810me2a
αTDRD3
←TDRD3
Coomassie Stain
←GST-TOP3B

Figure S3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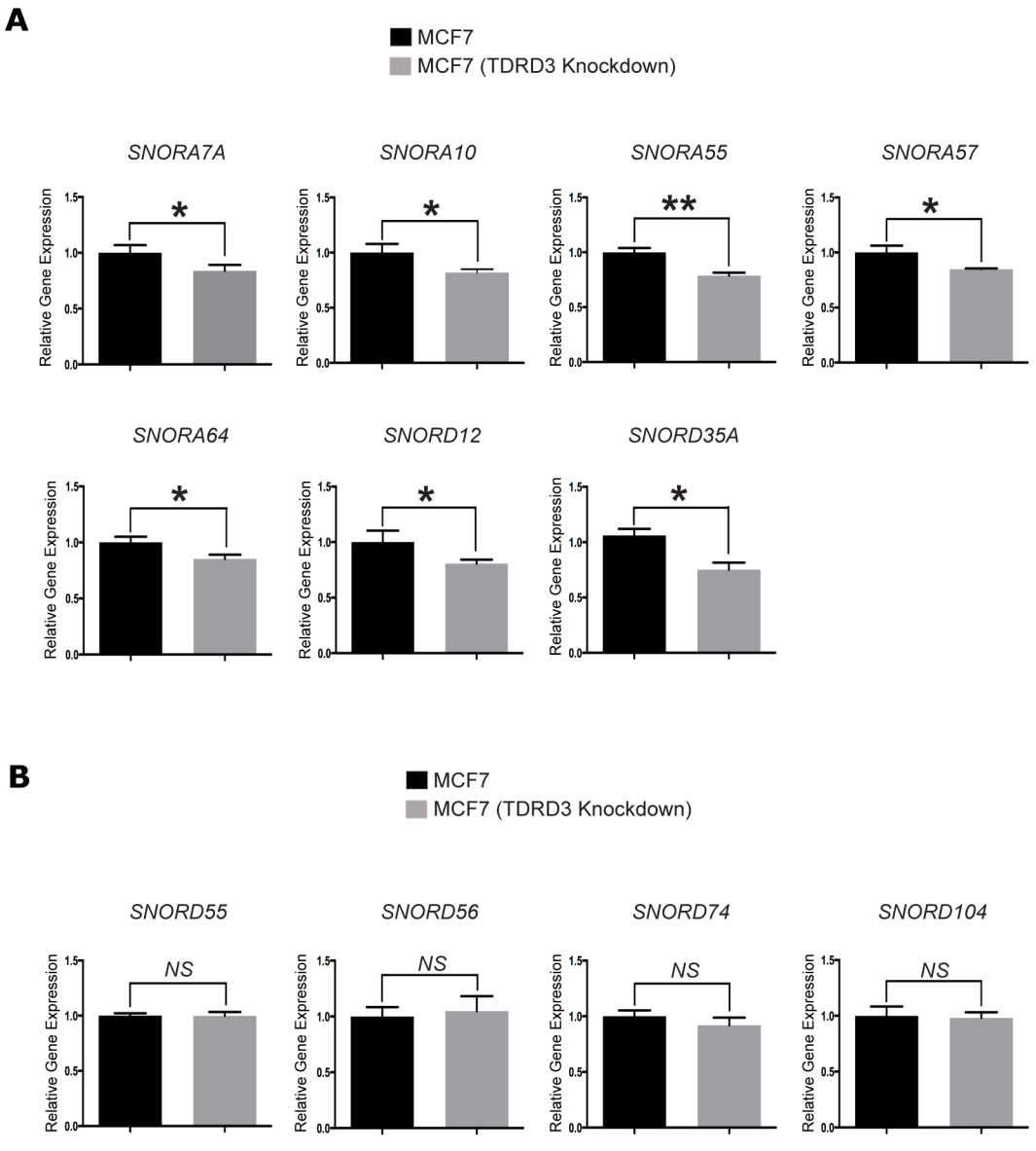
GFP ezRNA
TDRD3 ezRNA
αTOP3B
αTDRD3
αActin
B
Input
H4R3me2a Pull down
H3R17me2a Pull down
GST
GST-Tudor
GST-Tudor^E691K
αGST
Peptide Loading

Figure S4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MCF7
MCF7 (TDRD3 Knockdown)
SNORA7A
SNORA10
SNORA55
SNORA57
SNORA64
SNORD12
SNORD35A
Relative Gene Expression
*
**
B
MCF7
MCF7 (TDRD3 Knockdown)
SNORD55
SNORD56
SNORD74
SNORD104
NS

Figure S4 (continued)

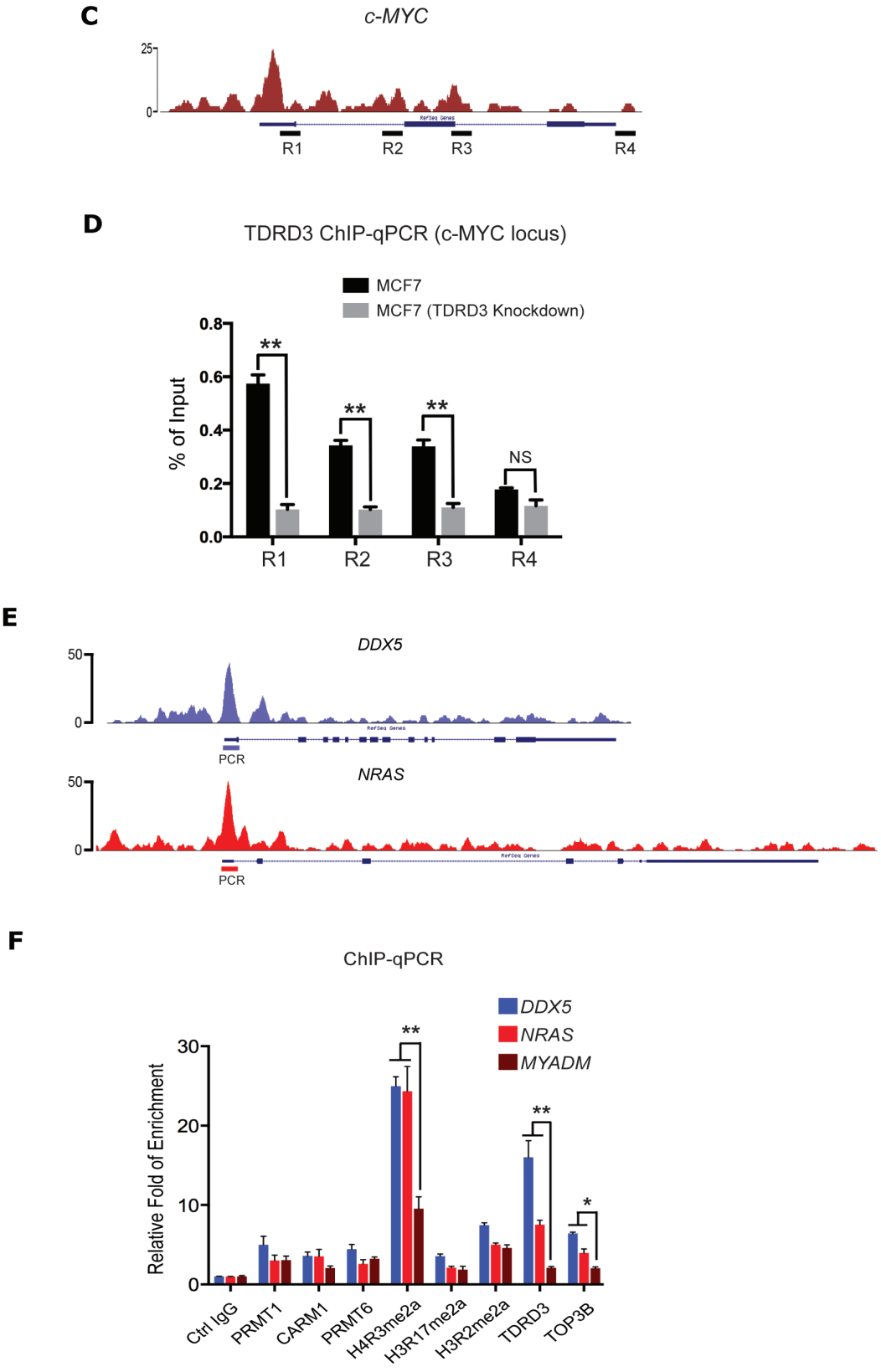
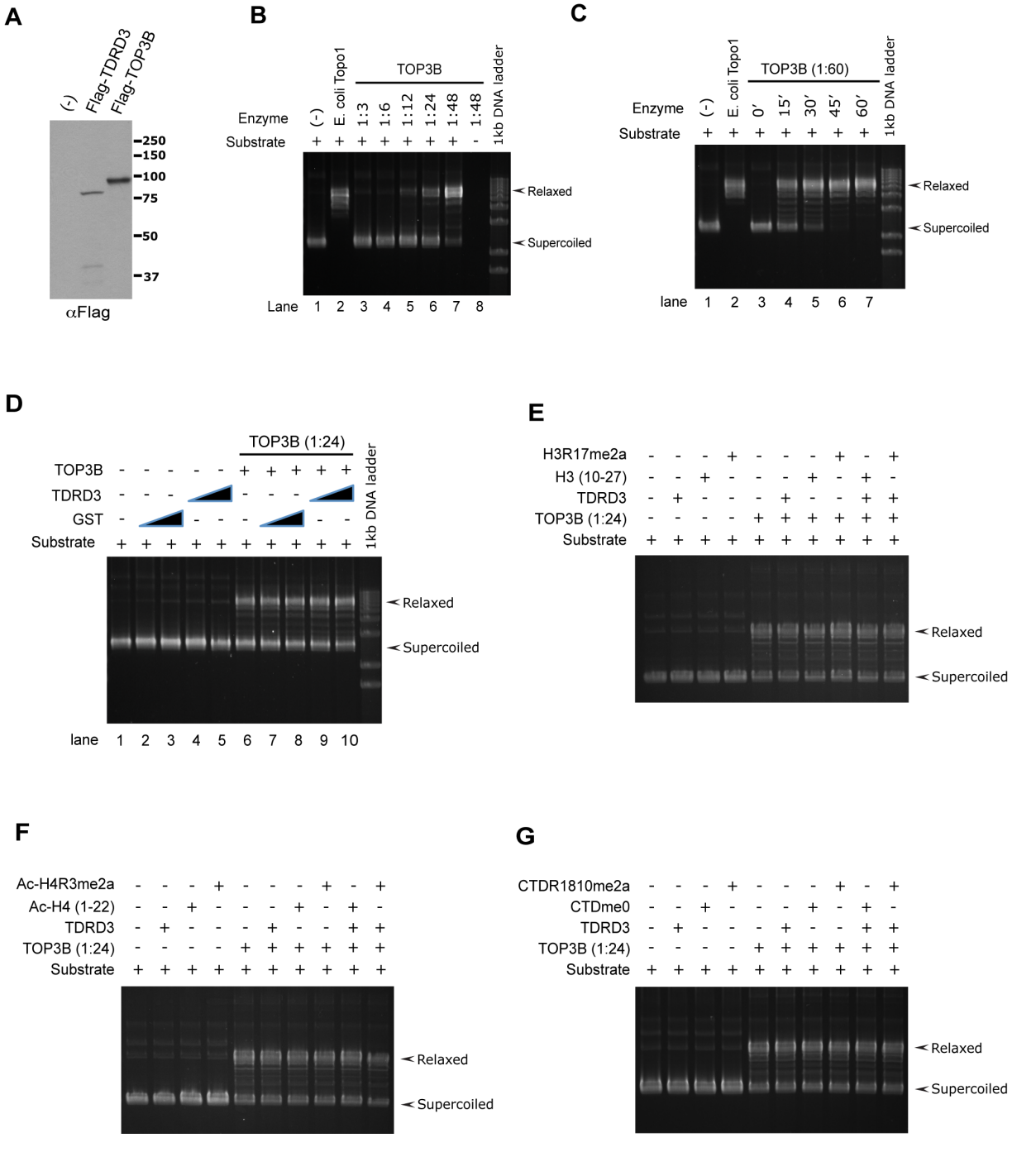


Figure S5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(-)
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Flag-TOP3B
250
150
100
75
50
37
αFlag
B
Enzyme
Substrate
(-)
E. coli Topo1
TOP3B
1:3
1:6
1:12
1:24
1:48
1:48
1kb DNA ladder
Relaxed
Supercoiled
Lane 1 2 3 4 5 6 7 8
C
Enzyme
Substrate
(-)
E. coli Topo1
TOP3B (1:60)
0′
15′
30′
45′
60′
1kb DNA ladder
Relaxed
Supercoiled
lane 1 2 3 4 5 6 7
D
TOP3B (1:24)
TOP3B
TDRD3
GST
Substrate
1kb DNA ladder
Relaxed
Supercoiled
lane 1 2 3 4 5 6 7 8 9 10
E
H3R17me2a
H3 (10-27)
TDRD3
TOP3B (1:24)
Substrate
Relaxed
Supercoiled
F
Ac-H4R3me2a
Ac-H4 (1-22)
TDRD3
TOP3B (1:24)
Substrate
Relaxed
Supercoiled
G
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CTDme0
TDRD3
TOP3B (1:24)
Substrate
Relaxed
Supercoiled

Figure S5 (continued)

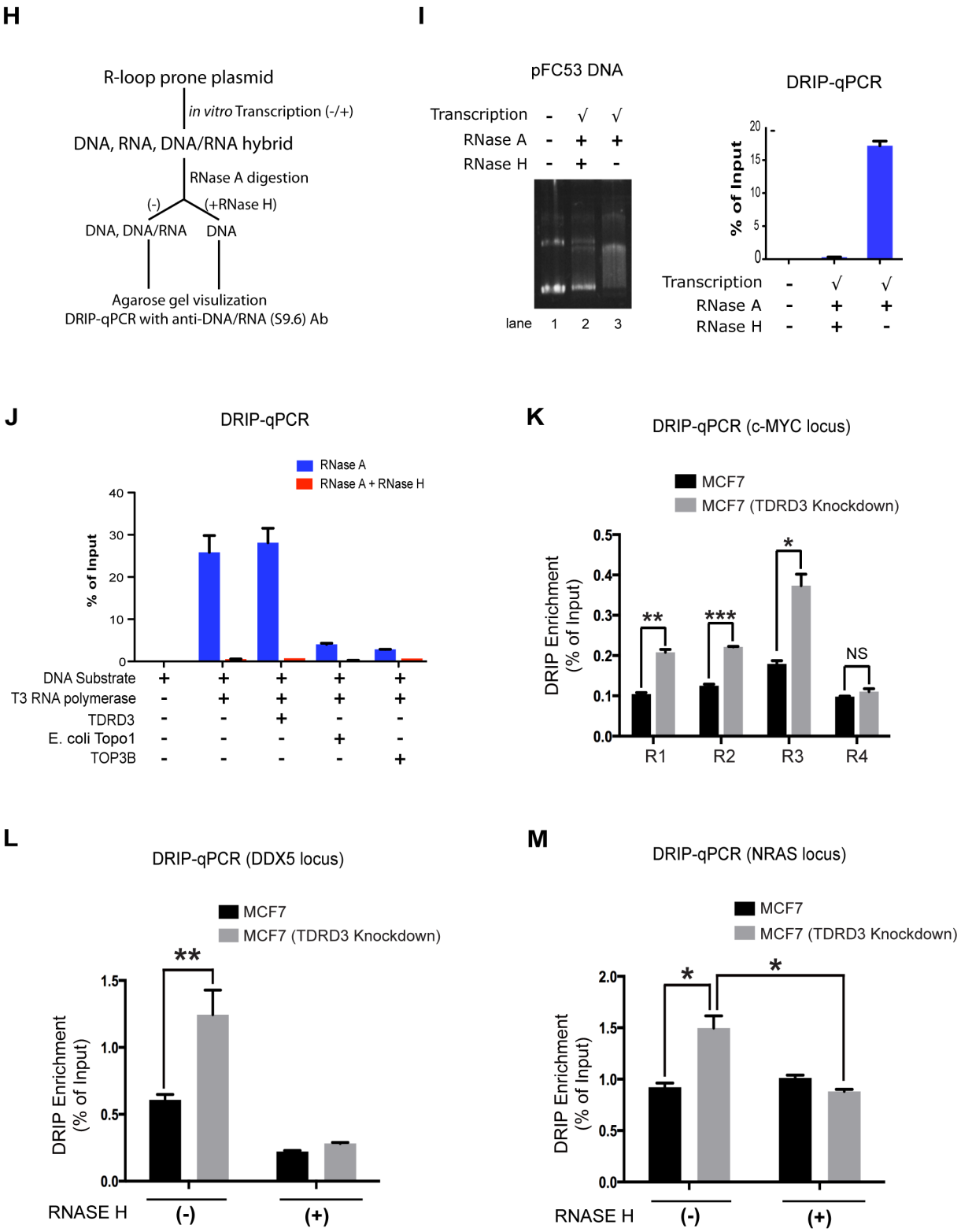


Figure S5 (continued)

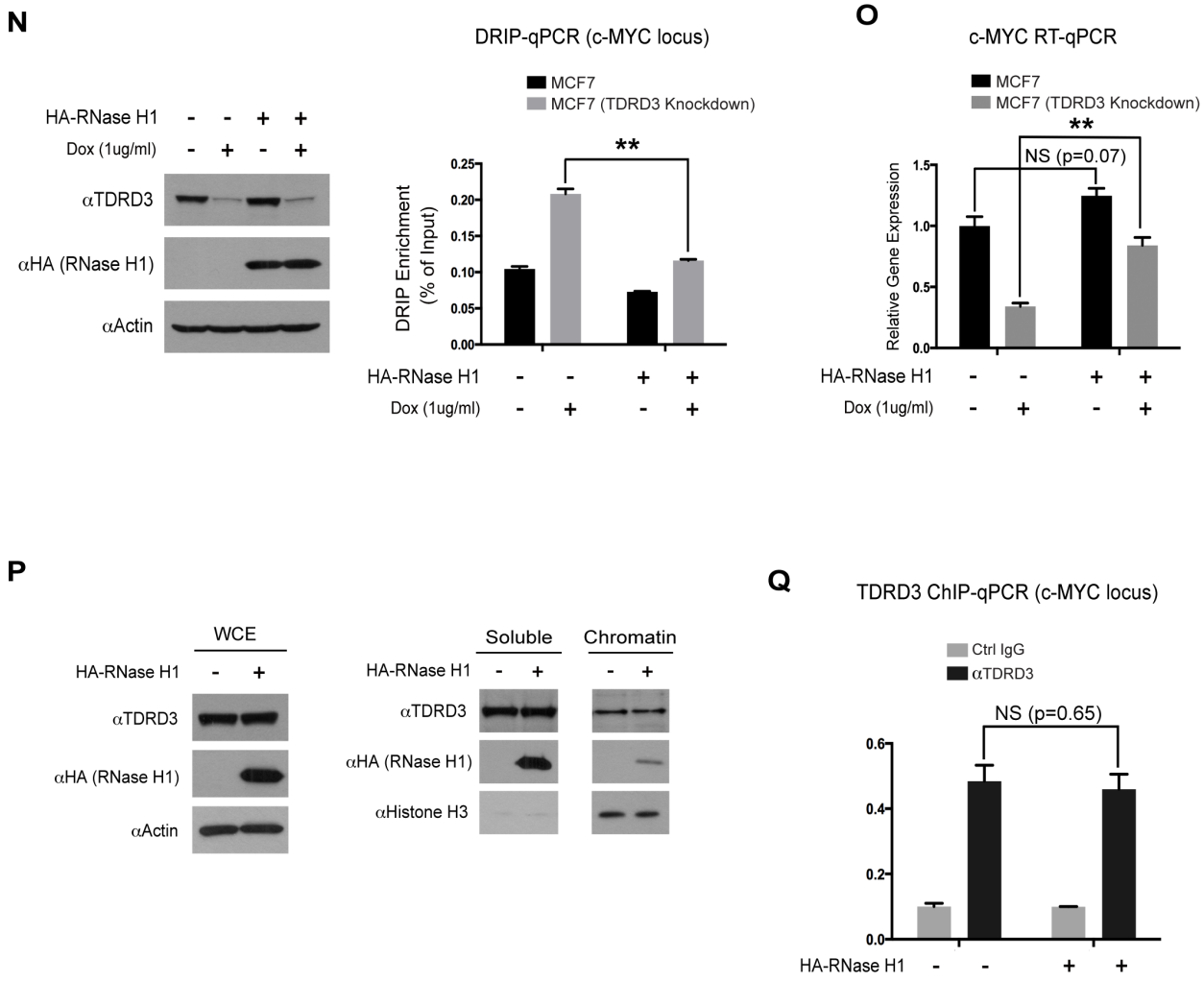
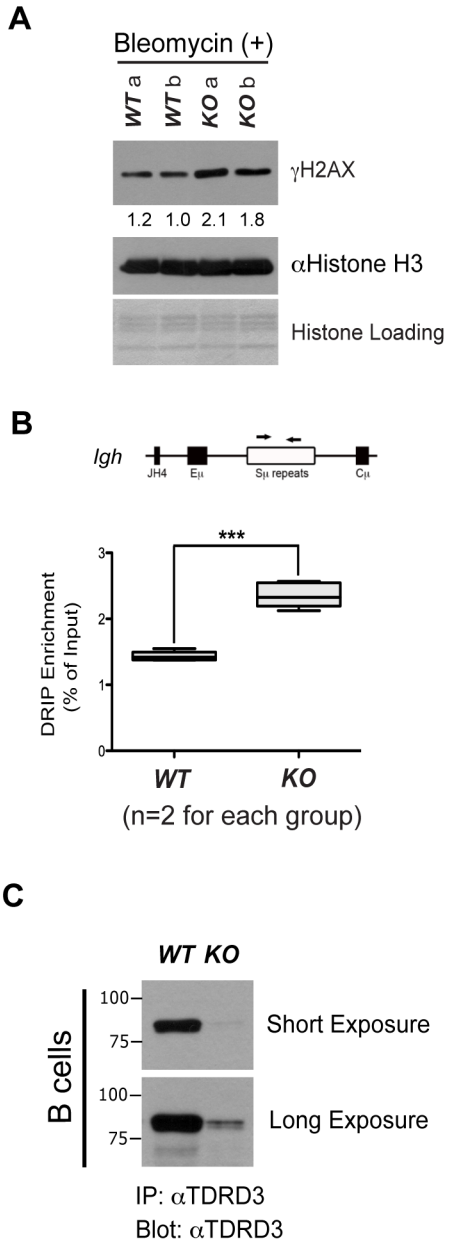


Figure S6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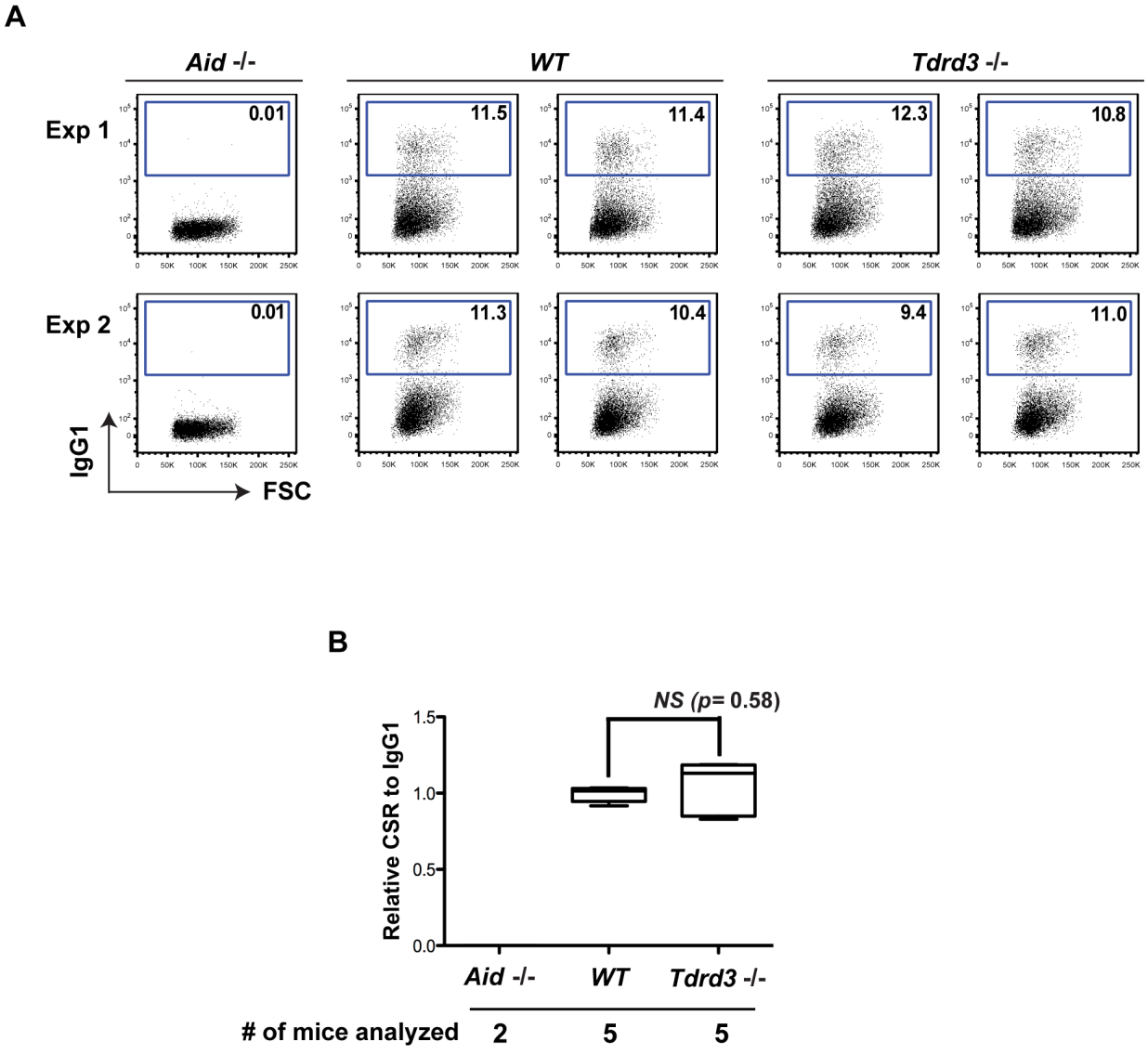
Bleomycin (+)
WT a
WT b
KO a
KO b
γH2AX
1.2 1.0 2.1 1.8
αHistone H3
Histone Loading
B
Igh
JH4
Eμ
Sμ repeats
Cμ
***
DRIP Enrichment (% of Input)
0
1
2
3
WT
KO
(n=2 for each group)
C
WT KO
B cells
100
75
Short Exposure
100
75
Long Exposure
IP: αTDRD3
Blot: αTDRD3

Figure S7


A
Aid -/-
WT
Tdrd3 -/-
Exp 1
Exp 2
0.01
11.5
11.4
12.3
10.8
0.01
11.3
10.4
9.4
11.0
IgG1
FSC
B
NS (p= 0.58)
Relative CSR to IgG1
1.5
1.0
0.5
0.0
Aid -/-
WT
Tdrd3 -/-
# of mice analyzed
2
5
5

Supplemental Figure Legends

Figure S1. TDRD3 interacts with TOP3B in the nucleus, Related to Figure 1.

MCF7 cells were subjected to nuclear and cytoplasm fractionation. The fractions were detected with the indicated antibodies. The nuclear fraction was subjected to immunoprecipitation using anti-TDRD3 antibody. The immunoprecipitated samples were detected with anti-TOP3B antibody.

Figure S2. Binding with methylated peptides does not affect the interaction between TDRD3 and TOP3B, Related to Figure 1.

GST pull-down assays were performed using recombinant GST-TOP3B and TDRD3 proteins with the addition of 15 ug of either unmodified or arginine-methylated peptides as indicated. Both input samples and pull-down samples were detected with anti-TDRD3 antibody (upper panel). The GST-TOP3B proteins in the pull-down samples were visualized by coomassie staining (lower panel).

Figure S3. Loss of TDRD3 destabilizes TOP3B, Related to Figure 3.

(**A**) Transient knock-down TDRD3 expression reduces TOP3B protein level. HeLa cells were transfected with either control ezRNA targeting GFP or TDRD3 ezRNA for 72 hours. Both TDRD3 and TOP3B levels are detected by Western blot.

(**B**) The TDRD3 Tudor domain mutant does not interact with methylated histone peptides. Biotinylated H3R17me2a and H4R3me2a peptides were incubated with purified GST, GST-Tudor and GST-Tudor (E691K) proteins. Pull-down samples were detected by Western blot using αGST antibody. Peptide loading was detected with straptavidin-HRP.

Figure S4. TDRD3 functions as a coactivator for gene transcription regulation, Related to Figure 4.

(**A**) and (**B**) The mRNA levels of 11 randomly selected snoRNAs genes, in control and TDRD3 knockdown MCF7 cells, were detected by RT-qPCR. Error bars represent standard deviation calculated from triplicate qPCR reactions.

(**C**) Raw reads of the ChIP-seq tracings for c-MYC gene. The regions (R1, R2, R3, R4) that were subjected to qPCR in (**B**) are depicted.

(**D**) Chromatin immunoprecipitation experiment was performed in control and TDRD3 knockdown MCF7 cells using αTDRD3 antibodies. The ChIP DNA was analyzed by qPCR with primers for the indicated regions in (**A**).

(**E**) Raw reads of the ChIP-seq tracings for DDX5 and NRAS genes (our published data, Yang et al., 2010). The regions that were subjected to qPCR in (**D**) are depicted.

(**F**) Chromatin fractions from MCF7 cells were immunoprecipitated with αPRMT1, αCARM1, αPRMT6, αH4R3me2a, αH3R17me2a, αH3R2me2a, αTDRD3, αTOP3B and control antibodies. The ChIP DNA was analyzed by qPCR with primers for the indicated regions in (**C**). Primers amplifying MYADM are used as negative control.

Figure S5. TOP3B resolves negative supercoiled DNA and reduces R-loop formation *in vitro*, Related to Figure 5.

(**A**) Purification of Flag-TDRD3 and Flag-TOP3B from insect Sf21 cells. The purified proteins are identified by Western blot (αFlag).

(**B**) TOP3B relaxes negatively supercoiled DNA. Reaction mixtures containing negatively supercoiled plasmid DNA (pUC19) were incubated with purified human TOP3B protein at various molar ratio (DNA:Enzyme from 1:3 to 1:48) at 37º C for 1 hour. *E. coli* Topo1 was used as positive control. The arrows indicate the negative supercoiled and relaxed forms of substrate DNA.

(**C**) Reaction mixtures containing negatively supercoiled plasmid DNA (pUC19) were incubated with purified human TOP3B protein at 1:60 molar ratio (DNA:Enzyme) for the indicated time points at 37º C. *E. coli* Topo1 was used as a positive control. The arrows indicate the negative supercoiled and relaxed forms of substrate DNA.

(**D**) TDRD3 does not influence TOP3B topoisomerase activity *in vitro*. Reaction mixtures containing negatively supercoiled plasmid DNA (pUC19) were incubated with purified GST, TDRD3 (DNA:protein from 1:30 to 1:60 in molar ratio), and TOP3B (DNA:enzyme at 1:24 molar ratio) proteins as indicated. The arrows indicate the negative supercoiled and relaxed forms of substrate DNA.

(**E**) (**F**) and (**G**) Methylated H3, H4 and RNA polymerase II CTD peptides do not influence TOP3B topoisomerase activity *in vitro*. Reaction mixtures containing negatively supercoiled plasmid DNA (pUC19) were incubated with purified TDRD3 (DNA:protein at 1:30 in molar ratio), either unmodified or arginine methylated peptides (TDRD3:peptide at 1:5 in molar ratio) and TOP3B protein (DNA:enzyme at 1:24 in molar ratio) as indicated. The arrows indicate the negative supercoiled and relaxed forms of substrate DNA.

(**H**) Work flow of *in vitro* R-loop detection using DRIP-qPCR. R-loop prone regions were cloned into pBluescript vectors. The plasmid was transcribed *in vitro* using T3 RNA polymerase under standard conditions. The transcription products, which contain DNA template, free RNA product and DNA/RNA hybrid, were then subjected to RNaseA digestion to remove RNA. The remaining samples were equally divided and either treated or not treated with RNaseH. The final products, together with untranscribed control, were run on an ethidium bromide gel for visualization or subjected to DRIP-qPCR with DNA/RNA hybrid antibody (S9.6) for quantification.

(**I**) An example of R-loop detection using DRIP-qPCR. The pFC53 plasmid, which contains *Airn* R-loop prone sequence (Ginno et al., 2012), was subjected to go through the work-flow as described in (**H**). On the left, ethidium bromide gel visualization of R-loop formation, showed a characteristic shift in mobility compared to untranscribed or RNase H-treated samples. On the right, DRIP-qPCR, using primers specific for *Airn* R-loop sequence, indicated the formation of R-loops and RNase H treatment disrupted these structures.

(**J**) TOP3B reduces co-transcriptional R-loops generated on a pFC53 DNA template. pFC53 plasmid was subjected to *in vitro* transcription in the presence of TDRD3, TOP3B and *E. coli* Topo1 as indicated. DRIP-qPCR was performed to quantify the co-transcriptional R-loop levels in individual samples. The immunoprecipitated DNA samples (DRIP DNA) were analyzed by qPCR. The experiments were independently performed three times. Error bars represent standard deviation calculated from triplicate qPCR reactions of one representative experiment.

(**K**) DRIP experiment was performed on genomic DNA from parental cells, and TDRD3 induce knockdown MCF7 cells. The DRIP DNA samples were analyzed by qPCR using primers indicated in **Figure S4C**.

(**L**) and (**M**) DRIP experiment was performed on RNase H treated or not treated (- or +) genomic DNA from parental cells, and TDRD3 induce knockdown MCF7 cells. The DRIP DNA samples were analyzed by qPCR using primers indicated in **Figure S4E**.

(**N**) TDRD3 inducible knockdown MCF7 cells were treated with either vehicle or Doxycycline (Dox) (1ug/ml) for 6 days before cells were transfected with HA-RNaseH1. Cell lysates were prepared 36 hours after transfection, and western blot was carried out to detect TDRD3 and RNase H1 expression (left). Genomic DNA from cells described above was subjected to DRIP experiment using S9.6 antibody. The DRIP DNA samples were analyzed by qPCR using primers described in **Figure 5E** (right).

(**O**) c-MYC mRNA expression level in the cells describe above was detected using RT-qPCR. Error bars represent standard deviation calculated from triplicate qPCR reactions.

(**P**) Total cell lysates (left panel), as well as soluble and chromatin fractions (right panel) were prepared from MCF7 cells transiently transfected with HA-RNaseH1. The expression levels of TDRD3 and HA-RNaseH1 were detected by Western blot analysis. αHistone H3 blotting shows the quality of soluble/chromatin fractions and equal loadings.

(**Q**) Chromatin fractions from the cells described above were subjected to ChIP experiment using control and TDRD3 antibody. The ChIP DNA samples were analyzed by qPCR with primers described in **Figure 4** (**C**).

Figure S6. *Tdrd3* knockout mouse and cell line analysis, Related to Figure 6.

(**A**) Two wild-type and two *Tdrd3*$^{-/-}$ primary MEF cells were treated with Bleomycin (20 ug/ml, 1 hour) to induce DNA damage. Histone extracts from these cells were subjected to Western blotting with an anti-γH2AX antibody. Western blot detection of Histone H3 serves as loading controls. Band intensities were quantified and normalized relative to their respective histone H3 bands. Quantification was expressed as folds of the lowest value.

(**B**) Elevated *Igh Su* R-loop in *Tdrd3*$^{-/-}$ B cells. R-loop DRIP experiments were carried out with naïve B cells cultured in IL-4 and LPS for 72 hours. *Su* R-loops were detected use the indicated PCR primers. Two mice from each group were tested. *** = p-value less than 0.001.

(**C**) *Tdrd3*$^{-/-}$ mice are hypomorphic. B cells from both wild-type and *Tdrd3*$^{-/-}$ mice were lysed and the cell lysates were immunoprecipitated with αTDRD3 antibody. The eluted samples were loaded on SDS-PAGE gel and subjected to Western analysis with an αTDRD3 antibody.

Figure S7. *Ig*-CSR in *Tdrd3* knockout mice, Related to Figure 7.

(**A**) Tdrd3 does not influence immunoglobulin class switch recombination. Representative flow cytometry dot plots of IgG1 expression on splenocytes from wild-type, *Tdrd3*$^{-/-}$ or *Aid*$^{-/-}$ mice following a 3-day culture with LPS and IL-4. The relative percentage of cells expressing IgG1 is in the upper right corner of each plot.

(**B**) Summary of relative CSR to IgG1 in cultured splenocytes. Averages represent triplicate cultures from five separate spleens in two independent experiments. P value (p=0.58) was determined by a two-tailed t test assuming unequal variance.

Table S2. Primers information

RT-qPCR primers	
NRAS	CTACAGGGAGCAGATTAAGCG
	TAACTCTTGGCCAGTTCGTG
DDX5	TGATTTGGAGAGAGGTGTGG
	TTCAAAGCCCATATCAAGCA
c-MYC	TTCTCTCCGTCCTCGGATTCTCTG
	TCTTCTTGTTCCTCCTCAGAGTCG
TOP3B	AGATTGATGCAGAGCTGGTG
	TTCCTCTTGAACACGTCCAG
SNORA7A	CGCATCTGGAGAGTGCCTA
	CTGTCGCAGAGTGTCTTCCA
SNORA10	TCTCAGCTCCGCTTAACCAC
	TTGTCGCACTCCTAGGAACA
SNORA55	AGCACCTGAATCTTTCCCATT
	CTGTAGAGACAGGCCCCAAG
SNORA57	CCCGTAATGTACGGAGGAAG
	GGCGTTTGAGGATAGAACCA
SNORA64	CTCGGCTCTGCATAGTTGC
	CAAGGAAAGAGAGGCCACAG
SNORD12	GCCTTTGCAGCTGATGATAC
	GCCAATGCATCAGACAAAAC
SNORD35A	GGCAGATGATGTCCTTATCTCA
	GGCATCAGCTAAGCCATTG
SNORD55	GTGTATGATGACAACTCGGTAATG
	AGCTCTCCAAGGTTGGCTTC
SNORD56	AATGTCAATAGTTTTCATCAACAGC
	CCACTCAGACCCAAAGTATCG
SNORD74	GCCTCTGATGAAGCCTGTGT
	CCACCATCAGAGCGGTTG
SNORD104	GGCCTGCTGTGATGACATT
	GCAGGCTCAGACTCCAGTTC

ChIP-qPCR and DRIP-qPCR primers	
c-MYC (human) R1	GAGGCTATTCTGCCCATTTG
	GGTGCTTACCTGGTTTTCCA
c-MYC (human) R2	CAAGCCGCTGGTTCACTAAG
	CCTTGCAGAGCTCTCCCCTA
c-MYC (human) R3	GGCTGGATACCTTTCCCATT
	AAGCCCAAGGTTTCAGAGGT
c-MYC (human) R4	GGCAAATATATCATTGAGCCAAA
	GAAGGGGCAATTGATGAAAA
DDX5	GTGTCATCGGTGTCCTTCCT
	ACTCGAATAACCCGACATGG
NRAS	CGTTTCACTGATGCCAGAAA
	TCCTTCCCATTCTCCCTTCT
MYADM	TGCATCTACATCCGCAAAAG
	AGAGTGGACGCTGCAGAAAT
EGR1	GAACGTTCAGCCTCGTTCTC
	GGAAGGTGGAAGGAAACACA
c-Myc (mouse)	GCGATCAGCTCTCCTGAAAA
	ACACAGGGAAAGACCACCAG
Igh Su (mouse)	TAGTTTAGCTTAGCGGCCCA
	GGGCTTCTCTGAGTGCTTCT
Translocation PCR primers (derivative chromosome 15)	
First Round	ACTATGCTATGGACTACTGGGGTCAAG
	GTGAAAACCGACTGTGGCCCTGGAA
Second Round	CCTCAGTCACCGTCTCCTCAGGTA
	GTGGAGGTGTATGGGGTGTAGAC
Southern Blot Probes (derivative chromosome 15)	
c-Myc	GGACTGCGCAGGGAGACCTACAGGGG
Igh	GAGGGAGCCGGCTGAGAGAAGTTGGG

Supplemental Experimental Procedures

Plasmids, Antibodies, Peptides

GFP-TDRD3 and its truncated constructs have been described before (Yang et al., 2010). NTAP-TDRD3 used for tandem affinity purification was generated by cloning TDRD3 cDNA into pCeMM NTAP(GS) vector (EUROSCARF). GFP-TDRD3 (E691K) was generated by introducing a Glu (E)-to-Lys (K) mutation at amino acid 691 site of TDRD3 using site-directed mutagenesis (Agilent Technologies). GST-TDRD3 and GST-TOP3B constructs used in the pull-down experiments were generated by cloning TDRD3 and TOP3B cDNA into pGEX-6P-1 vector (GE Healthcare Life Sciences). GFP-TOP3B and its truncated constructs were generated by cloning TOP3B cDNA into pEGFP-C1 vector (Clontech). Human RNase H1 cDNA devoid of Mitochondria localization signal was cloned into pcDNA3 vector. pFC53 and Myc R-loop prone plasmids were generated by cloning Airn and c-Myc genomic sequences into pBluescript vector (Agilent Technologies). Baculovirus expressing constructs were generated by cloning TDRD3 and TOP3B cDNA into pBacPAK8 vector (Clontech). H3R17me2a and H4R3me2a peptides have been described before (Yang et al., 2010).

Mice and Cell lines

Tdrd3 knockout mice were generated from a targeted mES clone RRK474 (obtained from BayGenomics) by the Genetically Engineered Mouse Core Facility of the M. D. Anderson Cancer Center. The *Aid* knockout mice have been described before (Ramiro et al., 2004). MCF7 cells, MCF7-tet-on-shCARM1, Human VMRC-LCD cells, and GFP/GFP-TDRD3 stably expressing LCD cells have been described before (Yang et al., 2010). Tet-on-shTDRD3 construct was generated by cloning TDRD3 targeting sequences 5'-AGCATCGAGGCAAGCTCTTATGGATAATG-3' into tetracycline-regulated pSUPERIOR vectors (OligoEngine). MCF7 cells were stably transfected with tet-on-shTDRD3 construct followed by selection of single clones using Western blotting analysis. TAP-TDRD3 was stably transfected into HEK293 cells to purify the protein complex. HeLa cells were used to map the interaction domains of TDRD3 and TOP3B, because they have a higher transfection efficiency than MCF7 cells. MCF7 cells were used for the ChIP and DRIP experiments, because TDRD3 was identified as a coactivator of the estrogen receptor. The TDRD3 ChIP-seq data had previously been generated in MCF7 cells. Wild type and Tdrd3 knockout MEF cells were generated from E12.5 mouse embryo following standard MEF generation protocol and the primary MEFs were cultured in complete DMEM media supplied with 10% FBS. Spontaneously immortalized MEFs

were created according to standard 3T3 protocol. Immortalized *Carm1* (flox/flox) MEF cells were transfected by a plasmid DNA encoding estrogen receptor-CRE fusion protein (ER-CRE) and a blasticidin selection marker. Stable cell lines were selected with 3 μg/ml of blasticidin. The stable clones were treated with the estrogen receptor ligand OHT to induce relocalization of the ER-CRE fusion protein and deletion of *Carm1* gene. ER-CRE stably transfected PRMT1$^{flox/-}$ MEF cells were kindly provided by Dr. Stephane Richard.

Tandem Affinity Purification

HEK293 cells were stably transfected with NTAP-TDRD3. A total of 3 x 10^8 cells were used for tandem affinity purification. In brief, cells were first lysed in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 125 mM NaCl, 5% glycerol, 0.2% NP-40, 1.5 mM MgCl2, 25mM NaF, 1 mM Na3VO4 and protease inhibitors. After centrifuge, the supernatant was incubated with rabbit-IgG sepharose (GE Healthcare Life Sciences) at 4º C for 4 hours. The bound beads were first washed with lysis buffer and then TEV-protease cleavage buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.2% NP-40). The bound protein complexes were eluted by the addition of 50 μg TEV protease (4º C, O/N). TEV-protease cleavage products were then incubated with Straptavidin agarose (Millipore) at 4º C for 2 hours. The bound proteins were eluted by boiling in SDS sample buffer. Samples were loaded on SDS-PAGE gel followed by either silver staining or SYPRO Ruby staining. After comparison with control samples, differential bands were cut from the gel and protein IDs were identified by LC-MS/MS.

Immunoprecipitation

The immunoprecipitation experiments were performed as described in (Yang et al., 2010), except that the co-immunoprecipitation of TDRD3 and TOP3B in the nucleus was carried out in buffer containing 350mM NaCl.

Chromatin Fractionation

LCD cells stably transfected with GFP, GFP-TDRD3 and GFP-TDRD3 (E691K) were harvested and lysed in buffer containing 10 mM Pipes, (pH 7.0), 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 0.1% Triton X-100, protease inhibitors and phosphatase inhibitors. After centrifuge at 1,300g for 5 minutes at 4º C, the pellet contained chromatin and associated factors. The pellet was then digested with Benzonase (Sigma) at 37º C for 20 minutes. The final samples were loaded on SDS-PAGE gel and detected by Western blots.

Histone Extraction

MEF cells were first lysed in NETN buffer (100 mM NaCl. 20 mM Tris-Cl (pH 8.0). 0.5 mM EDTA. 0.5% (v/v) NP-40) supplied with protease inhibitor cocktail (Roche) for 20 minutes at 4º C. Cell pellets were collected by centrifuge at 6000 rpm for 5 minutes, and dissolved in 0.8N HCl. After a brief sonication, the samples were placed on ice (or at 4º C) for 3 hours to help dissolve histones. The supernatant was then collected by centrifuge at maximum speed for 15 minutes and neutralized with 1.5M Tris-Cl (pH8.8).

Cytoplasm and Nuclear Fractionation

Cytoplasmic and nuclear fractions were prepared following the protocol as described in the instructions from Pierce Biotech.

Glycerol Gradient Sedimentation

Cells were lysed in buffer containing 50 mM Tris HCl, (pH 7.5), 300 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 1.5 mM MgCl2, 60 mM β-glycerophosphate, 25mM NaF, 1 mM Na3VO4, protease inhibitor (Roche), sonicated and centrifuged. The clarified protein lysates were loaded on a 15-35% continuous glycerol gradient prepared in 20mM Tris (pH8.0), 5mM MgCl2, 100mM KCl, 0.1%NP-40 and centrifuge in a swinging bucket SW 50.1 type rotor (Beckman) at 200,000g for 16-18 hours at 4º C. Protein complexes from all gradient fractions were analyzed by western blot analysis using anti-TDRD3 and anti-TOP3B antibodies.

GST Pull-down

GST, GST-TDRD3 and GST-TOP3B were expressed and purified from E.coli. GST tag on GST-TDRD3 was then cleaved by PreScission cleavage to get untagged TDRD3 protein. The pull-down was performed in buffer containing 50 mM Tris HCl, (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 1.5 mM MgCl2, 5% glycerol, protease inhibitor (Roche). Overnight bound proteins were then incubated with Glutathione Sepharose (GE Healthcare Life Sciences) for 1 hour at 4º C. The eluted samples were loaded on SDS-PAGE gel and detected by Western blots using anti-TDRD3 antibody.

In Vivo Ubiquitination Assay

Cells were treated with 10 uM MG132 for 16 hours prior to harvest. Cell pellets were lysed in 1x RIPA buffer (20 mM Tris HCl, (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor) at 4º C for 1 hour. The lysates were sonicated on ice and clarified

by centrifugation followed by preclearing with protein G agarose. The lysates were subsequently immunoprecipitated with the anti-TOP3B antibody. Immunoprecipitated proteins were analyzed by Western blots using anti-Ubiquitin, anti-TDRD3 antibodies. Anti-p53 blot was used as positive control for proteasome inhibition efficiency. Anti-Actin was used as equal loading control.

Chromatin Immunoprecipitation (ChIP)

The chromatin immunoprecipitatioin assay has been described before (Yang et al., 2010). In brief, MCF7 cells were crosslinked and lysed followed by chromatin immunoprecipitation with anti-PRMT1, anti-CARM1, anti-PRMT6, anti-H4R3me2a, anti-H3R17me2a, anti-H3R2me2a, anti-TDRD3 and anti-TOP3B antibodies. The primers used for qPCR are listed in the supplemental table.

In Vitro Transcription for R-loop formation

In vitro transcription of R-loop formation has been described before (Ginno et al., 2012). In brief, either pFC53 or Myc R-loop prone plasmids were in vitro transcribed with T3 RNA polymerase at 37º C for 1 hour before the reaction was inactivated at 65º C for 10 minutes. Samples were equally divided and either treated with RNase A plus RNase H or RNase A only at 37º C for 30 minutes followed by proteinase K treatment at 37º C for 30 minutes. Samples were then cleaned up with phenol/chloroform extraction. Precipitated DNA was dissolved in water.

DNA/RNA Immunoprecipitation (DRIP)

The DRIP approach was modified from published studies (Ginno et al., 2012; Skourti-Stathaki et al., 2011).

In vitro DRIP - *In vitro* transcription products were incubated with S9.6 antibody in binding buffer (10 mM NaPO4 pH 7.0, 140 mM NaCl, 0.05% Triton X-100) at 4º C overnight. The binding mixture was then incubated with protein A/G agarose for an additional 1 hour. The bound DNA was eluted with buffer containing 50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS and 300 μg of proteinase K at 50º C for 30 minutes. Samples were phenol/chloroform extraction and ethanol precipitation. Immunoprecipitated DNA was subjected to analysis by qPCR.

In vivo DRIP - Total nucleic acids were extracted from MCF7, MEFs, and activated mouse B cells by SDS/proteinase K treatment at 37º C overnight, followed by phenol/chloroform extraction and ethanol precipitation. DNA was fragmented using restriction enzymes HindIII, EcoRI, BsrG1, XbaI and Ssp1 and pretreated (where indicated) with RNase H overnight. After phenol/chloroform extraction and ethanol precipitation, 4 ug of each samples were subject to

immunoprecipitation with 10 ug of S9.6 antibody. Immunoprecipitated DNA was analyzed by qPCR using primers listed in the supplemental table.

Quantitative PCR (qPCR)

Quantitative PCR has been described before (Yang et al., 2010).

Southern Blot

Southern blot analysis was used for both mouse genotyping and detection of *c-Myc/Igh* translocations. Mouse embryonic stem cell (mES) clones obtained from BayGenomics were given to the Genetically Engineered Mouse Core Facility of the M. D. Anderson Cancer Center for blastocyst injection and chimeric mouse production. Approximately 600 bp of intronic TDRD3 sequence downstream of the trap cassette insertion site was used as a probe to detect differential signals between wild type and *Tdrd3* knockout mice. The mouse genomic DNA was isolated and digested with restriction enzyme Bgl II.

Southern blots were used to confirm amplified translocations. Southern blot probes were designed internally to the primers used in the PCR assay. Each of 10 ul PCR products were run on 1% agarose gel followed by transferring. The membrane was hybridized with either *Igh* or *c-Myc* probe at 42º C overnight followed by 3 times of washing at 62º C.

γH2AX Foci Counting

Both wild type and TDRD3 knockout primary MEFs were grown on glass coverslips to desired confluence before fixation. Cells were rinsed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. After block with 20% newborn calf serum, cells were incubated with anti-γH2AX antibody (Ser139) in 1:2000 at 4º C overnight. Cells were then stained with secondary antibody Alexa Fluor 555, and DAPI sequentially. The coverslips were then sealed and examined in a fluorescence microscope. At least 400 cells were imaged for each genotype and the data was processed with ImageJ software.

ezRNA Knockdown

ezRNA used for transient knockdown of Tdrd3 was designed using the website-http://cluster-12.mpi-cbg.de/cgi-bin/riddle/search. The preparation of dsRNA was followed according to the instructions of MEGAscript® RNAi Kit (Invitrogen). dsRNA was then digested with RNase III to

produce a blender of 18-30 bp siRNA mixture. After purification, the ezRNA was dissolved in H_2O and tested for knockdown efficiency at 6-well plate scale.

Baculovirus Expression and Protein Purification

Flag-tagged TDRD3 and TOP3B cDNA was cloned into a pBacPAK8 vector (Clontech) and cotransfected with linearized Baculovirus DNA (BD Biosciences) using Fugene 6 transfection reagent (Roche). The expression and purification of recombinant proteins were followed according to the instruction manual of Baculovirus Expression Vector System (6^{th} Edition, May 1999), except that Flag M2 beads and Flag peptide (Sigma) were used to bind and elute the recombinant proteins, respectively. Purified proteins were then dialyzed in 1X TBS buffer (50 mM Tris-Cl, pH 7.5. 150 mM NaCl).

In vitro Topoisomerase Assay

The reaction mixture for the DNA relaxation assay consisted of 40 mM Tris-Cl, pH 7.5, 1 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mg/ml BSA, and 400 ng of negative supercoiled pUC19 DNA in a 30-ul reaction system. The incubation was preceded at 37º C at the indicated time. The DNA: Enzyme ratios are indicated. The reaction was stopped with 4 ul of stop solution (1% SDS, 50% glycerol, 0.05% bromphenol blue). The sample was then loaded onto a 0.8% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV illumination.

Lymphocyte Cultures, Ig-CSR and Translocation Assays

B lymphocyte isolation, cultures and CSR to IgG1 analysis were as described previously (McBride et al., 2008). In brief, resting B cells were purified from mouse spleens by depletion with anti-CD43 beads (Miltenyi Biotec) and cultured in RPMI 1640 medium with 5 ng/ml IL-4 (R&D Systems) plus 25 ug/ml lipopolysaccharide (LPS) (Sigma-Aldrich) for 72 hours. For class switch analysis, cells were stained with APC labeled anti-mouse IgG1 (BD Biosciences) and analyzed by flow cytometry. Triplicate cultures from 5 individual spleens were evaluated for IgG1 expression in two independent experiments.

The translocation assay, primers, probes and amplification conditions were previously described (Gazumyan et al., 2011; Ramiro et al., 2004). Cells were isolated and cultured as in the CSR assay and genomic DNA was harvested after 72 hours. Forty-eight separate PCR reactions were performed, each with DNA from 10^5 cells and primers that amplify “derivative 15” *c-*

Myc/Igh translocations (Ramiro et al., 2004). Amplified translocations were confirmed by Southern blots with probes internal to the primers used in the PCR assay (Ramiro et al., 2004). The experiment was performed on cultured B cells from the spleens of six mice in three independent experiments. A lane was considered to have a translocation if an amplified band was positive for both *Igh* and *c-Myc* probes. A two-tailed Fisher's exact test was used to calculate p value.

Bioinformatics and Computational Analysis

Landscape of ChIP-Seq Data - Sequenced DNA tags were mapped to human genome hg18 using ELAND (Illumina Analysis Pipeline) and uniquely mapped tags were kept. To avoid PCR bias, for the multiple tags that were mapped to the same genomic location, only one copy was retained. Each tag was extended by 150bp to its 3' end. The number of tags on each genomic position was rescaled to normalize the total number of mapped tags to 10M and averaged over every 10bp window. The normalized values were displayed using UCSC genome browser (http://genome.ucsc.edu/).

Classification of TSS by its association with TDRD3 and CpG island - The RefSeq gene database was downloaded from UCSC Genome Browser (hg18) in August 2013. In totall, there are 28875 unique transcription start sites (TSSs) in chromosome 1-22, X and Y. For each TSS, it was classified as TDRD3-associated if TDRD3 peaks at TSSs, and CGI-associated if there is CpG island at TSSs. The list of CpG islands was downloaded from UCSC Genome Browser (hg18).

Statistics

Fisher exact test was performed to determine the p-value for translocation experiments. Student *t*-test was performed to determine the p-value for ChIP-qPCR and DRIP-qPCR experiments. Asterisks were used to describe values of statistical significance. * stands for p-value ranges between 0.01 to 0.05; ** stands for p-value ranges between 0.001 to 0.01 and *** stands for p-value less than 0.001.

Supplemental References

Gazumyan, A., Timachova, K., Yuen, G., Siden, E., Di Virgilio, M., Woo, E.M., Chait, B.T., Reina San-Martin, B., Nussenzweig, M.C., and McBride, K.M. (2011). Amino-terminal phosphorylation of activation-induced cytidine deaminase suppresses c-myc/IgH translocation. Mol Cell Biol *31*, 442-449.

Ginno, P.A., Lott, P.L., Christensen, H.C., Korf, I., and Chedin, F. (2012). R-loop formation is a distinctive characteristic of unmethylated human CpG island promoters. Mol Cell *45*, 814-825.

McBride, K.M., Gazumyan, A., Woo, E.M., Schwickert, T.A., Chait, B.T., and Nussenzweig, M.C. (2008). Regulation of class switch recombination and somatic mutation by AID phosphorylation. J Exp Med *205*, 2585-2594.

Ramiro, A.R., Jankovic, M., Eisenreich, T., Difilippantonio, S., Chen-Kiang, S., Muramatsu, M., Honjo, T., Nussenzweig, A., and Nussenzweig, M.C. (2004). AID is required for c-myc/IgH chromosome translocations in vivo. Cell *118*, 431-438.

Skourti-Stathaki, K., Proudfoot, N.J., and Gromak, N. (2011). Human senataxin resolves RNA/DNA hybrids formed at transcriptional pause sites to promote Xrn2-dependent termination. Mol Cell *42*, 794-805.

Yang, Y., Lu, Y., Espejo, A., Wu, J., Xu, W., Liang, S., and Bedford, M.T. (2010). TDRD3 is an effector molecule for arginine-methylated histone marks. Mol Cell *40*, 1016-1023.