A genome-wide RNA interference screen identifies putative chromatin regulators essential for E2F repression

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Supporting Information

Files in this Data Supplement:

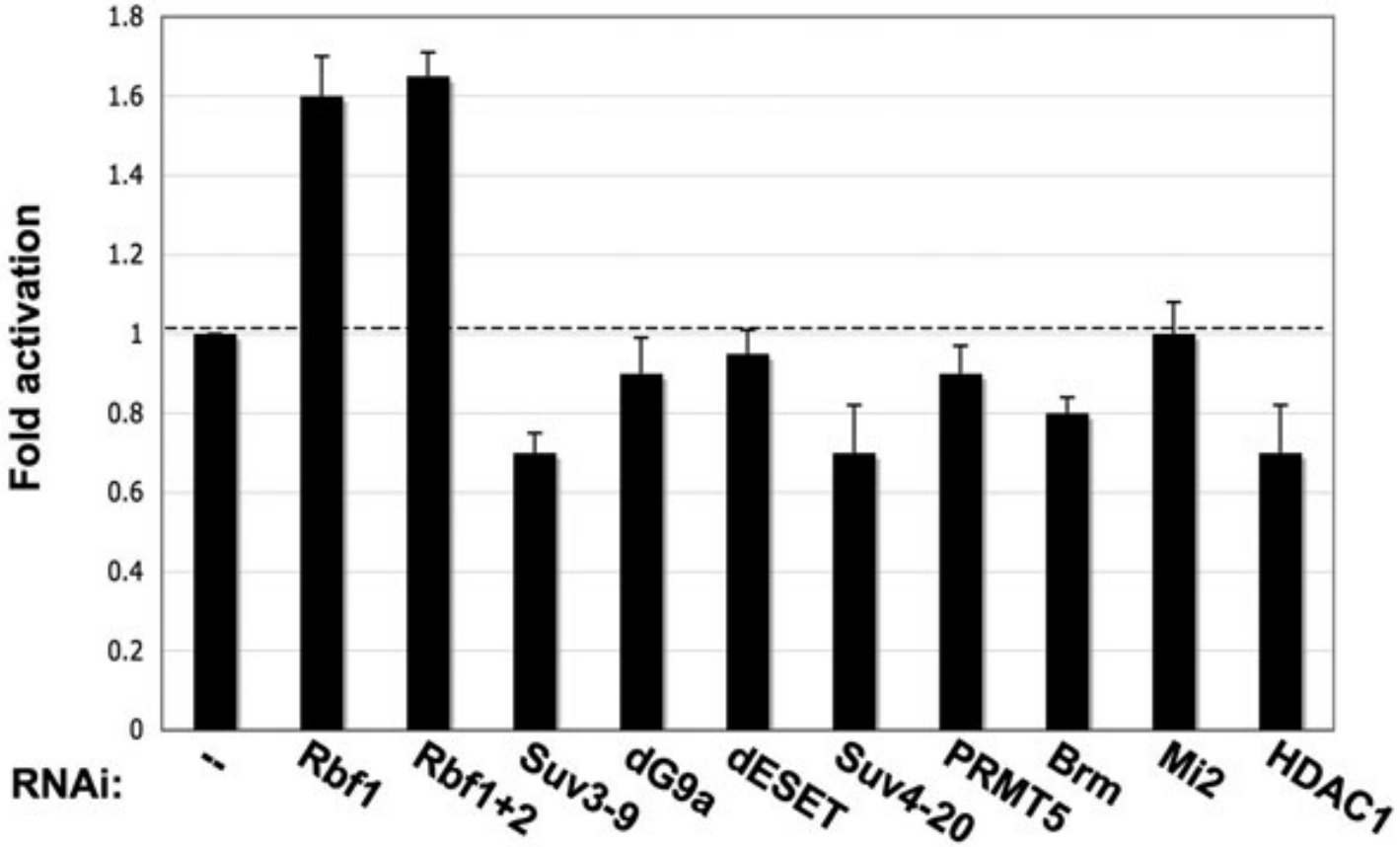

Fig. 6. Histogram shows the E2F reporter activity in cells incubated with dsRNAs of Rbfs and a variety of chromatin regulatory genes.

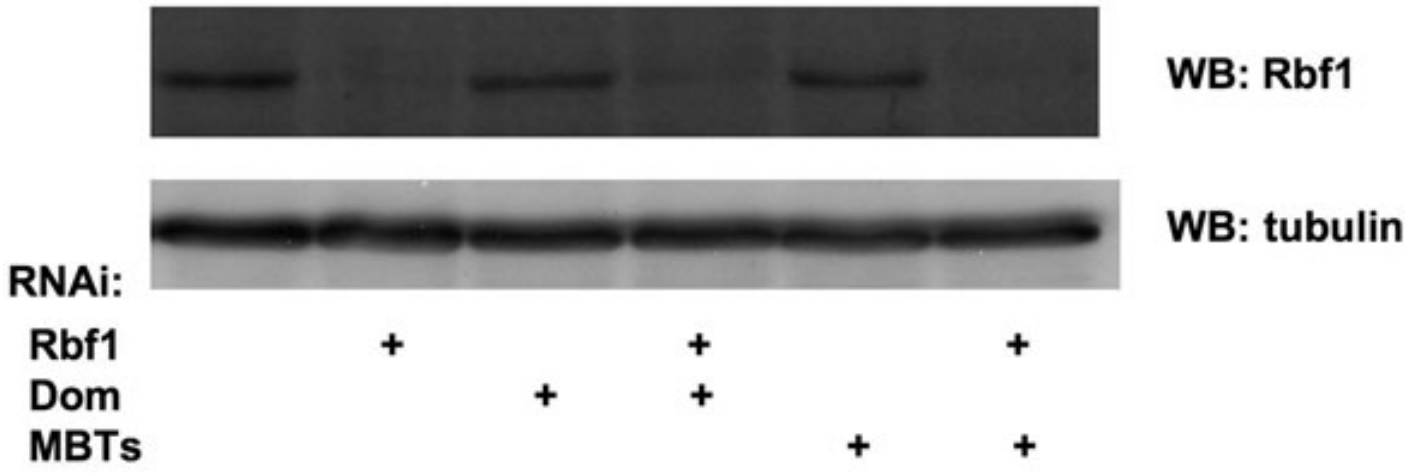

Fig. 7. Depletion of Rbf1 in S2* cells with RNAi treatment. S2* cells were incubated with a variety of dsRNAs for 6 days, and then subjected to Western blot analyses with the indicated antibodies.

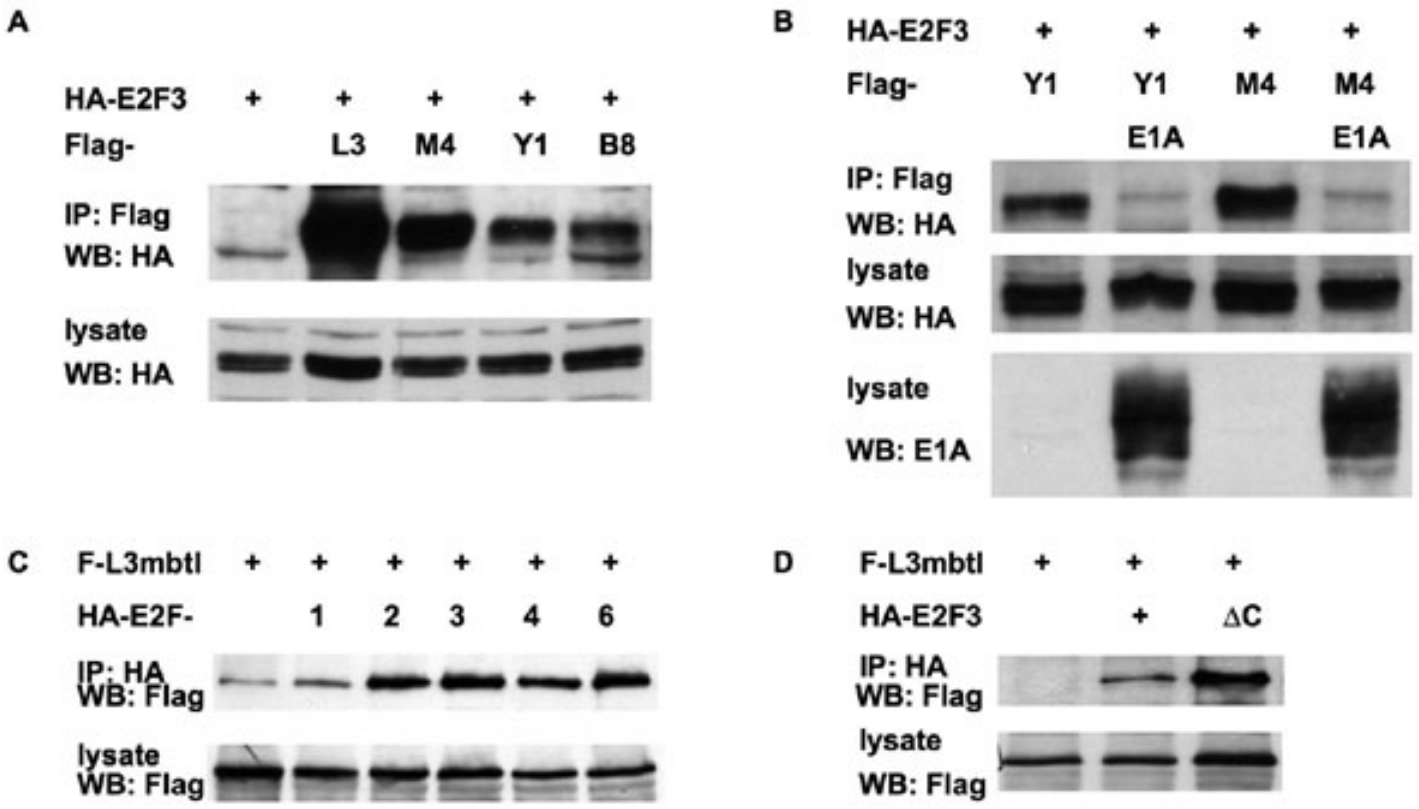

Fig. 8. Mammalian Dom and MBT proteins interact with E2F. (*A*) Mammalian MBT proteins and Dom complex associate with E2F. Whole-cell lysates made from COS7 cells transfected with HA-tagged E2F3 and Flag-tagged L3mbtl (L3), M4MBT (M4), YL1 (Y1), or Brd8 (B8) (see SI Table 1), were incubated with anti-Flag antibody and the pelleted material was examined for the presence of E2F3 by immunoblotting with HA antibody. (*B*) E1A disrupts interactions of E2F with MBT and Dom. E1A overexpression was included in experimental settings similar to *A*). (*C*) Multiple mammalian E2Fs interact with MBT. COS7 cells expressing Flag-L3mbtl together with HA-tagged E2F-1, -2, -3, -4, or -6, were subjected to immunoprecipitation with anti-HA antibody and Western blotting with anti-Flag Antibody. (*D*) The C-terminal Rb-binding domain of E2F is dispensable for its association with MBT. Cellular lysates from COS7 cells expressing Flag-L3mbtl and HA-tagged E2F3 or its truncated (DC) form, which lacks the entire C-terminal region including the Rb-binding motif, were immunoprecipitated with HA antibody. Co-precipitation of MBT protein was verified with anti-Flag immunoblotting

Table 1. Genes that negatively regulate the E2F-dependent reporter activity

Fly genes	Mammalian orthologs	Annotation
Group 1		
Rbf1	Rb	Tumor suppressor
Domino	SRCAP, p400	ATPase; chromatin remodeling complex

CG14514	Brd8	Bromo domain
CG4621	TCFL1/YL1	Suppression of transformation
L3mbt	L3mbtl, L3mbtl3, L3mbtl4	MBT domain; fly tumor suppressor
CG16975	Mbtd1, M4mbt/L3mbtl2	MBT domain
Group 2		
Cdc6	Cdc6	DNA replication
Cdc7	Cdc7	DNA replication
Dup	Cdt1	DNA replication
MCM7	MCM7	DNA replication
Group 3		
EAST		Nuclear endoskeleton assembly
CG7752		Zinc Finger; TRF2/DREF complex
CG9797		Zinc Finger
CG31329		FLYWCH Zinc Finger
CG3363		
CG12538		
CG17836		
AE003220_Sanger_7368		

Genes of Group 1 are highly conserved and implicated in transcriptional or chromatin regulation. To avoid potential off-target effect of RNAi, repression on E2F was verified with new sets of dsRNAs different from those used in the screen. As examined by FACS analysis, cells treated with RNAi of Rbf1, Dom, MBTs or the GFP control did not display notable changes in profiles of cell cycle distribution (not shown).

Genes of Group 2 are involved in initiation of DNA replication. RNAi of Dup results in strong growth arrest. E2F target genes are upregulated in fly *dup* mutants (1).
Genes of Group 3 are either nonconserved (no obvious mammalian orthologs) or unannotated.

Reference
1. Whittaker AJ, Royzman I, Orr-Weaver TL (2000) *Genes Dev* 14:1765-1776.

Table 2. List of known endogenous E2F target genes whose expression was upregulated after depletion of the MBT proteins (L3mbt and CG16975) or Dom complex (CG4621) in the *Drosophila* S2* cells

E2F targets	MBT RNAi	Dom RNAi	Annotation
Group A			
CG8484	1.6	-	Zinc finger
Group B			
CG14193	2.1	1.5	Chromo domain
Group C			
CG14545	23.7	4.4	
Group D			
CG6999	3.1	1.5	RNA binding
CG8399	4.2	-	
CG8316	15.8	5.7	
CG9427	7.0	4.2	
Group E			
Fcp3C/CG4015	3.5	2.6	Follicle cell protein 3C
CG14036	4.4	1.6	
Bgcn/CG30170	12.6	1.5	Helicase; germ cell development
CG4623	30.2	3.1	
CG2887	5.1	2.3	Heat shock protein, chaperone
Cyp28d1/CG10833	1.9	3.8	Cytochrome p450
CG9475	1.5	1.5	
Dip3/CG12767	6.0	4.3	Dorsal-interacting protein 3
CG3505	11.0	1.7	Chymotrypsin serine protease

CG7628	27.3	3.7	Phosphate transporter
CG3105	7.9	4.0	Protein serinethreonine kinase
CG17142	1.8	3.2	Calcium channel

Fly S2* cells were treated with RNAi for 6 days, and genome-wide changes in gene expression were monitored by a microarray assay with Affymetrix GeneChip (Drosophila Genome 2.0 Array). Grouping of E2F target genes was based on a previous report (1).

1. Dimova DK, Stevaux O, Frolov MV, Dyson NJ (2003) *Genes Dev* 17:2308-2320.

SI Text

RNAi screen

Drosophila S2* cells were maintained in Schneider's Drosophila Medium supplemented with 10% FBS and antibiotics. For the screen, S2* cells were transfected with the E2F-dependent and normalization control reporters and resuspended in serum-free Schneider's Medium at a density of 2.5-3.0 million cells/ml. Ten microliter of cells were aliquoted into 384-well dsRNA library plates using a MultiDrop liquid dispenser. Each well contained 5 ml of »0.05 mg/ul dsRNA in water. After 45 min of incubation at room temperature, 20 ml of Schneider's Medium supplemented with 15% serum was added. S2* cells grow in suspension, and they continue to divide even after 7 days of incubation with RNAi (generally about 30% of the cells remain in S phase). In our screen, the plates were incubated for 5 days at room temperature and cells were then subjected to luciferase assays (Dual-Glo kit, Promega). This time point was chosen for a balance between maximal depletion of targeted genes and minimal RNAi-induced changes in cell cycle distribution. The RNAi screen was carried out in duplicate.

RNAi treatment for luciferase reporter assays

Double-stranded RNAs were produced using a T7 RiboMax Express RNAi kit (Promega) with DNA templates amplified from *Drosophila* genomic DNA by PCR. Except for the screen, all RNAi experiments were performed in 24-well plates (each well was preloaded with about 1 m g dsRNA for each gene). Fly cells were transfected with the reporters before RNAi treatment and luciferase activity was determined using the Dual-Glo kit (Promega). All of the reporter assays were repeated more than four times, and similar reporter activation patterns were observed. Data were taken from duplicate experiments of 5-day incubation with dsRNAs.

Immunoprecipitation (IP) and western analysis

S2* cells, untransfected or two days after transfection with Flag-L3mbt, were lysed in IP buffer [20 mM Tris, pH 7.4/150 mM NaCl/0.5% Nonidet P-40/protease inhibitor mixture (Roche)]. Cleared lysates were used for IP and Western blot analyses by standard procedures. Flag antibody was purchased from Sigma.

Chromatin IP (ChIP) assay

ChIP assays were done essentially as previously described (1) and according to the instructions from an Upstate ChIP kit. About 2-5 ´ 10^7 S2* cells, untransfected or transfected with Flag-L3mbt, were cross-linked with formaldehyde for 10 min. at room temperature followed by 50 min. on ice. The cells

were washed with PBS, lysed in the lysis buffer provided in the kit and subjected to repeated sonication. The supernatants were immunoprecipitated by incubation with corresponding antibodies. The subsequent steps followed the ChIP kit manual. Antibodies for dimethylated H3K9 and acetylated H4 were purchased from Upstate.

1. Leach TJ, Mazzeo M, Chotkowski HL, Madigan JP, Wotring MG, Glaser RL (2000) *J Biol Chem* 275:23267-23272.