DREAM Interrupted: Severing MuvB from DREAM’s pocket protein in *Caenorhabditis elegans* impairs gene repression but not DREAM chromatin assembly

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Interrupting DREAM complex formation

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Abstract

The mammalian pocket protein family, which includes the Retinoblastoma protein (pRb) and Rb-like pocket proteins p107 and p130, regulates entry into and exit from the cell cycle by repressing cell cycle gene expression. Although pRb plays a dominant role in mammalian systems, p107 and p130 represent the ancestral pocket proteins. The Rb-like pocket proteins interact with the highly conserved 5-subunit MuvB complex and an E2F-DP transcription factor heterodimer, forming the DREAM (for Dp, Rb-like, E2F, and MuvB) complex. DREAM complex formation on chromatin culminates in direct repression of target genes mediated by the MuvB subcomplex. Here, we examined how the Rb-like pocket protein contributes to DREAM formation by disrupting the interaction between the sole Caenorhabditis elegans pocket protein LIN-35 and the MuvB subunit LIN-52 using CRISPR/Cas9 targeted mutagenesis. Disrupting the LIN-35-MuvB association did not affect DREAM chromatin occupancy but did cause a highly penetrant synthetic multivulval (SynMuv) phenotype, indicating that blocking DREAM assembly impairs MuvB function. Some DREAM target genes became derepressed, indicating that for those genes MuvB chromatin binding alone is not sufficient for gene repression and that direct LIN-35-MuvB association potentiates MuvB’s innate repressive activity. In a previous study we showed that in worms lacking LIN-35, E2F-DP and MuvB chromatin occupancy is reduced genome-wide. With LIN-35 present, this study demonstrates that the E2F-DP-LIN-35 interaction promotes E2F-DP’s chromatin localization, which we hypothesize supports MuvB chromatin occupancy indirectly through DNA. Altogether, this study highlights how the pocket protein family may recruit
regulatory factors like MuvB to chromatin through E2F-DP to facilitate their transcriptional activity.

**Introduction**

Members of the mammalian Retinoblastoma (Rb) protein family, pRb, p107, and p130, collectively called pocket proteins, serve key roles in regulating transcription during the cell cycle (Classon and Dyson 2001; Classon and Harlow 2002; Cobrinik 2005; Burkhart and Sage 2008; Dick and Rubin 2013). In mammalian cells, pRb interacts with activating E2F-DP transcription factor heterodimers (in mammals, E2F1/2/3-DP1/2), sequestering E2F-DP and preventing E2F-DP-mediated activation of early cell cycle genes (Helin et al. 1992; Lees et al. 1993; Liban et al. 2016). In contrast, the Rb-like proteins p107 and p130 interact with repressive E2F-DPs (in mammals, E2F4/5-DP1/2) and a highly conserved 5-subunit MuvB subcomplex (in mammals, LIN9, LIN37, LIN52, LIN54, and RBAP48), forming the 8-subunit DREAM transcriptional repressor complex (Korenjak et al. 2004; Lewis et al. 2004; Harrison et al. 2006; Litovchick et al. 2007; Schmit et al. 2007). When associated with the DREAM complex, MuvB mediates transcriptional repression of early and late cell cycle genes (Litovchick et al. 2007; Goetsch et al. 2017; Muller et al. 2017). DREAM functionally overlaps with pRb to repress cell cycle gene expression during cellular quiescence (G0) (Hurford et al. 1997; Litovchick et al. 2007; Muller et al. 2017). Upon progression into the cell cycle, pRb and the Rb-like pocket proteins are phosphorylated by CDK4/6-cyclin D, releasing their respective interaction partners and triggering activation of cell cycle genes (Telesco et al. 2002; Pilkinton et al. 2007; Burke et al. 2010). Thus, the association and dissociation of pocket proteins from their respective transcriptional
repressive complexes governs the switch between cell cycle quiescence and cell cycle progression.

The Rb-like homologs p130 and p107 represent the ancestral pocket proteins and likely the conserved components that mediate cell cycle control among eukaryotes (CAO et al. 2010; LIBAN et al. 2017). In C. elegans, LIN-35 is the sole pocket protein, most closely resembling p130/p107 (LU AND HORVITZ 1998). The pocket protein-associated complex MuvB was isolated in Drosophila melanogaster (KORENJAK et al. 2004; LEWIS et al. 2004) and Caenorhabditis elegans (HARRISON et al. 2006) before homologs were identified in mammals (LITOVCHICK et al. 2007; PILKINTON et al. 2007; SCHMIT et al. 2007). The C. elegans complex, called DRM, regulates cell cycle genes and requires MuvB to mediate gene repression (BOXEM AND VAN DEN HEUVEL 2002; GOETSCH et al. 2017). DRM also regulates cell fate specification by antagonizing Ras signaling during vulval development (MYERS AND GREENWALD 2005; CUI et al. 2006; HARRISON et al. 2006) and by protecting somatic cells from expressing germline genes (WANG et al. 2005; PETRELLA et al. 2011).

Extensive biochemical analyses have demonstrated how the DREAM complex forms on chromatin (Figure 1A) (LITOVCHICK et al. 2007; PILKINTON et al. 2007; SCHMIT et al. 2007; GUILEY et al. 2015). E2F-DP and LIN54, a MuvB component, direct site-specific chromatin localization (ZWICKER et al. 1995; SCHMIT et al. 2009; MULLER AND ENGELAND 2010; MULLER et al. 2012; MARCEAU et al. 2016). The Rb-like pocket protein serves as a bridge between the 2 DNA-binding DREAM components (GUILEY et al. 2015). The mammalian LIN52 protein interacts with the pocket protein via an “LxCxE motif” in LIN52. In mammals, the LxCxE motif is instead a suboptimal LxSxExL
sequence that is rendered optimal by phosphorylation of a nearby serine residue (S28) (GUILEY et al. 2015) (Figure 1B). S28 phosphorylation by DYRK1A kinase induces formation of mammalian DREAM (LITOVCHICK et al. 2011). In C. elegans, the conserved lin-52 gene encodes the optimal LxCxE sequence (Figure 1B). C. elegans lacks a DYRK1A homolog and its corresponding consensus motif RxSP in LIN-52 (Figure 1B), suggesting that in C. elegans a phospho-switch likely does not induce DRM formation (LITOVCHICK et al. 2011; GUILEY et al. 2015). Importantly, the LxCxE binding motif mediates the high-affinity interaction that is employed by the human papillomavirus (HPV) viral oncoprotein E7 to disrupt association of LIN52 with mammalian pocket protein (GUILEY et al. 2015).

Here, we assessed how the Rb-like pocket protein contributes to DREAM complex formation and function on chromatin. We previously reported that the absence of LIN-35 results in a genome-wide decrease in chromatin occupancy of both E2F-DP and MuvB, illustrating how DRM/DREAM disassembly likely proceeds during cell cycle progression (GOETSCH et al. 2017). The model of DREAM complex assembly centers on reintroduction of the pocket protein associations with E2F-DP and MuvB as cells finish the cell cycle. To test this model, we used CRISPR/Cas9-mediated genome editing of the C. elegans LIN-52 subunit of MuvB to sever the association of the pocket protein with the MuvB subcomplex. Disrupting the C. elegans LIN-35-MuvB association caused a highly penetrant synthetic multivulval (SynMuv) phenotype, indicating that DRM antagonization of Ras signaling in the developing vulva, and thus its transcription repressive activity, was impaired. Chromatin immunoprecipitation revealed that the chromatin association of E2F-DP-LIN-35 and MuvB was not impaired by loss of LIN-35-
MuvB association. Finally, transcript analyses revealed that only 2 of 10 tested target genes became derepressed, suggesting that direct LIN-35-MuvB association potentiates, but is not required for, MuvB-mediated transcriptional repression. Together, this study highlights how the scaffolding activity of the pocket proteins facilitates transcriptional repression of target genes.

**Materials and Methods**

**Worm strains**

Strains were cultured on Nematode Growth Medium (NGM) agarose plates with *E. coli* OP50 and incubated at 20°C. The following strains were used:

- **Wild type N2 (Bristol)**
- **SS1240:** *lin-52*(bn132(*lin-52p::TagRFP-T^SEC^3xFLAG::lin-52 3' UTR*)) III / *hT2G [bli-4(e937) let-?(q782) qIs48] (I:III)*
- **SS1241:** *lin-52*(bn133(*lin-52p::TagRFP-T::3xFLAG::lin-52 3' UTR*)) III / *hT2G [bli-4(e937) let-?(q782) qIs48] (I:III)*
- **SS1325:** *lin-52*(bn138(*lin-52::GFP^SEC^3xFLAG*)) III
- **SS1256:** *lin-52*(bn139(*lin-52::GFP::3xFLAG*)) III
- **SS1273:** *lin-52*(bn150(*lin-52[C44A]::GFP::3xFLAG*)) III
- **SS1276:** *lin-52*(bn151(*lin-52[L42A,C44A,E46A]::GFP:3xFLAG*)) III
- **MT10591:** *lin-8*(n2731)
- **MT1806:** *lin-15A*(n767)

**CRISPR/Cas9-mediated genome editing**

For all genomic edits, 20 nucleotide crDNA targeting sequences were identified using the MIT CRISPR design tool ([http://crispr.mit.edu](http://crispr.mit.edu)). Single guide RNA sequences
were cloned into the PU6::unc119_sgRNA vector (Addgene plasmid #46169) using the overlapping PCR fragment method described in (FRIEDLAND et al. 2013) or were cloned into pDD162 (Addgene plasmid #47549) using the Q5 Site Directed Mutagenesis Kit (New England Biolabs), as described in (DICKINSON et al. 2013). Homologous repair templates were cloned into pDD282 (Addgene plasmid #66823) or pDD284 (Addgene plasmid #66825) using Glibson Assembly (New England Biolabs) (GIBSON et al. 2009), as described in (DICKINSON et al. 2015). CRISPR/Cas9 component plasmids were co-injected with marker plasmids (FROKJAER-JENSEN et al. 2008) to identify strains with an extra-chromosomal array instead of a mutated endogenous gene. For targeted mutagenesis, dpy-10(cn64) sgRNA (pJA58, Addgene plasmid #59933), and dpy-10(cn64) ssDNA template, dpy-10(cn64) guide and ssDNA template were co-injected to select for positive CRISPR activity in injectant progeny, as described in (ARRIBERE et al. 2014). Additional details are provided in Supplemental Materials and Methods.

Microscopy

L4 larvae were mounted on a 10% agarose pad and immobilized in a 1-2 µL suspension of 0.1 µm polystyrene beads (Polysciences), as described in (KIM et al. 2013). Fluorescence images were acquired using a Solamere spinning-disk confocal system with µManager software (EDELSTEIN et al. 2014). The microscope setup was as follows: Yokogawa CSUX-1 spinning disk scanner, Nikon TE2000-E inverted stand, Hamamatsu ImageEM X2 camera, solid state 405-, 488-, and 561-nm laser lines, 435–485, 500–550, and 573–613 fluorescent filters, and Nikon Plan Fluor 40x air objective. Images were processed using Image J (SCHNEIDER et al. 2012).

C. elegans phenotype scoring
For brood size analyses, L4 individuals were cloned to fresh plates every 24 hours and all progeny were counted. For SynMuv phenotype scoring, 3 replicate plates per strain were set up with 5-10 adults that were allowed to lay eggs for 6 hours. Progeny were incubated at 20°C for 3 days, then scored for the presence or absence of pseudovulvae. The percentages of multivulva worms in each replicate population were averaged, and the standard deviation was calculated.

**Immunoblotting and co-immunoprecipitation (coIP)**

For immunoblotting whole worm lysates, 200 adults from each strain were picked into SDS gel-loading buffer (50 mM pH 6.8 Tris-Cl, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 100 mM β-mercaptoethanol). For coIP, embryos collected after bleaching gravid worms were aged for 3.5 hours and then frozen in liquid nitrogen, and lysates were prepared as described in (Goetsch et al. 2017). For each IP, 8 mg of protein lysate was mixed with antibody-conjugated Dynabeads (ThermoFisher) and incubated for 2 hours at 4°C. Proteins were separated by SDS/PAGE, and western blot analysis was performed using a 1:1,000-1:5000 dilution of primary antibody and 1:2,000 dilution of an appropriate HRP-conjugated secondary antibody. Serial western blot analysis was performed by stripping the blot with buffer containing 0.2M pH 2.2 glycine, 0.1% SDS, and 1% Tween-20 between antibody probings. Additional details are provided in Supplemental Materials and Methods.

**Chromatin immunoprecipitation (ChIP) and sequential ChIP**

Embryos collected after bleaching gravid worms were aged for 3.5 hours and then frozen in liquid nitrogen. Lysates were prepared by grinding, crosslinking for 10 minutes in 1% formaldehyde, and sonicating to an average size of 250 base pairs in FA
buffer (50 mM HEPES/KOH pH 7.5, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 150 mM NaCl) using a Bioruptor (Diagenode) on the high setting with 60 rounds of 30 seconds on and 1 minute rest. Protein concentrations of lysates were determined using a Qubit fluorometer. ChIP and sequential ChIP experiments were performed as described in (GOETSCH et al. 2017) and in Supplemental Materials and Methods. Quantitative PCR was performed using SYBR green reagents on an Applied Biosystems ViiA 7 Real-Time PCR System (ThermoFisher).

**Analysis of transcript levels by RT-qPCR**

Embryos collected after bleaching gravid worms were aged for 3.5 hours and then frozen in Trizol for RNA isolation. A total of 1 μg RNA was treated with DNase and reverse transcribed using the High Capacity cDNA Kit (Applied Biosystems). qPCR was performed using SYBR green reagents on an Applied Biosystems ViiA 7 Real-Time PCR System (ThermoFisher). The relative quantity of experimental transcripts was calculated with act-2 as the control gene using the ΔCt method.

**Quantification and statistical analysis**

For brood size analysis, significance was determined using a Wilcoxon-Mann-Whitney test comparing CRISPR/Cas9-genome edited strains to wild type (N2). For ChIP-qPCR and transcript level analysis by RT-qPCR, significance was determined using a student’s t-test between lin-52(WT) and lin-52(1A) and lin-52(3A) transgenic strains.

**Data and reagent availability**

Requests for information, strains, and reagents should be directed to and will be fulfilled by Paul D. Goetsch (pdgoetsc@mtu.edu). Primers used for cloning, ChIP-
qPCR, and RT-qPCR are available in Supplemental Table S1. All data necessary for confirming the conclusions present are represented fully within the article. Supplemental material is available at FigShare: XXX

Results

Targeted mutagenesis to disrupt DREAM complex formation

Structural studies previously demonstrated that MuvB interacts with the pocket protein via the LIN52 subunit (Figure 1A) (GUILEY et al. 2015). Using the self-excising cassette (SEC) method for C. elegans CRISPR/Cas9 genome editing (DICKINSON et al. 2015), we generated a lin-52(KO) strain (lin52(bn133[lin-52p::TagRFP-T::3xFLAG]) by completely replacing the lin-52 gene with TagRFP-T coding sequence (Figure 2A). We observed that lin-52(KO) rendered worms sterile (Figure 2C), as previously observed in the lin-52(n3718) protein null strain (CEOL et al. 2006; HARRISON et al. 2006). This resembles loss of other MuvB components, as loss of LIN-9, LIN-53 (C. elegans RBAP48), or LIN-54 in protein null strains also renders worms sterile and affects the levels of other MuvB subunits, suggesting that MuvB components require co-expression for assembly/stability of the complex (HARRISON et al. 2006). Loss of LIN-37 does not cause sterility and does not affect assembly of the rest of MuvB in either C. elegans or mammalian cells (HARRISON et al. 2006; MAGES et al. 2017). We next replaced the TagRFP-T coding sequence with lin-52 tagged with a C-terminal GFP-3xFLAG coding sequence, generating the lin-52(WT) strain (lin-52(bn139[lin-52::GFP::3xFLAG]), Figure 2A). We observed that lin-52(WT) completely rescued fertility (Figure 2C), indicating that the GFP tag does not disrupt LIN-52 function.
Since LIN-52 is essential for *C. elegans* fertility, we sought to disrupt the LIN-35-LIN-52 interaction without affecting protein integrity. We directed targeted mutagenesis of the LIN-52 LxCxE sequence (Figure 1B) using CRISPR/Cas9-mediated genomic editing. We generated 2 mutants of the LxCxE binding motif in *lin-52(WT)* using the *dpy*-10 co-CRISPR method of small oligo homology-directed repair (ARRIBERE et al. 2014). We generated the *lin-52(1A)* single-alanine mutation strain (*lin-52(bn150[lin-52[C44A]::GFP::3xFLAG])*) and the *lin-52(3A)* triple-alanine mutation strain (*lin-52(bn151[lin-52[L42A,C44A,E46A]::GFP::3xFLAG])*) (Figure 2B) with the intent to completely disrupt LIN-52’s interaction with the *C. elegans* pocket protein LIN-35. Additional silent mutations were included in the oligo repair templates to generate new restriction enzyme cut sites to aid in genotyping (Figure 2B).

Full loss of *C. elegans* DREAM activity causes sterility, as observed in protein null mutants of worm E2F-DP (*dpl-1* and *efl-1*) and worm MuvB (*lin-9, lin-52, lin-53, and lin-54*) (BEITEL et al. 2000; CHI AND REINKE 2006; TABUCHI et al. 2011). Since the C-terminally GFP-tagged *lin-52* coding sequence completely rescued *lin-52(KO)* sterility, we were able to test whether *lin-52(1A)* and *lin-52(3A)* disrupt DREAM function. We observed that neither the 1A nor 3A mutation in the LIN-52 LxCxE sequence caused a significant reduction in brood size (Figure 2C). Using western blot analysis of selected DREAM components from *lin-52(WT)* and mutant lysates, we observed that DREAM component protein levels were unaffected compared to wild type (N2) (Figure 2D, Figure S1). Similarly, using live image analysis of *lin-52(WT), lin-52(1A),* and *lin-52(3A)* L4 larvae, we observed that LIN-52 level and localization appeared normal in mutants (Figure 2A). Together, these results demonstrate that mutation of the LIN-52 LxCxE
sequence does not cause a lin-52 null phenotype and does not alter the levels and
tissue distribution of MuvB components.

**Blocking DREAM complex formation causes the classic SynMuv mutant phenotype**

*C. elegans* DREAM components were initially identified in genetic screens for a
Synthetic Multivulval (SynMuv) phenotype *(LU AND HORVITZ 1998; CEOL AND HORVITZ
2001; CEOL *et al.* 2006; HARRISON *et al.* 2006)*. All 8 components of DREAM were
classified as SynMuv B genes; double mutant worms bearing a mutation in a SynMuv B
gene along with a mutation in a SynMuv A gene have multiple vulvae along their ventral
body instead of the usual single vulva *(FAY AND YOCHEM 2007)*. We hypothesized that if
DREAM function was affected by mutation of LIN-52’s LxCxE sequence, then pairing
our 1A and 3A LIN-52 mutations with a SynMuv A mutation should cause a SynMuv
phenotype. When paired with SynMuv A allele lin-8(n2731) *(HARRISON *et al.* 2007)* or
lin-15A(n767) *(HUANG *et al.* 1994), lin-52(3A) but not with lin-52(1A) resulted in a
SynMuv phenotype (Figure 3A). These results indicate that the 3A substitution in LxCxE
impairs DREAM function.

To test whether the 3A substitution in fact impairs pocket protein-MuvB
association, we performed co-immunoprecipitations (co-IPs) from protein extracts
prepared from *lin-52(WT)*, *lin-52(1A)*, and *lin-52(3A)* late embryos. We pulled down LIN-
35 and tested for LIN-52 association using the GFP epitope, and we pulled down LIN-52
using either the GFP or FLAG epitope and tested for LIN-35 association (Figure 3B,
Figure S2). In both co-IP experiments, we observed that LIN-52 association with LIN-35
was lost in *lin-52(3A)* extracts but not in *lin-52(1A)* extracts. These results demonstrate
that the LIN-52 3A substitution successfully severed the protein-protein association between LIN-52 and LIN-35, effectively blocking formation of an intact DREAM complex.

**E2F-DP-LIN-35 and MuvB subcomplexes independently co-occupy chromatin sites**

In the absence of LIN-35, E2F-DP and MuvB do not associate with one another and their chromatin occupancy is reduced genome-wide (GOETSCH et al. 2017). In our *lin-52(3A)* worm strain, LIN-35 is present, but its association with MuvB is severed. We tested the impact of this severing on the chromatin localization of DREAM components using chromatin immunoprecipitation (ChIP). We chose 4 genes, *set-21, mis-12, polh-1,* and *air-1,* as representative DREAM target genes; in *lin-35* null embryos, the chromatin occupancy of DREAM components was greatly diminished at each of their gene promoters and was undetectable at the *air-1* promoter (GOETSCH et al. 2017). We observed that all tested DREAM components remained similarly enriched at the 4 selected promoters in *lin-52(3A)* as compared to *lin-52(WT)* (Figure 4A). An additional 6 DREAM target gene promoters were tested and showed similar DREAM occupancy profiles (Figure S3A). The DREAM components tested included E2F-DP (DPL-1 and EFL-1) and LIN-35, suggesting that the chromatin association of the repressive E2F-DP transcription factor heterodimer is stabilized by its interaction with the pocket protein.

To test whether MuvB and E2F-DP-LIN-35 co-occupy DREAM target regions, we performed sequential ChIP analysis. We first ChIPed LIN-52 via its FLAG tag and then ChIPed LIN-35. We observed no significant difference in LIN-35 co-occupancy in *lin-52(3A)* extracts versus *lin-52(WT)* extracts (Figure 4B). Our results indicate that,
although the interaction of LIN-35 and MuvB is disrupted, DREAM components nevertheless co-localize at target promoters through their respective protein-DNA interactions.

**Severing the LIN-35-MuvB connection impairs transcriptional repression of some but not all DREAM target genes**

MuvB dissociation from E2F-DP-LIN-35 resulted in no observed decline in chromatin occupancy of DREAM at the 10 gene promoters tested (Figure 4A, Figure S3A). Each of the gene products targeted by the 4 selected promoter regions in Figure 4A was upregulated in the *lin-35* null strain (KIRIENKO AND FAY 2007; GOETSCH et al. 2017). We performed gene expression analysis of these 4 genes in *lin-52*(WT), *lin-52*(1A), and *lin-52*(3A) late embryos using RT-qPCR (Figure 4C). We observed that 2 genes, *set-21* and *polh-1*, were significantly upregulated in both *lin-52* mutant strains, while 2 genes, *mis-12* and *air-1*, were not up-regulated. Transcript levels of the gene products targeted by the 6 selected promoter regions in Figure S3A were not affected (Figure S3B). Importantly, in the *lin-35* null strain, DREAM was dissociated from the *air-1* promoter and *air-1* was upregulated (GOETSCH et al. 2017). In our study, DREAM (with a severed LIN-35-LIN-52 connection) was associated with the *air-1* promoter and *air-1* was not upregulated. Thus, MuvB chromatin occupancy is necessary but not sufficient for repression of DREAM target genes.

**Discussion**

The trio of pocket proteins, pRb, p107, and p130, govern cell cycle exit and reentry through targeted transcriptional repression of cell cycle genes. We analyzed how the sole *C. elegans* Rb-like pocket protein LIN-35 contributes to the formation and
function of the DREAM complex, which relies on the recruitment of the highly conserved
and essential 5-subunit MuvB complex to direct repression of target genes. Using
CRISPR/Cas9-mediated targeted mutagenesis, we generated a mutant *C. elegans*
strain in which MuvB’s LIN-35-interacting subunit LIN-52 was rendered incapable of
interacting with LIN-35. Blocking DREAM assembly in worms impaired DREAM
function, causing the classic SynMuv phenotype and upregulation of some DREAM
target genes. However, even though LIN-35 and MuvB association was abolished, all of
the DREAM components tested showed unimpaired association with chromatin. Thus,
our findings reveal that the LIN-35-MuvB association potentiates MuvB-mediated
transcriptional repression but is not required.

Our analysis provides important insight into how assembly of the mammalian
DREAM complex establishes repression of cell cycle genes. DREAM assembly is
triggered by DYRK1A phosphorylation of LIN52, initiating MuvB association with
p107/p130 (LITOVCHICK et al. 2011; GUILEY et al. 2015). Mammalian MuvB’s function
switches between transcriptional repression in the DREAM complex during quiescence
and transcriptional activation after associating with the B-Myb transcription factor and
forming the Myb-MuvB (MMB) complex during the late cell cycle (LEWIS et al. 2004;
DYRK1A-mediated LIN52 phosphorylation also inhibits MuvB association with B-Myb
(LITOVCHICK et al. 2011), even though the 2 interaction interfaces are not exclusive
(GUILLEY et al. 2018). Our data demonstrate that MuvB localizes to chromatin sites and
represses gene targets without direct association with the pocket protein. Thus, we
propose that the pocket protein’s primary role in DREAM complex assembly is to protect MuvB’s function as a transcriptional repressor.

The delicate switch between MuvB-associated cell cycle gene repression and activation is hijacked in cancer cells. All 3 mammalian pocket proteins are inactivated by the E7 viral oncoprotein present in high-risk human papillovirus (HPV) (ZHANG et al. 2006; HUH et al. 2007). E7 interacts with the mammalian pocket proteins through the high-affinity LxCxE binding motif, disrupting MuvB association in DREAM (GUILEY et al. 2015). HPV E7 concurrently coaxes MuvB into its transcriptional activator function by stimulating MMB assembly (PANG et al. 2014). However, cancer cells resist cytotoxic chemotherapy by temporarily exiting the cell cycle (BOICHUK et al. 2013), suggesting that MuvB’s capacity for transcriptional repression is retained. Based on our findings that MuvB does not require direct association with the pocket protein to target gene repression, we propose that MuvB’s function in cancer cells requires closer scrutiny.

We previously observed that E2F-DP and MuvB chromatin association is severely affected by loss of LIN-35 (GOETSCH et al. 2017). By severing LIN-35-MuvB association, this study reveals that LIN-35 directly stabilizes E2F-DP chromatin occupancy. However, we also observed that MuvB chromatin occupancy is not impaired even though MuvB no longer associates directly with E2F-DP-LIN-35. Importantly, in vitro analysis of heterodimeric mammalian E2F-DP complex DNA binding characteristics identified a distinct induction of DNA bending, especially in the case of the homologues of C. elegans EFL-1-DPL-1 (E2F4-DP1/2) (TAO et al. 1997). We propose that DREAM-associated E2F-DP heterodimers promote MuvB co-occupancy through a DNA bending-dependent mechanism. Together, our results suggest a model...
in which the LIN-35 pocket protein promotes E2F-DP chromatin occupancy, which in turn promotes MuvB chromatin occupancy.

Our results support an exciting model for how local E2F-DP-mediated alterations to DNA shape enhanced by their interaction with a pocket protein promote MuvB co-occupancy. Even with evolutionary divergence from the ancestral pocket protein, this model may also apply to pRb function. Many histone deacetylases and chromatin remodeling complexes associate with pRb through the LxCxE binding cleft, although many of these associations have only limited support thus far from structural/biochemical interaction studies (Dyson 2016). Variation in pRb monophosphorylation events that can alter pRb structure and recognition of binding partners offer one explanation for how pRb can potentially interact with >300 individual protein partners (Rubin 2013; Narasimha et al. 2014). Our data provide an alternative, but not exclusive, possibility, namely that direct and stable pRb association with these myriad protein partners may be unnecessary. Perhaps pRb association with a few partners such as E2F-DPs promotes localization of multi-protein complexes to genomic sites. Additional dissection of DREAM and pRb structure and function will shed light on how the pocket proteins mediate their essential cellular roles.

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Figure 1

(A) Model of the *C. elegans* DREAM complex bound to DNA: E2F-DP (blue), the pocket protein LIN-35 (purple), and the 5-subunit MuvB subcomplex (green). The highlighted region shows the target region for this study: an LxCxE binding motif in the MuvB subunit LIN-52 that interacts directly with the LIN-35 pocket protein.

(B) Alignment of *H. sapiens* LIN52 and *C. elegans* LIN-52 sequences. The human LxSxExL and worm LxCxE sequences are highlighted in yellow, and the human DYRK1A consensus phosphorylation sequence is highlighted in orange. Arrows indicate residues involved in the interaction with the pocket protein.
**Figure 2**

(A) Live worm fluorescence images of $\text{lin-52(KO)}$, $\text{lin-52(WT)}$, $\text{lin-52(1A)}$, and $\text{lin-52(3A)}$ L4 larvae. Composites were artificially straightened. Scale bar, 100µM.

(B) Sanger sequencing of the $\text{lin-52}$ LxCxE coding region in $\text{lin-52(WT)}$, $\text{lin-52(1A)}$, and $\text{lin-52(3A)}$.

(C) Strip chart of the brood sizes of wild-type (N2) worms and $\text{lin-52(KO)}$, $\text{lin-52(WT)}$, $\text{lin-52(1A)}$, and $\text{lin-52(3A)}$ transgenic worms. Significance (** p-value < 0.01) was determined by a Wilcoxon-Mann-Whitney test comparing the indicated strains to wild type (N2).

(D) Western blot analysis of DREAM subunits LIN-52 (via GFP tag), EFL-1, LIN-35, and LIN-37 using lysates from wild-type (N2) worms and $\text{lin-52(WT)}$, $\text{lin-52(1A)}$, and $\text{lin-}$
52(3A) transgenic worms separated by SDS/PAGE. Antibodies used are indicated on the right. Alpha-tubulin was used as a loading control. Full blots are shown in Figure S1.
**Figure 3**

(A) Table indicating the percentage Synthetic Multivulval (SynMuv) of *lin-52(WT)*, *lin-52(1A)*, and *lin-52(3A)* in combination with SynMuv A mutant alleles *lin-8(n2731)* or *lin-15A(n767)* with standard deviation indicated. The population size (n) is indicated in parentheses.

**Figure 3** Blocking DREAM complex formation recapitulates the classic SynMuv phenotype
(B) Late embryo extracts from *lin-52(WT)*, *lin-52(1A)*, and *lin-52(3A)* (each tagged with GFP and FLAG) were immunoprecipitated with anti-LIN-35, anti-GFP, and anti-FLAG antibodies, with no antibody serving as a negative control. Proteins bound (B) and unbound (UB) were separated by SDS/PAGE, and western blot analysis was performed using the antibodies indicated on the right. 5% of Input (In) is shown on the left. Asterisks indicate non-specific bands. Full blots are shown in Figure S2.
Figure 4

(A) ChIP-qPCR of 5 DREAM subunits DPL-1, EFL-1, LIN-37, LIN-35, and LIN-52 (via GFP tag) from lin-52(WT) (white) and lin-52(3A) (black) late embryo extracts at 4 DREAM target genes. IgG was used as a negative control. Signals are presented as percentage of Input DNA. Error bars indicate standard error of the mean. Additional target genes are shown in Figure S3.

Figure 4 Analysis of chromatin association with and repression of DREAM target genes
(B) Sequential ChIP-qPCR of LIN-52 (via FLAG tag) followed by LIN-35 or IgG from \textit{lin-52}(WT) (white) and \textit{lin-52}(3A) (black) late embryo extracts at 4 DREAM target genes. Signals are presented as percentage of FLAG IP DNA. Error bars indicate standard error of the mean.

(C) RT-qPCR analysis comparing transcript levels of 4 DREAM target genes in \textit{lin-52}(WT) (white) \textit{lin-52}(1A) (grey), and \textit{lin-52}(3A) (black) late embryos. Expression values from 2 independent experiments each consisting of 4 biological replicates were averaged and are presented as the relative quantity (Rq) compared to \textit{act-2}. Error bars indicate standard error of the mean, and significance was determined by a student’s T-test between transcript levels in mutant (3A or 1A) vs WT (** p-value < 0.01). Additional target genes are shown in Figure S3.
References


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Zhang, B., W. Chen and A. Roman, 2006 The E7 proteins of low- and high-risk human papillomaviruses share the ability to target the pRB family member p130 for degradation. Proc Natl Acad Sci U S A 103: 437-442.

Supplemental Information

DREAM Interrupted: Severing MuvB from DREAM’s pocket protein in *Caenorhabditis elegans* impairs gene repression but not DREAM chromatin assembly

Paul D. Goetsch and Susan Strome
**Supplemental Materials and Methods:**

**CRISPR/Cas9-mediated genome editing**

To generate *lin-52(KO)*, 2 Cas9 target sites were identified near the 5’ and 3’ ends of the *lin-52 gene*. Single guide RNA sequences were cloned into the PU6::unc119_sgRNA vector. The *lin-52* KO homologous repair template was generated by amplifying homology arms containing the *lin-52* promoter and *lin-52* 3’ UTR and cloned into the N-terminal tag digested pDD284 vector. The following injection mix was microinjected into the germline of ~50 N2 young adults: 50 ng / µL Cas9 expression plasmid (pDD162, Addgene #47549), 2.5 ng / µL *Pmyo-2::mCherry::unc-54utr* (pCJF90, Addgene #19327), 5 ng / µL *Pmyo-3::mCherry::unc-54utr* (pCFJ104, Addgene #19328), 10 ng / µL *Prab-3::mCherry::unc-54utr* (pGH8, Addgene #19359), 50 ng / µL *lin-52* 5’ sgRNA (pPDG14), 50 ng / µL *lin-52* 3’ sgRNA (pPDG18), and 10 ng µL *Plin-52::TagRFP-T^SEC^3xFLAG::lin-52utr* (pPDG13). CRISPR/Cas9-positive progeny were treated with hygromycin and screened for the Roller phenotype and absence of fluorescent co-injection marker expression (the latter enables extrachromosomal arrays to be distinguished from edited endogenous genes). Individuals from 1 positive selection plate were selected and balanced to create the strain SS1240 *lin-52(bn132(*lin-52p::TagRFP-T^SEC^3xFLAG::lin-52 3’ UTR*)) III / hT2G [bli-4(e937) let-? (q782) qIs48] (I:III). The self-excising cassette (SEC) was removed by a 4-5 hour heat-shock of L1 larvae at 32°C. Non-Roller F1 progeny were isolated to create the strain SS1241 *lin-52(bn133(*lin-52p::TagRFP-T::3xFLAG::lin-52 3’ UTR*)) III / hT2G [bli-4(e937) let-? (q782) qIs48] (I:III).
To generate *lin-52(WT)*, 2 Cas9 target sites were identified near the 5’ and 3’ ends of the *TagRFP-T-3xFLAG* coding sequence. Single guide RNA sequences were cloned into pDD162. The *lin-52* WT homologous repair template was generated by amplifying homology arms containing the *lin-52* promoter with the gene’s coding sequence and the *lin-52* 3’ UTR and cloned into the C-terminal tag digested pDD282 vector. The following CRISPR/Cas9 and co-injection marker plasmid mix was microinjected into the germline of ~50 SS1241 young adults: 50 ng / µL *TagRFP-T* 5’ sgRNA-Cas9 vector (pPDG21), 50 ng / µL *TagRFP-T* 3’ sgRNA-Cas9 vector (pPDG22), 2.5 ng / µL pCJF90, 5 ng / µL pCFJ104, and 10 ng / µL *Plin-52::lin-52 CDS-GFP^SEC^3xFLAG::lin-52utr* (pPDG17). CRISPR/Cas9-positive progeny were treated with hygromycin and screened for the Roller phenotype and absence of fluorescent co-injection marker expression. Individuals from 2 of 3 positive selection plates were selected and made homozygous to create strains SS1325 and SS1326 *lin-52(bn138(lin-52::GFP^SEC^3xFLAG)) III*. The SEC was removed by heat-shock, and non-Roller F1 progeny were isolated to create the strains SS1256 and SS1257 *lin-52(bn139(lin-52::GFP::3xFLAG)) III*. SS1256 was backcrossed 6 times to generate strain SS1272, which was used in downstream experiments.

To generate *lin-52(1A)* and *lin-52(3A)*, 1 Cas9 target site was identified near the LxCxE coding sequence and cloned into the pDD162 vector. Single strand DNA templates included at least 40 base pairs of homology flanking the LxCxE coding sequence and silent mutations to aid in genotyping, as illustrated in Figure 2B. The following CRISPR/Cas9 and co-injection marker plasmid mix was microinjected into the germline of 6 (for 1A) and 10 (for 3A) SS1256 young adults: 40 ng / µL *lin-52 LxCxE sgRNA-*
Cas9 vector (pPDG59), 2.5 ng / µL pCJF90, 5 ng / µL pCFJ104, 20 ng / µL lin-52 mutagenesis ssDNA template (1A or 3A), 40 ng / µL dpy-10(cn64) sgRNA (pJA58, Addgene plasmid #59933), and dpy-10(cn64) ssDNA template. dpy-10(cn64) guide and ssDNA template were co-injected to select for positive CRISPR activity in injectant progeny. Injected adults were cloned onto individual plates, and F1 progeny were screened for presence of a Roller (Rol) and/or Dumpy (Dpy) phenotype. Individual Rol and/or Dpy progeny were genotyped, resulting in 3 independent lin-52(1A) and 2 independent lin-52(3A) strains. Each strain was backcrossed 6 times to create SS1273-SS1275 lin-52(bn150[lin-52[C44A]:GFP::3xFLAG]) III, and SS1276 and SS1277 lin-52(bn151[lin-52[L42A,C44A,E46A]:GFP::3xFLAG]) III. SS1273 and SS1276 were used in downstream experiments.

Immunoblotting and co-immunoprecipitation (coIP)

CoIP lysates were prepared by grinding frozen embryos using a mortar and pestle, resuspending in lysis buffer (25 mM HEPES pH 7.6, 150 mM NaCl, 1mM DTT, 1mM EDTA, 0.5 mM EGTA, 0.1% Nonidet P-40, 10% glycerol) with Complete EDTA-free Protease Inhibitors (Roche), and sonicating twice for 30 seconds. Lysates were clarified and precleared using a mix of Protein A and Protein G Dynabeads (ThermoFisher). Protein concentrations of coIP lysates were determined using a Qubit fluorometer (ThermoFisher). For each IP, 5 µg of anti-FLAG was crosslinked to Protein G Dynabeads and 2 µg of anti-GFP or anti-LIN-35 was crosslinked to Protein A Dynabeads using dimethyl pimelimidate in 0.2 M trimethylamine pH 8.2. Crosslinking was stopped using 0.1M Tris pH 8.0, and beads were washed with 0.1 M glycine pH 2.8 before being stored in phosphate buffered saline pH 7.2 with 0.05% Tween-20. Each IP
was washed with lysis buffer, and eluted with 50 μL 2x SDS gel-loading buffer for 5 minutes at 98°C

**Chromatin immunoprecipitation (ChIP) and sequential ChIP**

For ChIP, chromatin extracts were precleared with Protein A Dynabeads. ChIPs were performed with 2 mg of extract and 1 μg of antibody, with 2% of the extract set aside for an input reference control. ChIPs were incubated overnight at 4°C with 1% sarkosyl. Protein A Dynabeads equilibrated in 20 μL FA buffer were added and incubated for 2 hours at 4°C. ChIPs were washed with the following buffers: once with FA buffer containing 1 M NaCl, once with FA buffer containing 0.5 M NaCl, once with TEL buffer (10 mM Tris-HCl pH 8.0, 0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA), and twice with TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). 2 elutions of 50 μL elution buffer containing TE plus 1% SDS and 250 mM NaCl were incubated at 55°C. Eluted ChIP and input samples were incubated with proteinase K for 1 hour at 55°C. Crosslinks were reversed overnight at 65°C. DNA was purified by phenol-chloroform extraction and ethanol precipitation using glycogen as a carrier.

For sequential ChIP, chromatin extracts were precleared with Protein G Dynabeads and 4 parallel ChIPs per replicate were performed with 2.5 mg of extract and 2.5 μg of anti-FLAG antibody, with 2% of the extract set aside for an input reference control. ChIPs were incubated overnight at 4°C with 1% sarkosyl. Protein G Dynabeads equilibrated in 20 μL FA buffer were added and incubated for 2 hours at 4°C. ChIPs for each replicate were washed as described above and pooled. 2 elutions of 50 μL 0.1M NaHCO₃ plus 1% SDS were incubated at 55°C for 15 minutes. Elutions were divided, diluted with FA buffer with 1% sarkosyl, and incubated with anti-LIN-35 or IgG as a
negative control, with 10% of the elution set aside as a reference control. The 2nd ChIP was incubated overnight at 4°C. Protein A Dynabeads equilibrated in 20 μL FA buffer were added and incubated for 2 hours at 4°C. ChIPs were washed and eluted twice with 50 μL elution buffer with incubation at 55°C. Eluted ChIP, reference, and input samples were incubated with proteinase K for 1 hour at 55°C. Crosslinks were reversed overnight at 65°C. DNA was purified by phenol-chloroform extraction and ethanol precipitation using glycogen as a carrier.
**Figure S1**

Full western blots of DREAM subunits LIN-52 (via GFP tag), EFL-1, LIN-35, and LIN-37 using whole worm lysates from Bristol (N2), *lin-52(WT)*, *lin-52(1A)*, and *lin-52(3A)* separated by SDS/PAGE. Antibodies used are indicated below each blot. Alpha-tubulin was used as a loading control. Membranes were cut at the 75 kDa band. Arrows indicate blot regions presented in Figure 2D.
Figure S2

Full western blots of late embryo extracts from *lin-52(WT)*, *lin-52(1A)*, and *lin-52(3A)* that were immunoprecipitated with anti-LIN-35, anti-GFP, and anti-FLAG antibodies, with no antibody serving as a negative control. Proteins bound (B) and unbound (UB) were separated by SDS/PAGE and transferred to PVDF membranes that were cut at the 75 kDa band (indicated by dashed line). Antibodies used are indicated below each blot. 5% of Input (In) was included. Arrows indicate blot regions presented in Figure 3B.
Figure S3

(A) ChIP-qPCR of 5 DREAM subunits DPL-1, EFL-1, LIN-37, LIN-35, and LIN-52 (via GFP tag) from *lin-52(WT)* (white) and *lin-52(3A)* (black) late embryo extracts at 6 DREAM target genes. IgG was used as a negative control. Signals are presented as percentage of Input DNA. Error bars indicate standard error of the mean.

(B) RT-qPCR analysis comparing transcript levels of the 6 DREAM target genes in *lin-52(WT)* (white) *lin-52(1A)* (grey), and *lin-52(3A)* (black) late embryos. Expression values from 2 independent experiments each consisting of 4 biological replicates were averaged and are presented as the relative quantity (Rq) compared to *act-2*. Error bars indicate standard error of the mean.
### Table S1

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**RT-qPCR primers**

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