MicroRNAs reinforce repression of PRC2 transcriptional targets independently and through a feed-forward regulatory network with PRC2

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Abbreviations

PRC2, Polycomb repressive complex 2; TF, transcription factor; iCLIP, individual crosslinking and immunoprecipitation; UTR, untranslated region; RISC, RNA-induced silencing complex; Dox, doxycycline mESCs, mouse embryonic stem cells; GBM, Glioblastoma multiforme; TSS, Transcription start site(s).

Abstract

Gene expression can be regulated at multiple levels, but it is not known if and how there is broad coordination between regulation at the transcriptional and post-transcriptional levels. Transcription factors and chromatin regulate gene expression transcriptionally, while microRNAs (miRNAs) are small regulatory RNAs that function post-transcriptionally. Systematically identifying the post-transcriptional targets of miRNAs and the mechanism of transcriptional regulation of the same targets can shed light on regulatory networks connecting transcriptional and post-transcriptional control. We used iCLIP (individual crosslinking and immunoprecipitation) for the RISC (RNA-induced silencing complex) component AGO2 and global miRNA depletion to identify genes directly targeted by miRNAs. We found that PRC2 (Polycomb repressive complex 2) and its associated histone mark, H3K27me3, is enriched at hundreds of miRNA-repressed genes. We show that these genes are directly repressed by PRC2 and constitute a significant proportion of direct PRC2 targets. For just over half of the genes co-repressed by PRC2 and miRNAs, PRC2 promotes their miRNA-mediated repression by increasing expression of the miRNAs that are likely to target them. miRNAs also repress the remainder of the PRC2 target genes, but independently of PRC2. Thus, miRNAs post-transcriptionally reinforce silencing of PRC2-repressed genes that are inefficiently repressed at the level of chromatin, by either forming a feed-forward regulatory network with PRC2 or repressing them independently of PRC2.

Introduction

Transcription factors (TFs) and miRNAs together form the largest components of gene regulatory networks and can regulate gene expression through both distinct and coordinated regulatory mechanisms. One of the ways in which TFs can coordinate their regulatory impact with miRNAs is by forming a feed-forward regulatory network. In such a network, a TF regulates a miRNA and both co-regulate a common target. In a coherent feed-forward regulatory network, the outcomes of both direct and indirect regulation by a TF are consistent. Many such TF-miRNA feed-forward regulatory networks have been shown to functionally impact several processes in development and disease (O'Donnell et al. 2005; Hobert 2008; Tsang et al. 2007; Lin et al. 2015; Gerloff et al. 2014; Polioudakis et al. 2013).

One potential function of coherent TF-miRNA feed-forward regulatory networks is to reinforce transcriptional regulation at the post-transcriptional level. In particular, this can help suppress residual transcripts produced from leaky transcription of transcriptionally silenced genes. This is most crucial during switches in transcriptional states in response to stress, developmental transitions, cell cycle stages or other external stimuli (Farh et al. 2005; WU et al. 2015; del Rosario et al. 2016).

PRC2 is an epigenetic regulator complex that transcriptionally silences genes through modification of histone H3 with tri-methylation at lysine 27 (H3K27me3). PRC2 plays a critical role in maintaining the silenced state of genes involved in

development and several cancers (Aranda et al. 2015; Di Croce and Helin 2013). In this study, our goal was to determine if the transcriptional regulation by PRC2 and post-transcriptional regulation by miRNAs occur through distinct or coordinated mechanisms (Mote et al. 2017). To test this, we first identified transcriptome-wide targets of miRNAs using AGO2 iCLIP and global knockdown of miRNAs. We then identified genes under direct transcriptional repression of PRC2 and asked if the same targets were post-transcriptionally repressed by miRNAs. We show that miRNAs post-transcriptionally repress a significant number of genes that are transcriptionally repressed by PRC2, both through mechanisms independent of PRC2 and coordinately, by forming a feed-forward network with PRC2. Overall, these findings point to a broad role of miRNAs where they independently strengthen and also reinforce the silencing of PRC2-repressed genes post-transcriptionally.

Results

Transcriptome-wide identification of miRNA targets

miRNAs primarily target mRNAs through interactions between their 5' seed region and the 3' untranslated region (3' UTR) of the mRNA, mediated by the RNA-induced silencing complex (RISC). To identify the transcriptome-wide targets of miRNAs, we first performed RNA individual crosslinking and immunoprecipitation (iCLIP) for the RISC component AGO2 in glioblastoma multiforme (GBM) cells (König et al. 2010). Several observations indicate that the AGO2-RNA interactions we identified using iCLIP were direct and specific. First, AGO2-RNA complexes were crosslinking- and RNase-treatment-specific (Fig. 1A). Second, reads from

iCLIP were enriched at 3' UTRs as expected from the binding of RISC (Fig. 1B). Third, reads mapping to genes showed high correlation and were reproducible across independent biological replicate experiments (Fig. 1C). We identified 45362 peaks mapping to 5896 protein-coding genes that were common across two independent biological replicate experiments.

In addition to mRNA targets, the AGO2 iCLIP experiment also recovered miRNAs that AGO2 interacts with, allowing for more detailed analysis of functional miRNA-mRNA interactions. Although enrichment of miRNAs in AGO2 iCLIP showed high correlation to their expression levels, miRNAs with the highest expression were not necessarily the most enriched (Supplemental Fig. S1A). We found that the region spanning 200 bases around AGO2 iCLIP peaks was significantly enriched for the seed sequences of several of the top AGO2-interacting miRNA families (Fig. 1D and Supplemental Fig. S1A). In addition, the predicted mRNA target sites of the top 10 highly enriched miRNAs were significantly more likely to occur proximal to AGO2 iCLIP peaks than background mRNAs (Supplemental Fig. S1B). This suggests that the miRNAs associated with AGO2 bind to a significant proportion of the AGO2-enriched mRNAs, further attesting to the ability of the iCLIP experiment to broadly identify the targets of active miRNAs.

To further support the miRNA-mRNA interactions we identified, we validated the regulation of PPIF by miR-23a-3p. miR-23a-3p was among the most enriched miRNAs in our AGO2 iCLIP dataset. We selected PPIF as a target of miR-23a-3p as it had a predicted binding site close to an AGO2 iCLIP peak (Supplemental Fig. S2A). Several lines of evidence show that miR-23a-3p directly represses PPIF

expression. First, treatment of cells with an inhibitor of miR-23a-3p led to an induction of both PPIF transcript and protein levels (Fig. 1E,F). Second, we checked if the repression of PPIF was through a direct interaction between the 3' UTR of PPIF and the miR-23a-3p seed region. We performed luciferase reporter assays where we cloned the 3' UTR of PPIF and compared luciferase expression to constructs that were mutated at the sites of interaction. We found that the effect of inhibitor treatment on regulation of the PPIF 3' UTR was abolished upon deletion of the miR-23a-3p binding sites (Supplemental Fig. S2B). Third, both deletion of the miRNA binding sites and disruption of miRNA-mRNA interactions through base substitutions led to a similar rescue in luciferase activity (Supplemental Fig. S2C).

miRNA-repressed genes are enriched for PRC2 binding and H3K27me3

Although AGO2 iCLIP identified all the interaction targets of RISC, the AGO2-RISC complex can regulate gene expression in both a miRNA-dependent and independent manner (Leung et al. 2011). To specifically identify miRNA-dependent genes, we adopted a strategy to globally deplete miRNAs, followed by expression profiling. Ectopic overexpression of the Vaccinia Virus protein VP55 has been previously shown to polyadenylate and degrade miRNAs, causing a global loss of miRNAs (Backes et al. 2012; Aguado et al. 2015). We cloned VP55 into a Sleeping Beauty transposon system under a doxycycline-inducible promoter and stably integrated this construct into T98G cells to generate a GBM cell line capable of doxycycline-responsive loss of miRNAs (Kowarz et al. 2015). miR-21, the most abundant miRNA in these cells, was almost completely depleted within

24 hours of doxycycline treatment (Fig. 2A). PCR-amplified cDNA libraries prior to size-selection for miRNA-seq showed a significant depletion of miRNAs (Supplemental Fig. S3A). miRNA-seq showed a global depletion of approximately 90% of all cellular miRNAs (Fig. 2B). To identify the genes regulated by miRNAs genome-wide, we performed mRNA-seq from doxycycline treated (+ Dox) and untreated cells (- Dox). 3844 protein-coding transcripts were up-regulated and thus repressed by miRNAs, and 3668 transcripts were down-regulated upon loss of miRNAs (Supplemental Fig. S3B). Some of the most strongly miRNA-repressed genes included several genes from the histone gene family. This is consistent with VP55-responsive genes identified previously in HEK293T cells (Aguado et al. 2015).

37% of the miRNA-repressed transcripts interacted directly with AGO2 based on iCLIP, suggesting that these were direct functional targets of miRNAs. Thus, miRNA-repressed genes include direct as well as indirect targets which could be regulated at the level of transcription (Gosline et al. 2016). To check if the miRNA-repressed genes were also regulated by specific transcription factors, we checked for the enrichment of TF binding sites proximal to their promoters. We used Enrichr to detect TF regulatory signatures in the ranked list of miRNA-repressed genes (Chen et al. 2013). Surprisingly, we found an enrichment for H3K27me3 (Fig. 2C) and a corresponding enrichment for SUZ12, a member of the PRC2 complex which adds H3K27me3 (Fig. 2D), at miRNA-repressed genes. This suggested the possibility that many miRNA-repressed genes could additionally be regulated at the level of chromatin by PRC2 and H3K27 methylation. To ascertain whether

PRC2 does in fact bind to miRNA-repressed genes and deposit H3K27me3 at miRNA-repressed genes, we performed ChIP-seq for EZH2, the catalytic component of PRC2, as well as for H3K27me3. The level of H3K27me3 positively correlated with that of EZH2 binding at genes containing an EZH2 peak (Supplemental Fig. S4). Consistent with the Enrichr analysis, we found EZH2 binding at more than a thousand miRNA-repressed genes (27% of all miRNA-repressed genes, Fig. 2E). To determine if this could be a general mode of dual regulatory control in other cell types, we analyzed data from mouse embryonic stem cells (mESCs) with a knockout of the miRNA-processing factor DICER, which also results in loss of miRNAs (Zheng et al. 2014). Similar to GBM cells, we found that genes repressed by miRNAs in mESCs also showed enrichment for H3K27me3 and PRC2 (Supplemental Fig. S5A,B).

miRNAs repress hundreds of genes directly repressed by PRC2

Based on the above results, we hypothesized that many genes that are post-transcriptionally repressed by miRNAs are also transcriptionally repressed by PRC2. To test this hypothesis, we generated a CRISPR-Cas9 knockout of EZH2 in GBM cells. H3K27me3 levels, as well as levels of SUZ12, were reduced in EZH2 -/- cells, signifying loss of the PRC2 complex (Supplemental Fig. S6A) (Pasini et al. 2004). 1519 protein-coding genes were down-regulated and 1834 protein-coding genes were up-regulated (derepressed) upon loss of EZH2 (Fig. 3A). Genes derepressed upon loss of EZH2 (EZH2-repressed genes) showed EZH2 binding and H3K27me3 (Fig. 3B and Supplemental Fig. S6B). Consistent with previous studies, EZH2-repressed genes also showed occupancy by H3K4me3

(Fig. 3B and Supplemental Fig. S6B), although the enrichment for EZH2 and H3K27me3 was anti-correlated with H3K4me3 (Fig. 3C) (Jadhav et al. 2016; Abou El Hassan et al. 2015). 959 of the 1834 EZH2-repressed genes had an EZH2 chromatin peak close to their transcription start site (TSS) and were thus likely directly repressed by EZH2.

We then checked if the miRNA-repressed genes bound by EZH2 (shown in Fig. 2E) were regulated by EZH2. Of these 1035 genes, 299 genes were significantly regulated by EZH2, with 71% of them (213/299) being directly repressed (Fig. 3D). These 213 genes that were jointly and directly repressed by EZH2 and by miRNAs constituted 22% of all direct EZH2 targets. We verified derepression upon loss of miRNAs or upon loss of EZH2 of multiple genes using qRT-PCR (Fig. 3E). A significant proportion (34.7%) of genes co-repressed by EZH2 and miRNAs also showed direct interaction with AGO2 by iCLIP (P = 2.9e-24, Fig. 3F and Supplemental Fig. S6C). miRNAs thus post-transcriptionally reinforce transcriptional repression by PRC2 of at least one-fifth of all PRC2 targets.

miRNAs repress PRC2 target genes through a feed-forward regulatory network with PRC2

PRC2 and miRNAs were recently shown to independently co-repress endocytosis genes in mESCs (Mote et al. 2017). To test if miRNAs and PRC2 repressed a common set of genes independently in GBM cells, we depleted miRNAs globally by overexpressing VP55 in EZH2 -/- T98G cells and performed miRNA-seq and mRNA-seq. Most miRNAs were depleted within 24 hours of doxycycline treatment

(Fig. 4A and Supplemental Fig. S7A). Both WT and EZH2 -/- cells showed similar responses to doxycycline treatment in terms of the number of miRNAs depleted and the extent of depletion (Supplemental Fig. S7B), indicating that VP55 overexpression was equally effective in WT and EZH2 -/- cells. If miRNAs and EZH2 repressed a common set of genes independently of one another, we would expect that these co-repressed genes would be repressed by miRNAs even in the absence of EZH2, and therefore show derepression upon miRNA depletion even in EZH2 -/- cells. Of the 213 genes co-repressed by EZH2 and miRNAs, 54% (116/213) showed significantly reduced derepression in response to miRNA depletion in EZH2 -/- cells compared to WT cells (Fig. 4B), suggesting that PRC2 and miRNAs work coordinately, rather than independently, to repress these genes.

This coordination between PRC2 and miRNAs can be accounted for by a feed-forward regulatory network where EZH2 promotes the miRNA-mediated repression of a subset (54%) of its direct targets, by activating miRNAs that repress those targets. Consistent with the heatmap (Fig. 4B), these feed-forward regulated genes showed derepression in response to miRNA depletion in WT but not in EZH2 -/- cells (Fig. 4C). We confirmed this finding by qRT-PCR for several genes (Fig. 4D). Further analysis of this data helped rule out alternative explanations for this behavior. One alternative explanation for the coordinated feed-forward regulation we observed is that miRNAs somehow promote PRC2 function, perhaps by promoting the binding of PRC2 to its repression targets (Graham et al. 2016). However, closer analysis of our data does not support this possibility. First, if miRNAs promoted PRC2 repressive function, derepression of miRNA targets

would be abolished in EZH2 -/- cells. Instead, 23% (27/116) of these genes showed derepression even in EZH2 -/- cells, although significantly lower than in WT cells (Supplemental Fig. S8). Second, if miRNAs promoted PRC2 repressive function in addition to directly repressing feed-forward regulated genes, derepression of those genes would be greater upon loss of miRNAs than upon loss of PRC2 function. However, the expression levels of these genes were significantly higher in EZH2 -/- cells than in WT cells depleted of miRNAs (Fig. 4 C,D). For these reasons, it is unlikely that the coordinate feed-forward regulation entails miRNAs promoting PRC2 binding or repression activity at those genes. Moreover, the impaired derepression upon miRNA depletion in the absence of EZH2 is also not due to genes attaining maximally derepressed expression levels in EZH2 -/- cells. The remaining 47% of the genes co-repressed by EZH2 and miRNAs that were not part of the feed-forward network showed significant derepression in response to miRNA loss both in WT and EZH2 -/- cells, indicating that multiple levels of repression are detectable for genes that are not part of the coordinated feed-forward regulatory network (Supplemental Fig. S9).

Our data thus suggests a model where PRC2 transcriptionally represses hundreds of genes in GBM cells and for a significant fraction of these genes, it further promotes additional repression by activating miRNAs that post-transcriptionally repress those genes. To identify miRNAs regulated by PRC2, we performed miRNA-seq in WT and EZH2 -/- cells and identified 31 miRNAs that were activated and 84 miRNAs that were repressed by EZH2 (Fig. 4E). We found no EZH2 binding

to chromatin around the EZH2-activated miRNAs, suggesting that EZH2 activated them indirectly.

To determine whether the feed-forward regulated genes as a group were likely to be targeted by EZH2-activated miRNAs, we tested whether the number of predicted interactions between them were higher than expected by random chance. There were 342 unique interactions predicted between 14 EZH2-activated miRNAs (conserved miRNAs with mRNA target predictions) and 85 of the 116 feed-forward regulated genes (Fig. 4F), which was significantly higher than the 255 interactions seen between a randomly selected set of 14 miRNAs and a random set of 116 genes ($P < 0.01$, binomial test). This implies that EZH2 indirectly promotes expression of the miRNAs that are likely to regulate its direct targets.

PRC2 regulates FRMD4B expression through a feed-forward regulatory network with let-7i

To verify the feed-forward regulatory network for a selected example in detail, we analyzed the repression of FRMD4B by PRC2. Consistent with direct transcriptional regulation by PRC2, both H3K27me3 and EZH2 were enriched near the promoter of FRMD4B (Fig. 5A). To verify that PRC2 promoted miRNA-mediated repression of FRMD4B, we measured its transcript levels in WT and EZH2 -/- cells, with and without miRNA depletion. Loss of EZH2 caused derepression of FRMD4B, whereas depletion of miRNAs caused derepression of FRMD4B expression in WT but not in EZH2 -/- cells (Fig. 5B), verifying that PRC2 promotes miRNA-mediated regulation of FRMD4B. To test if PRC2 promotes

expression of a specific miRNA that targets FRMD4B, we first identified let-7i as an EZH2-activated miRNA with a predicted interaction site near an AGO2 iCLIP site on the FRMD4B transcript (Fig. 5C and Supplemental Fig. S10A). We then analyzed the regulation of FRMD4B by let-7i using luciferase assays with a WT or mutant FRMD4B 3' UTR in WT and EZH2 -/- cells. Deletion of the let-7i binding site led to a marked increase in luciferase signal in WT but a much smaller increase in EZH2 -/- cells (P = 0.0098, paired t-test, Fig. 5D). Thus, FRMD4B is more strongly repressed by let-7i in the presence of its activator EZH2.

To address how EZH2 indirectly activates let-7i, we searched for likely repressors of let-7i among the genes directly repressed by EZH2. We found that LIN28B, a known repressor of the let-7 family of miRNAs, showed chromatin marks characteristic of EZH2 activity (Supplemental Fig. S10B). Our RNA-seq data showed that LIN28B was significantly derepressed upon loss of EZH2 (FDR corrected $P < 0.05$), suggesting that it was directly repressed by EZH2. LIN28B is an RNA-binding protein that inhibits the biogenesis of let-7 miRNAs (Balzeau et al. 2017). Since LIN28B is known to be a common post-transcriptional repressor of the let-7 family, EZH2 repression of LIN28B might be expected to activate multiple members of the let-7 family. Indeed, several other let-7 miRNAs also showed derepression in EZH2 -/- cells, though only let-7i and let-7f-3p met the stringent threshold for statistical significance we used earlier to identify derepressed miRNAs (Supplemental Fig. S10C). This data strongly suggests that EZH2 activates let-7i and other let-7 miRNAs by directly repressing LIN28B (Fig. 5E).

Taken together, these results confirm that PRC2 represses FRMD4B expression through a feed-forward regulatory network with let-7i.

Discussion

Transcription factors and miRNAs work in a highly coordinated manner, forming feed-back and feed-forward networks to regulate diverse processes. Using a combination of crosslinking based immunoprecipitation (iCLIP) and global miRNA depletion followed by RNA-seq, we identified miRNA-regulated genes genome-wide in GBM cells. This approach however only identified genes whose transcript levels are lowered due to miRNA function and thus likely underestimates the number of miRNA-repressed genes. Surprisingly, hundreds of miRNA-repressed genes nonetheless showed enrichment for PRC2 binding and H3K27me3 marks. We found that miRNAs post-transcriptionally reinforce the transcriptional repression of a significant fraction of PRC2 target genes, either independently of PRC2, or coordinately, by forming a feed-forward regulatory network with PRC2.

Overall, genes repressed by miRNAs and by PRC2 constituted 22% of all direct PRC2-repressed genes. 54% of these genes showed much less derepression in response to miRNA depletion in the absence of EZH2, indicating that PRC2 contributed to the repression function of some of these miRNAs. The difference in derepression in EZH2 -/- cells was not due to reduced miRNA depletion by VP55 in EZH2 -/- cells since the extent and the number of miRNAs depleted in WT and EZH2 -/- cells were almost identical (Supplemental Fig. S7B). We propose that many genes that are transcriptionally repressed by PRC2 continue to produce

transcripts at low levels due to inefficient repression by PRC2 at the chromatin and transcriptional level. These transcripts are then further repressed or destabilized by miRNAs which thus reinforce the repressive function of PRC2 in one of two modes. In the coordinate mode, miRNAs that repress PRC2 targets are themselves indirectly activated by EZH2, forming feed-forward regulatory networks. In the independent mode, miRNAs and PRC2 co-repress a common set of target genes independently (Fig. 6). This model would be in agreement with the notion that miRNAs are broadly involved in reinforcing transcriptional programs (Ebert and Sharp 2012). We found that PRC2 binds and tri-methylates H3K27 to repress genes that also harbor active H3K4me3 marks. These genes could produce residual transcripts as a result of leaky transcription, and indeed we found this to be the case. Genes co-repressed by PRC2 and miRNAs showed significantly higher expression upon depletion of miRNAs than genes silenced by PRC2 alone, even though PRC2 showed equal occupancy at both sets of genes (Supplemental Fig. S11). In an alternate model, miRNAs promote PRC2 function or binding, and global loss of miRNAs therefore leads to changes in PRC2 binding and consequent changes in expression of miRNA-repressed genes.

Four lines of evidence support the first model. First, genes co-repressed by PRC2 and miRNAs showed significant derepression in response to loss of miRNAs in EZH2 -/- cells, although lower than in WT cells (Supplemental Fig. S8). Second, mRNA targets of EZH2-activated miRNAs were significantly over-represented among feed-forward regulated genes. Third, we could verify direct repression of FRMD4B by PRC2 as well as by the EZH2-activated miRNA let-7i, which fits the

first model. PRC2 directly represses LIN28B, a let-7 miRNA repressor, causing an indirect activation of let-7i expression (Supplemental Fig. S10B). Finally, if miRNAs promote repression by PRC2 in addition to repressing genes directly, we would expect derepression in response to miRNA loss to be greater than derepression due to loss of EZH2. However derepression due to loss of miRNAs was significantly lower than derepression due to loss of EZH2 (Fig. 4 C,D and Fig. 5B). Our results thus favor the first model but do not completely rule out the second model and therefore it is possible that a feed-back network might also exist between PRC2 and miRNA mediated repression.

It is likely that this coordination between PRC2 and miRNA repression occurs in other cellular contexts, given the significant overlap in the genes regulated by PRC2 and miRNAs in mESCs (Supplemental Fig. S5). Our findings not only uncover a novel type of coordination between an epigenetic and post-transcriptional regulator of gene expression but could also have a broad impact on PRC2's regulatory function in a wide range of disease and developmental pathways.

Methods

Cell lines and reagents

GBM cell lines T98G and U87MG (ATCC-CRL-1690 and ATCC-HTB14) were grown in EMEM with 10% FBS (Gibco). All cell lines were maintained at 37°C and 5% CO_2. Antibodies used were – EZH2 (Abcam 186006), AGO2 (Abcam, ab 57113), SUZ12 (Active motif 39877), H3K27me3 (Millipore 07-449) and PPIF

(Abcam, ab 110324). pCMV(CAT)T7-SB100 was a gift from Zsuzsanna Izsvak (Addgene plasmid # 34879). pSBtet-GP was a gift from Eric Kowarz (Addgene plasmid # 60495).

RNA-seq and iCLIP

RNA-seq experiments were performed on poly-A selected mRNA (Bioo 512980) as previously described (Hall et al. 2018). AGO2 iCLIP experiments were performed as previously described (König et al. 2010). Small RNA-seq libraries were prepared using NEBNext small RNA library preparation kit (NEB E7330). Following library preparation, the miRNA size fraction was extracted from 6% TBE gel as per manufacturer's instructions.

Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted using Trizol and reverse transcribed by Superscript III reverse transcriptase (Thermo Fisher Scientific 18080085) using random hexamer primers. PCR was then performed on cDNA using Power SYBR Green master mix (Thermo Fisher Scientific 4367659). Transcript levels were normalized to 18S RNA levels and fold changes were quantified using the ΔΔCt method (Livak and Schmittgen 2001).

Chromatin immunoprecipitations

Cells were crosslinked with 1% formaldehyde for 20 min and quenched with 2.5 M glycine. The rest of the protocol was performed as previously described (Hall et al. 2018).

mRNA-seq and miRNA-seq analysis

Reads were aligned to the human genome (UCSC version hg38) using Hisat (Kim et al. 2015). FeatureCounts was then used to count reads mapping to genes and differential gene expression analysis was performed using DESeq2 with default parameters (Liao et al. 2014). Significantly differentially expressed genes were identified at a threshold of FDR corrected $P < 0.05$ and $\log_2$ fold-change >= 0.5 or <= -0.5. For miRNA-seq, reads mapping to mature miRNAs (miRBase hg38) were counted using bedtools (Quinlan and Hall 2010). Differentially expressed miRNAs were then identified using DESeq2 as described above, using the same thresholds (Love et al. 2014).

ChIP-seq analysis

ChIP-seq reads were aligned to the human genome (UCSC version hg38) using bwa (Li and Durbin 2009). MACS2 was then used to call peaks at the cut-off of *Q*-value < 0.01 (Zhang et al. 2008). For the heat maps plotted in Fig. 2E, Fig. 3B and Supplemental Fig. S4, signal represents enrichment scores ($-\log_{10}$ *Q*-value) output by MACS2. ChIP-seq data for H3K4me3 in GBM cells was obtained from our recently published study (Hall et al. 2018).

iCLIP analysis

iCLIP reads were first processed to remove duplicate reads using FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and then aligned to the human genome (UCSC version 19) using bowtie2. Reads mapping to repeat regions were filtered out using Repeat Masker. Peaks were then called using CLIPper using default parameters and filtered based on reproducibility across replicates (Lovci et al. 2013). MiRNA seed enrichments in sequences spanning 200 bases around peaks

were determined using HOMER software (Heinz et al. 2010). Prior to intersections with other datasets from RNA-seq, peak coordinates were converted to hg38 from hg19 using LiftOver.

CRISPR-Cas9 knockout of EZH2

EZH2 sgRNA was designed using http://crispr.mit.edu, and EZH2 -/- T98G cells were generated using the GeneArt CRISPR-Cas kit (Thermo Fisher Scientific A21175) following manufacturer's instructions. Knockout clones were verified by immunoblots.

Generation of VP55 expressing stable cell line

The VP55 coding sequence was PCR-amplified from a vector containing the codon-optimized VP55 sequence (a gift from Christopher Sullivan and Benjamin tenOever) and cloned into pSBtet-GP, a Tetracycline/Doxycycline-inducible expression vector containing the Sleeping Beauty transposase-specific inverted terminal repeats flanking the cloning site. Introduction of pSBtet-GP-VP55 and the Sleeping Beauty transposase expressing vector (SB100X) into cells allows the transposase-mediated genomic integration of the DNA sequence from pSBtet-GP-VP55 that is flanked by the inverted terminal repeats. SB100X and pSBtet-GP-VP55 constructs were transfected into T98G cells using Lipofectamine 2000 as per manufacturer’s instructions (Thermo Fisher Scientific 11668030). Clonal cells were then selected through puromycin selection at 2.5 μg/ml (Gibco A1113803). Expression of VP55 was induced with Doxycycline (D3072) at 5 μg/ml.

Luciferase reporter assays

The 3' UTR of target mRNAs spanning at least 500 bp around the predicted binding site of selected miRNAs were cloned into the psi-CHECK2 vector downstream of the Renilla luciferase gene. Mutagenesis was performed using the QuikChange II site directed Mutagenesis Kit (Agilent, 200523). Cells were transfected with WT or mutant 3' UTR with or without miRNA inhibitors and harvested after 48 hours. Luciferase activity was then measured using the Promega dual luciferase kit and calculated as the ratio of luminescence from Renilla to Firefly.

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Data Access

Primary sequencing data generated in this study is available at NCBI's GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112242).

Scripts used in this manuscript are available on github (https://github.com/haridh/PRC2-miRNA-manuscript).

Disclosures

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. Identification of miRNA targets by AGO2 iCLIP. (A) Autoradiograph showing AGO2-crosslinked ribonucleoprotein complexes from 2 replicates. The red box represents the region that was processed to make cDNA libraries. (B) Genomic enrichment of AGO2-crosslinked reads mapping to mRNAs. The plot shows total reads mapped to a genomic feature, normalized to the total length of the feature in the genome. (C) Correlation of the total reads mapping to genes between the two independent replicates of AGO2 iCLIP (left panel). Genome browser images showing AGO2 iCLIP peaks at 3' UTRs for two protein-coding genes (right panel). (D) AGO2-interacting miRNAs plotted in descending order of read coverage (left panel). Sequences enriched proximal to AGO2 iCLIP peaks on mRNAs that significantly match the binding sequence for AGO2-interacting miRNAs. Corresponding miRNA seed sequences are also shown (right panel). (E)

PPIF transcript levels in U87MG cells treated with negative control inhibitor or miR-23a-3p inhibitor. Error bars represent standard error across 2 independent replicates. * indicates $P < 0.05$ (paired t-test). (F) PPIF protein levels in U87MG cells treated with negative control inhibitor or miR-23a-3p inhibitor. The immunoblot showing two independent replicates (left panel) was quantified using ImageJ (right panel). The Y-axis represents average PPIF signal normalized to Actin. Error bars represent standard error across 2 independent replicates. * indicates $P < 0.05$ (paired t-test).

Figure 2. miRNA-repressed genes are enriched for PRC2 binding and H3K27me3. (A) miR-21 expression levels in response to VP55 induction. Dox = Doxycycline. (B) Volcano plot of differentially expressed miRNAs in response to VP55 induction. X-axis represents $\log_2$ fold difference between expression of protein-coding genes in VP55 induced and non-induced cells. Y-axis represents $-\log_2$ *P*-value of the expression difference calculated using DESeq2. (C,D) Enrichment for H3K27me3 (C) and SUZ12 (D) at genes derepressed in miRNA depleted cells (miRNA-repressed genes). Enrichments were calculated using Enrichr (Chen et al. 2013). (E) ChIP-seq enrichment scores ($-\log_{10}$ *Q*-value calculated using MACS2) for EZH2 and H3K27me3 at miRNA-repressed genes that also show EZH2 binding. Each row represents a miRNA-repressed gene with a significant peak for EZH2 and the columns represent 50 bp bins spanning 10 kb around the gene TSS.

Figure 3. miRNAs repress hundreds of genes directly repressed by PRC2. (A) Heat map showing differential expression of protein-coding genes in response to EZH2 knockout. Genes are ranked in increasing order of $\log_2$ fold change (≤ -0.5

and ≥ 0.5 log_2 fold change) in WT/EZH2 -/-. (B) Heat map showing enrichment scores (-log_{10} *Q*-value calculated using MACS2) for EZH2, H3K27me3 and H3K4me3 for genes plotted in and ordered as in A. (C) Average EZH2, H3K27me3 and H3K4me3 ChIP-seq signal for genes plotted in A. (D). Heat map showing log_2 fold values (WT/EZH2 -/-) of miRNA-repressed genes that are also significantly regulated by EZH2, ordered in increasing order of log_2 fold change(≤ -0.5 and ≥ 0.5 log_2 fold change in WT/EZH2 -/-). (E) qRT-PCR validation of genes repressed by both EZH2 and miRNAs, plotted as in Fig. 1E. * indicates $P < 0.05$ (paired t-test). (F) Correlation of transcript levels (X-axis) and AGO2 iCLIP enrichment (Y-axis)for four sets of protein-coding genes as indicated in the key (left panel). Overlap of AGO2-interacting genes repressed by miRNAs and genes co-repressed by EZH2 and miRNAs (right panel). Significance of overlap was calculated using the hypergeometric test.

Figure 4. miRNAs repress PRC2 target genes through a feed-forward regulatory network with PRC2. (A) Depletion of miR-21 in response to VP55 induction in EZH2 -/- cells. (B) Heat map showing derepression of gene expression in response to miRNA loss (+/- Dox) in WT (WT, +/- Dox) and EZH2 -/- cells (EZH2 -/-, +/- Dox) for genes co-repressed by EZH2 and miRNAs. Only 146 of the 213 co-repressed genes that had a significant difference in derepression between WT and EZH2 -/- cells are shown. Genes are ordered in decreasing order of WT, +/- Dox/EZH2 -/-, +/- Dox values. (C) Box plot comparing expression of feed-forward regulated genes in miRNA depleted (+ Dox) and non-depleted (- Dox) WT and EZH2 -/- cells. * indicates $P < 0.05$ and NS indicates not significant. (D) qRT-PCR showing

transcript levels in miRNA-depleted WT and EZH2 -/- cells, plotted as in Fig. 1E. (E) Heat map showing differential expression of miRNAs in response to EZH2 knockout. Rows are ordered from low to high $\log_2$ fold change between WT and EZH2 -/- miRNA counts. (F) Heat map showing interactions predicted by DIANA between EZH2-activated miRNAs and feed-forward regulated genes (Paraskevopoulou et al. 2013). Predicted interactions are indicated by filled cells.

Figure 5. PRC2 regulates FRMD4B expression through a feed-forward regulatory network with let-7i. (A) Genome browser view of FRMD4B showing tracks for EZH2 and H3K27me3 ChIP-seq. (B) qRT-PCR showing expression changes of FRMD4B in miRNA-depleted (+ Dox) and non-depleted (- Dox) WT and EZH2 -/- cells, plotted as in Fig. 1E. * indicates $P < 0.05$. (C) Genome browser view of let-7i in two replicates of WT and EZH2 -/- cells. (D) Luciferase assay comparing miRNA inhibition between WT and mutant FRMD4B 3' UTR (seed deletion) transfected WT and EZH2 -/- cells. (E) Feed-forward regulatory network formed by PRC2, LIN28B, let-7i and FRMD4B.

Figure 6. Model for reinforcement of PRC2 repression by miRNAs. Co-repression of target genes occurs transcriptionally by PRC2 and post-transcriptionally by miRNAs, either in a coordinated or independent manner.

Supplemental Figure Legends

Supplemental Figure S1. AGO2 binding sites identified by iCLIP are enriched for binding sites of highly expressed miRNAs. (A) Correlation of miRNA enrichment in AGO2 iCLIP data (X-axis) with their expression (Y-axis). AGO2 iCLIP enriched

miRNAs shown in Fig 1D (right) are indicated. (B) Enrichment for predicted mRNA targets of AGO2-interacting miRNAs among protein-coding genes interacting with AGO2.

Supplemental Figure S2. miR-23a-3p directly represses PPIF expression. (A) Genome browser view showing AGO2 iCLIP peaks at the 3' UTR of PPIF. Green bars represent predicted miRNA binding sites with miR-23a-3p binding site highlighted in red. (B) Luciferase assays showing rescue of miRNA inhibition on treatment with miR-23a-3p inhibitor or negative control inhibitor for WT 3' UTR and mutant 3' UTR (seed deletion, Mut-1) transfected U87MG cells. (C) Changes in luciferase signal on deletion of miR-23a-3p binding site (Mut-1) or disruption of miRNA-PPIF interactions through base substitutions (Mut-2) in T98G cells.

Supplemental Figure S3. Identification of miRNA-repressed genes by global depletion of miRNAs. (A) cDNA libraries prepared from miRNA depleted (+ Dox) and non-depleted (- Dox) T98G cells. (B) Volcano plot of differentially expressed protein-coding genes in response to VP55 induction.

Supplemental Figure S4. EZH2 and H3K27me3 ChIP-seq enrichment scores for protein-coding genes containing at least one significant EZH2 ChIP peak. Each row represents a gene containing at least one EZH2 ChIP-seq peak and the columns are binned into 50 bp windows spanning 10 kb around the TSS.

Supplemental Figure S5. miRNA-repressed genes in mESCs are enriched for H3K27me3 and PRC2. (A,B) Enrichr analysis showing enrichment for H3K27me3

(A) and SUZ12 (B) at genes up-regulated in miRNA-depleted mESCs (DICER knockout mESCs).

Supplemental Figure S6. Genes derepressed on loss of EZH2 are enriched for PRC2. (A) Immunoblots for EZH2, SUZ12 and H3K27me3 in WT and EZH2 -/- cells. (B) Normalized density of EZH2, H3K27me3 and H3K4me3 ChIP enrichment scores in the 10 kb region spanning the TSS of EZH2-regulated genes plotted in Fig. 3A. (C) Genome browser view of LAMA5, a gene enriched with EZH2 and H3K27me3 on chromatin, and whose transcript is also bound by AGO2.

Supplemental Figure S7. Global miRNA depletion in EZH2 -/- cells is comparable to WT cells. (A) cDNA libraries prepared from EZH2 -/- cells with (+ Dox) and without depletion of miRNAs (- Dox). (B) Total miRNA counts (Log_{10} total reads mapping to miRNAs) in WT and EZH2 -/- cells with and without depletion of miRNAs.

Supplemental Figure S8. Some feed-forward regulated genes show derepression in response to miRNA depletion in EZH2 -/- cells. Box plot comparing expression of a subset of feed-forward regulated genes (27 genes) in miRNA depleted and non-depleted EZH2 -/- cells.

Supplemental Figure S9. Non feed-forward regulated genes show derepression in response to miRNA loss in EZH2 -/- cells and WT cells. Box plot comparing expression of non-feed-forward regulated genes in miRNA depleted and non-depleted WT and EZH2 -/- cells.

Supplemental Figure S10. EZH2 activates let-7 miRNAs by directly repressing LIN28B. (A) Genome browser view of FRMD4B showing tracks for H3K27me3 ChIP-seq, EZH2 ChIP-seq and AGO2 iCLIP, highlighting the binding site for let-7i. (B) Genome browser view of LIN28B showing tracks for EZH2 and H3K27me3 ChIP-seq. (C) Heatmap showing expression change of all let-7 miRNAs in WT and EZH2 -/- cells. Rows are ranked by low to high $\log_2$ fold change (WT/EZH2 -/-).

Supplemental Figure S11. Genes co-repressed by EZH2 and miRNAs are less tightly repressed by EZH2 than genes silenced by EZH2 alone. (A) Box plot showing average expression in Dox treated (miRNA depleted) and untreated WT cells for two sets of genes - PRC2-repressed genes that are not co-repressed by miRNAs (non-miRNA-repressed) and genes that are co-repressed by PRC2 and miRNAs (miRNA-repressed). (B) Normalized density of EZH2 ChIP enrichment scores in a 10 kb region spanning the TSS of PRC2 target genes that are either non-miRNA-repressed or miRNA-repressed.

Figure 1

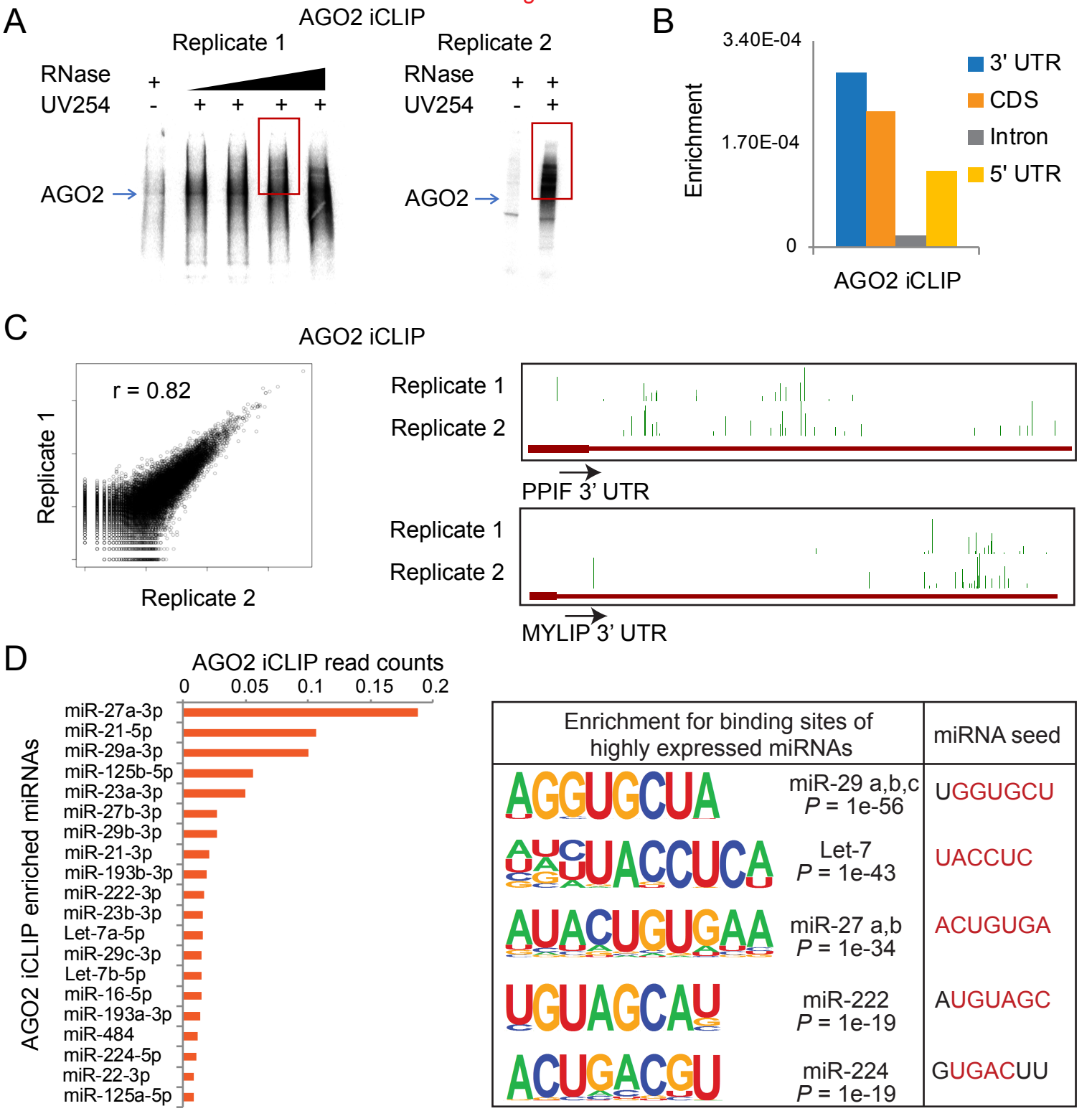

A
AGO2 iCLIP
Replicate 1
Replicate 2
RNase
UV254
AGO2
B
3.40E-04
1.70E-04
0
Enrichment
3' UTR
CDS
Intron
5' UTR
AGO2 iCLIP
C
AGO2 iCLIP
r = 0.82
Replicate 1
Replicate 2
PPIF 3' UTR
MYLIP 3' UTR
D
AGO2 iCLIP read counts
0
0.05
0.1
0.15
0.2
AGO2 iCLIP enriched miRNAs
miR-27a-3p
miR-21-5p
miR-29a-3p
miR-125b-5p
miR-23a-3p
miR-27b-3p
miR-29b-3p
miR-21-3p
miR-193b-3p
miR-222-3p
miR-23b-3p
Let-7a-5p
miR-29c-3p
Let-7b-5p
miR-16-5p
miR-193a-3p
miR-484
miR-224-5p
miR-22-3p
miR-125a-5p
Enrichment for binding sites of highly expressed miRNAs
miRNA seed
miR-29 a,b,c
P = 1e-56
UGGUGCU
Let-7
P = 1e-43
UACCUC
miR-27 a,b
P = 1e-34
ACUGUGA
miR-222
P = 1e-19
AUGUAGC
miR-224
P = 1e-19
GUGACUU
E
1.E-01
6.E-02
0
Normalized transcript level
*
Negative control
miR-23a-3p inhibitor
PPIF
F
miR-23a-3p inhibitor
Replicate
PPIF
Actin
5
2.5
0
Normalized protein level
*
Negative control
miR-23a-3p inhibitor
PPIF

Figure 2

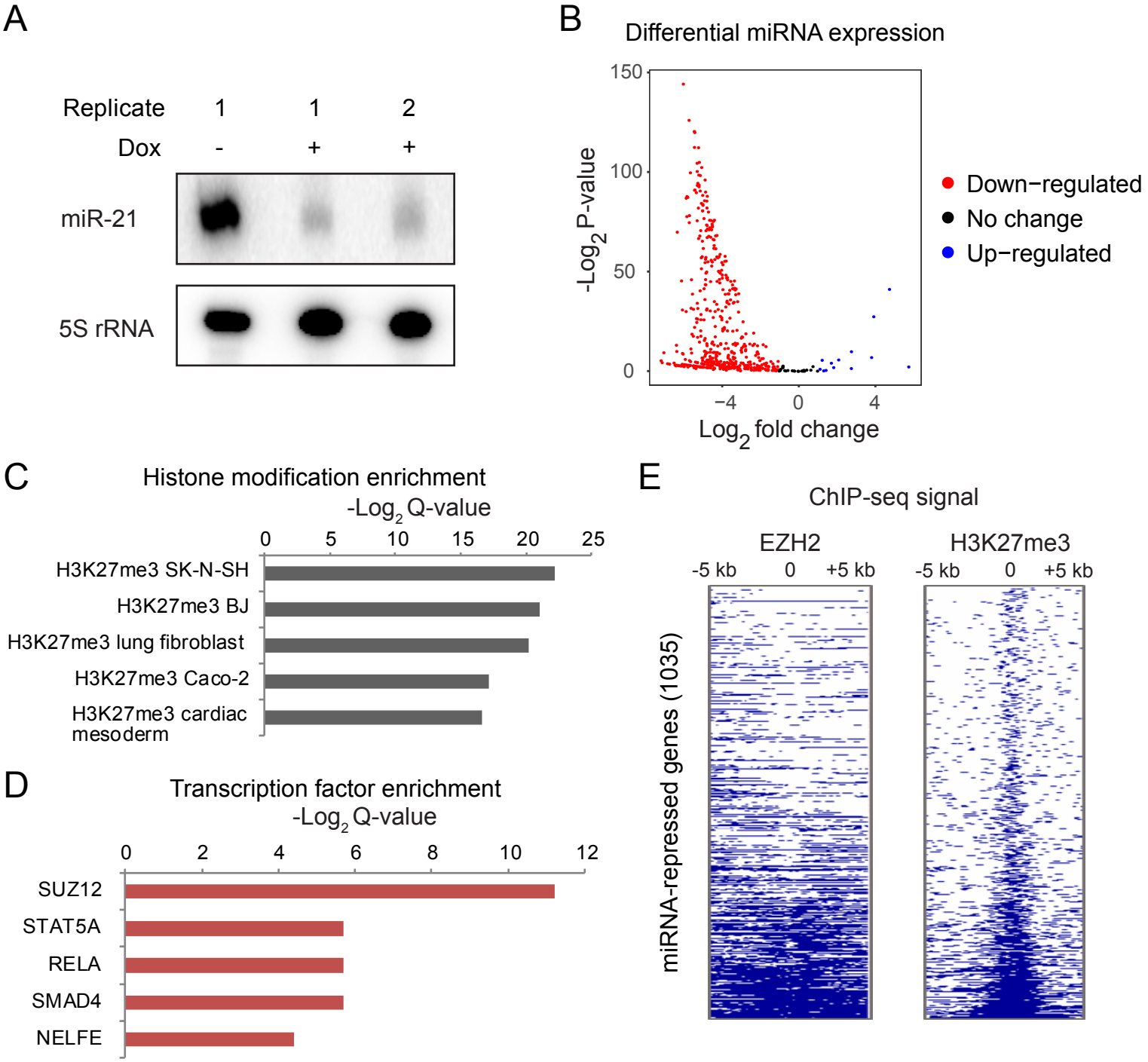

A
Replicate 1 1 2
Dox - + +
miR-21
5S rRNA
B
Differential miRNA expression
-Log2 P-value
Log2 fold change
Down−regulated
No change
Up−regulated
C
Histone modification enrichment
-Log2 Q-value
H3K27me3 SK-N-SH
H3K27me3 BJ
H3K27me3 lung fibroblast
H3K27me3 Caco-2
H3K27me3 cardiac mesoderm
D
Transcription factor enrichment
-Log2 Q-value
SUZ12
STAT5A
RELA
SMAD4
NELFE
E
ChIP-seq signal
EZH2
H3K27me3
-5 kb 0 +5 kb
miRNA-repressed genes (1035)

Figure 3

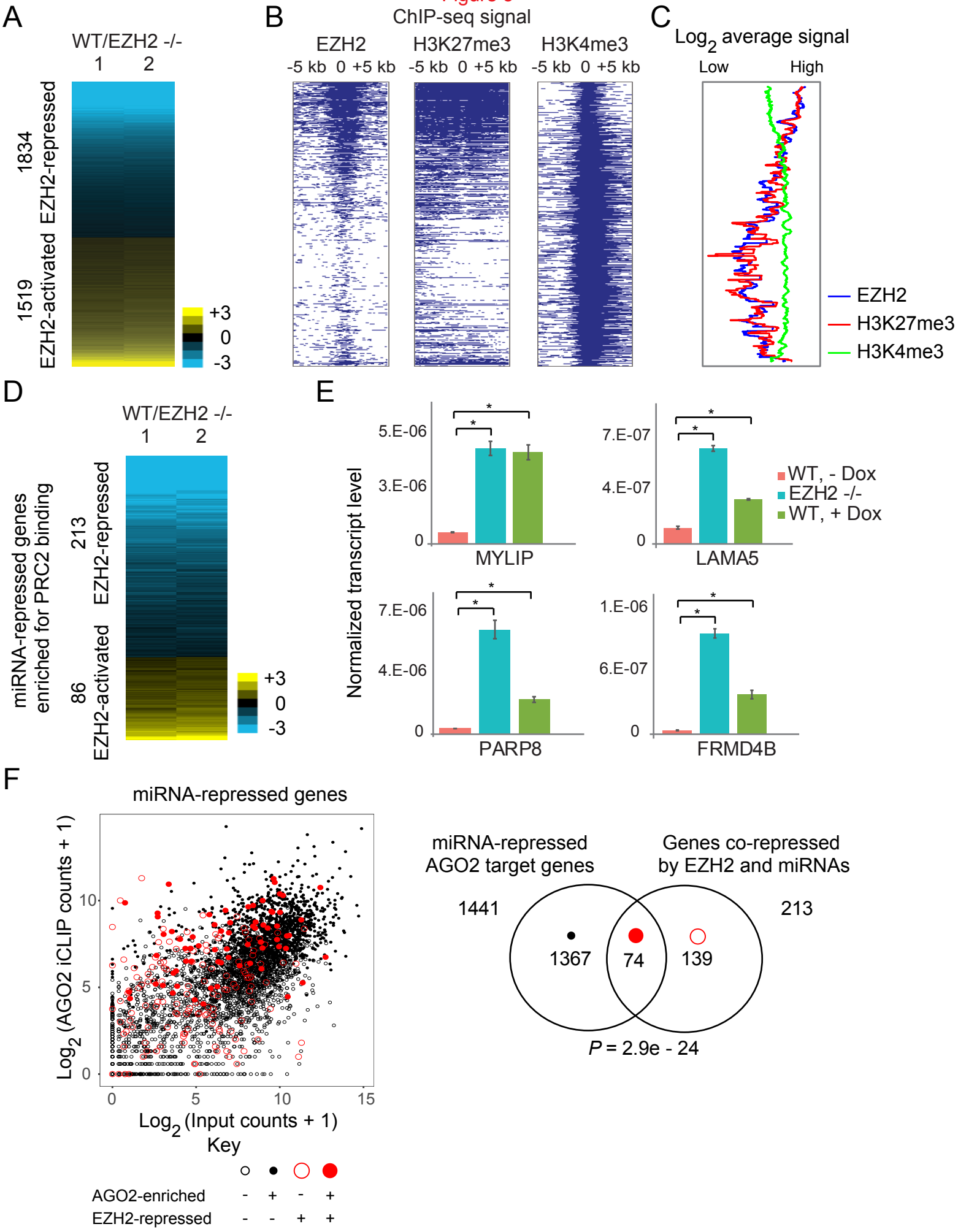

A
WT/EZH2 -/-
1
2
1834
EZH2-repressed
1519
EZH2-activated
+3
0
-3
B
ChIP-seq signal
EZH2
H3K27me3
H3K4me3
-5 kb 0 +5 kb
-5 kb 0 +5 kb
-5 kb 0 +5 kb
C
Log2 average signal
Low
High
EZH2
H3K27me3
H3K4me3
D
WT/EZH2 -/-
1
2
miRNA-repressed genes enriched for PRC2 binding
213
EZH2-repressed
86
EZH2-activated
+3
0
-3
E
Normalized transcript level
5.E-06
3.E-06
0
MYLIP
7.E-07
4.E-07
0
LAMA5
7.E-06
4.E-06
0
PARP8
1.E-06
6.E-07
0
FRMD4B
WT, - Dox
EZH2 -/-
WT, + Dox
F
miRNA-repressed genes
Log2 (AGO2 iCLIP counts + 1)
Log2 (Input counts + 1)
0
5
10
15
Key
AGO2-enriched - + - +
EZH2-repressed - - + +
miRNA-repressed AGO2 target genes
Genes co-repressed by EZH2 and miRNAs
1441
213
1367
74
139
P = 2.9e - 24

Figure 4

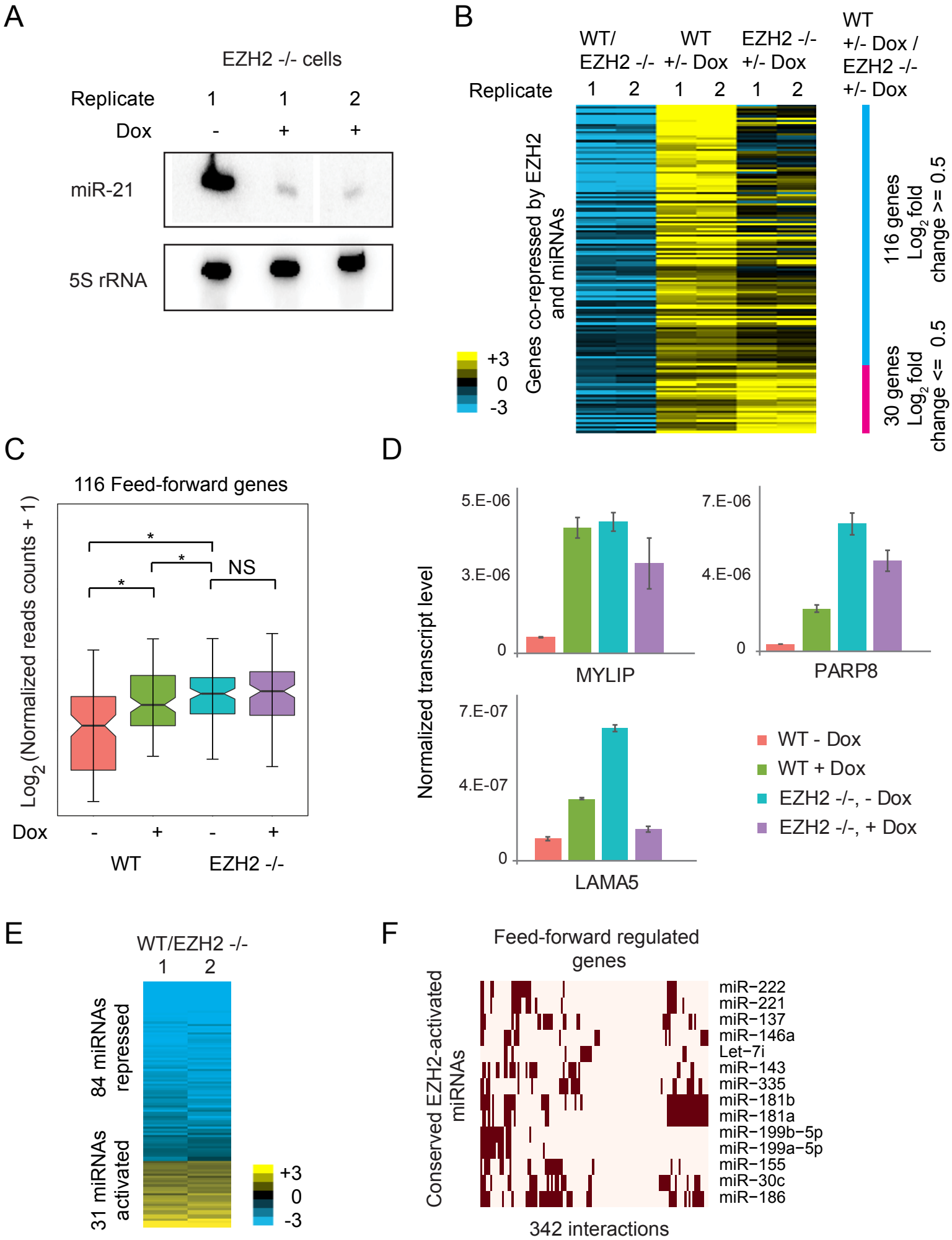
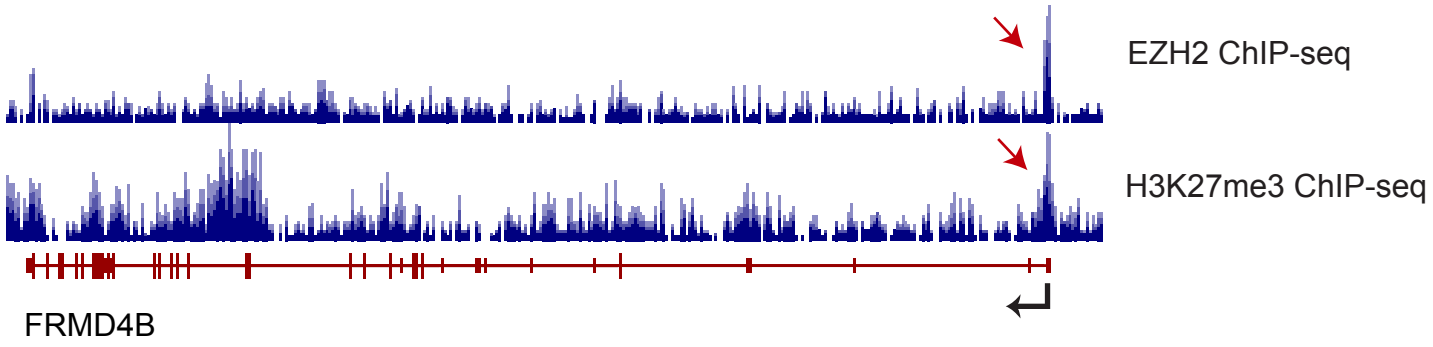
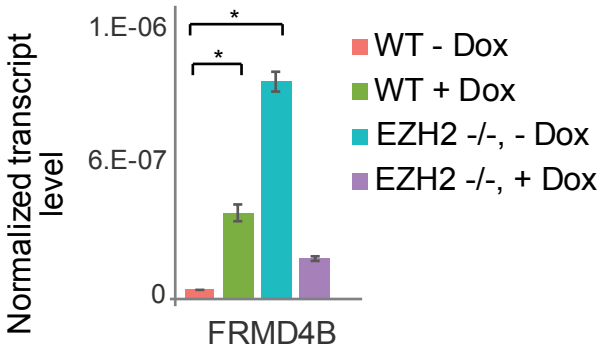

A
EZH2 -/- cells
Replicate 1 1 2
Dox - + +
miR-21
5S rRNA
B
WT/ EZH2 -/-
WT +/- Dox
EZH2 -/- +/- Dox
WT +/- Dox / EZH2 -/- +/- Dox
Replicate 1 2 1 2 1 2
Genes co-repressed by EZH2 and miRNAs
+3
0
-3
116 genes Log2 fold change >= 0.5
30 genes Log2 fold change <= 0.5
C
116 Feed-forward genes
Log2 (Normalized reads counts + 1)
*
*
*
NS
Dox - + - +
WT
EZH2 -/-
D
Normalized transcript level
5.E-06
3.E-06
0
MYLIP
7.E-06
4.E-06
0
PARP8
7.E-07
4.E-07
0
LAMA5
WT - Dox
WT + Dox
EZH2 -/-, - Dox
EZH2 -/-, + Dox
E
WT/EZH2 -/-
1 2
84 miRNAs repressed
31 miRNAs activated
+3
0
-3
F
Feed-forward regulated genes
Conserved EZH2-activated miRNAs
miR−222
miR−221
miR−137
miR−146a
Let−7i
miR−143
miR−335
miR−181b
miR−181a
miR−199b−5p
miR−199a−5p
miR−155
miR−30c
miR−186
342 interactions

Figure 5

A



B



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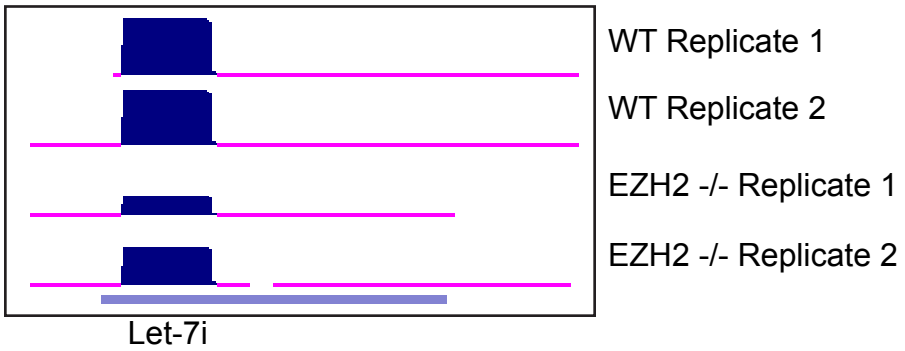

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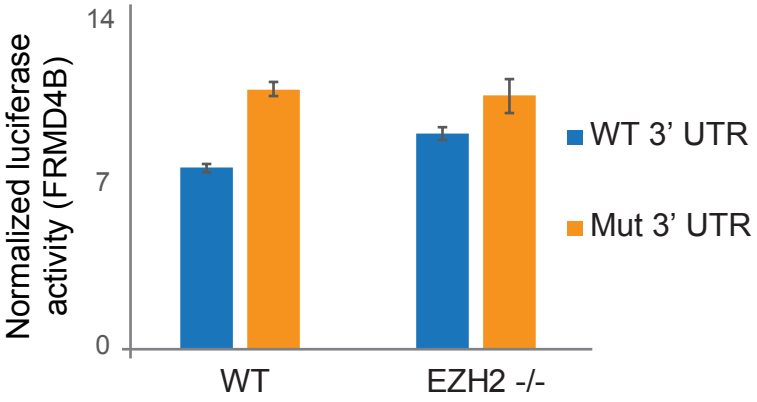


E

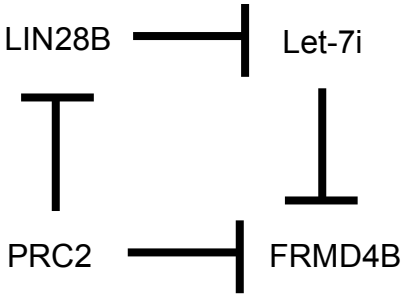
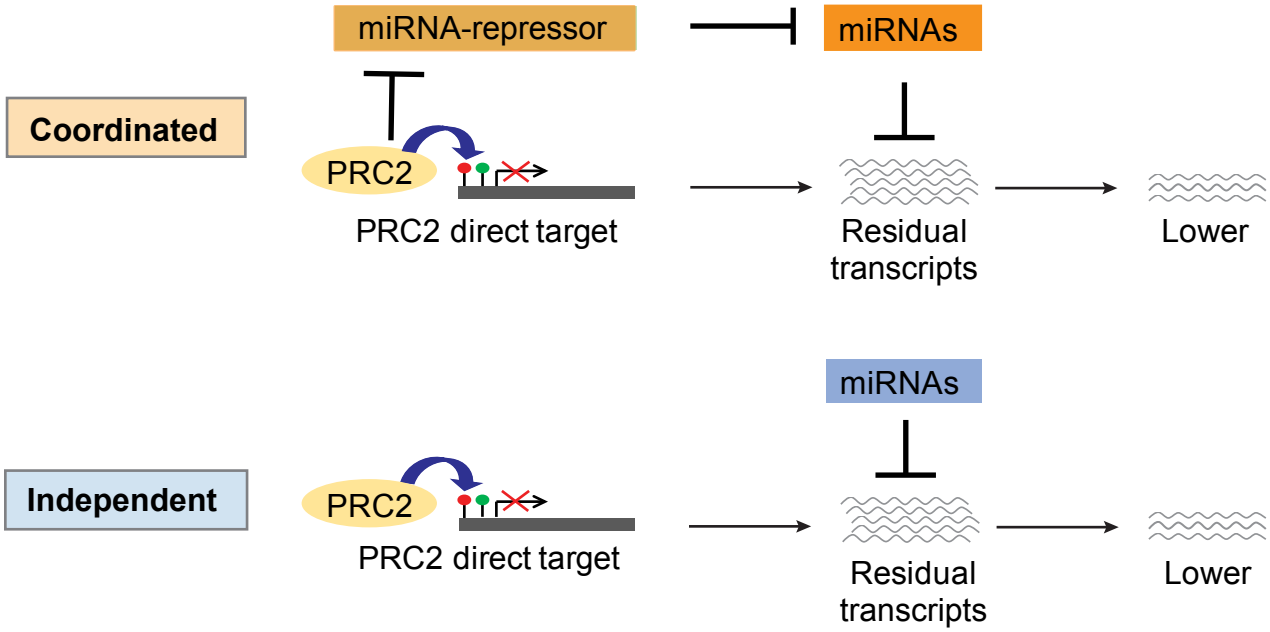
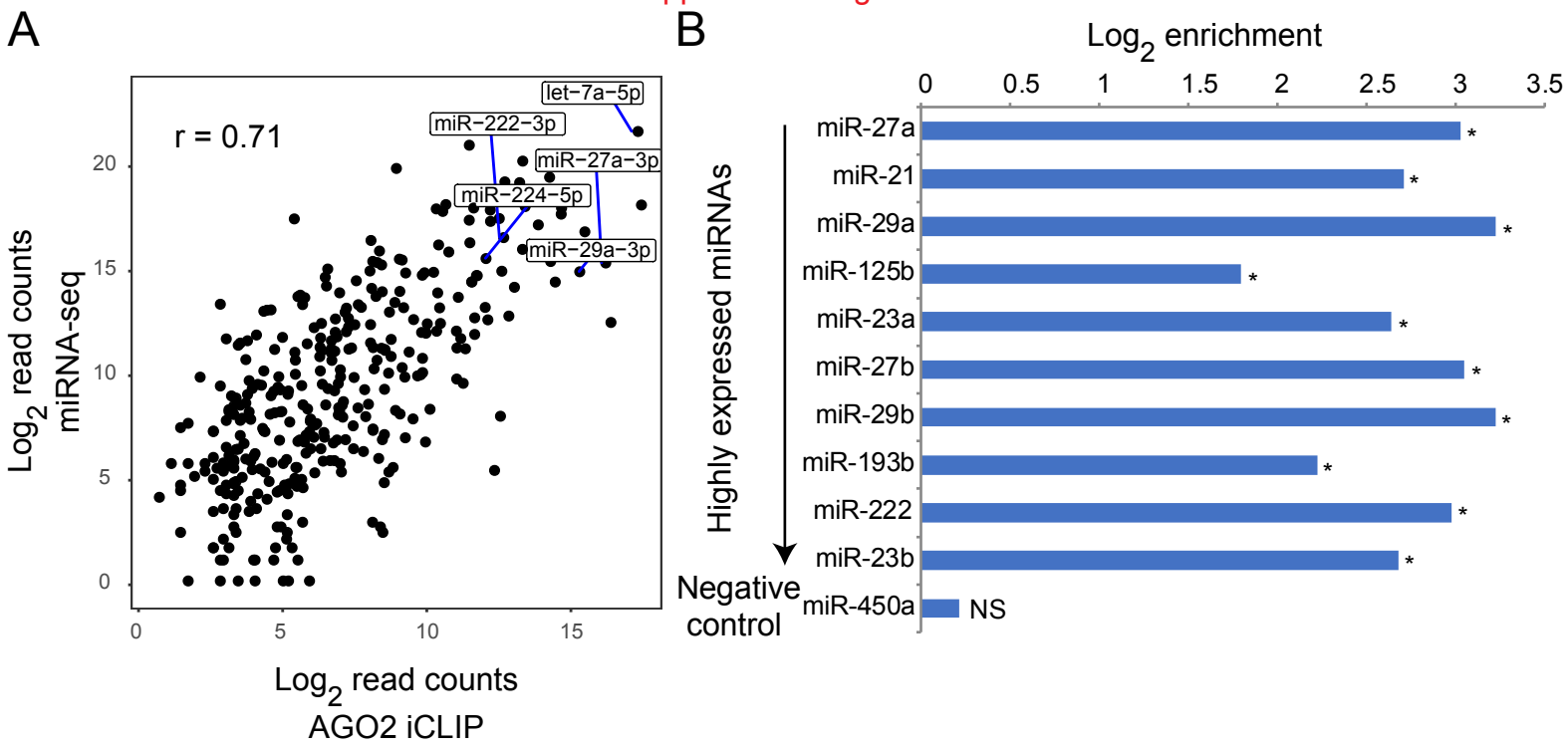


Figure 6

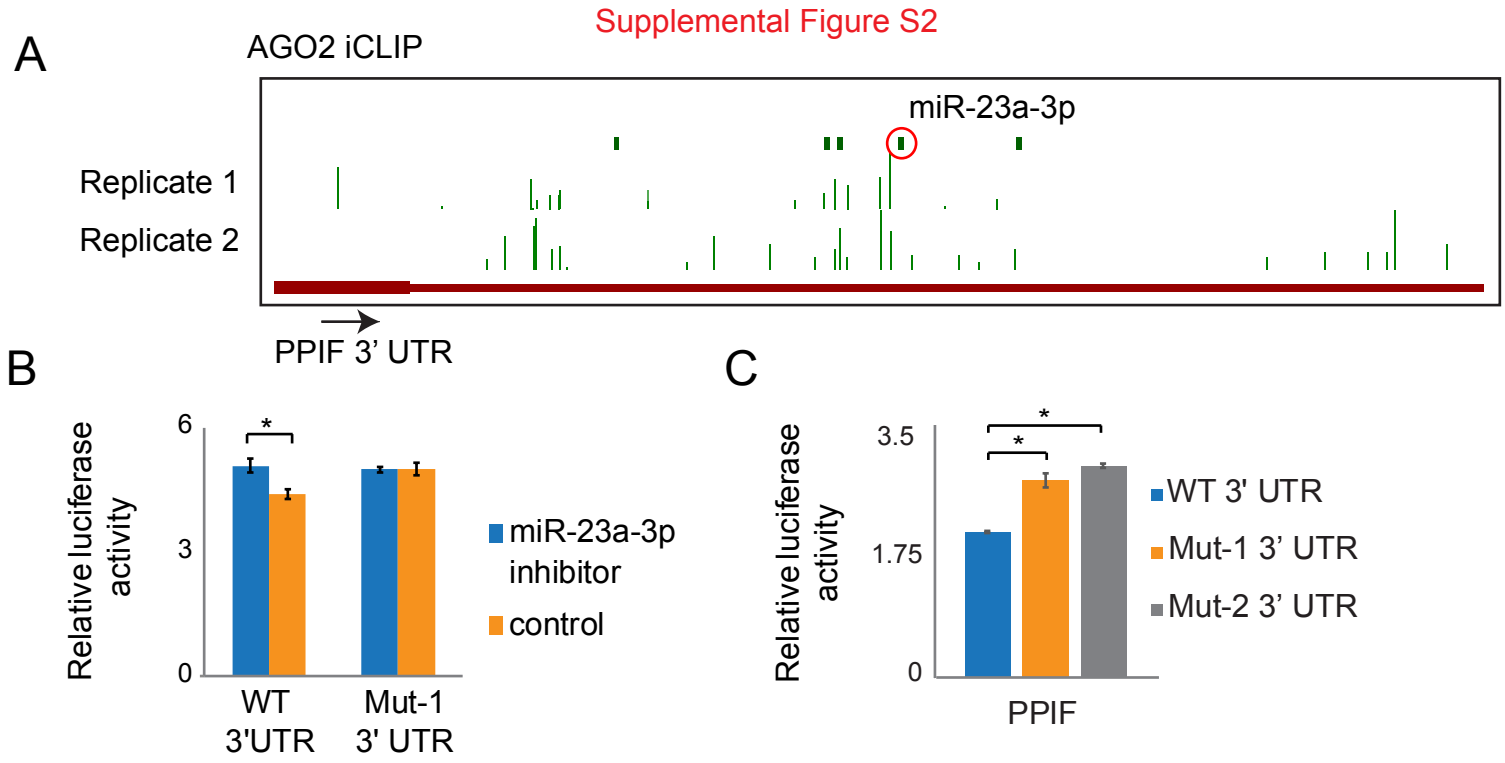
Reinforcement of PRC2 repression by miRNAs



Supplemental Figure S1

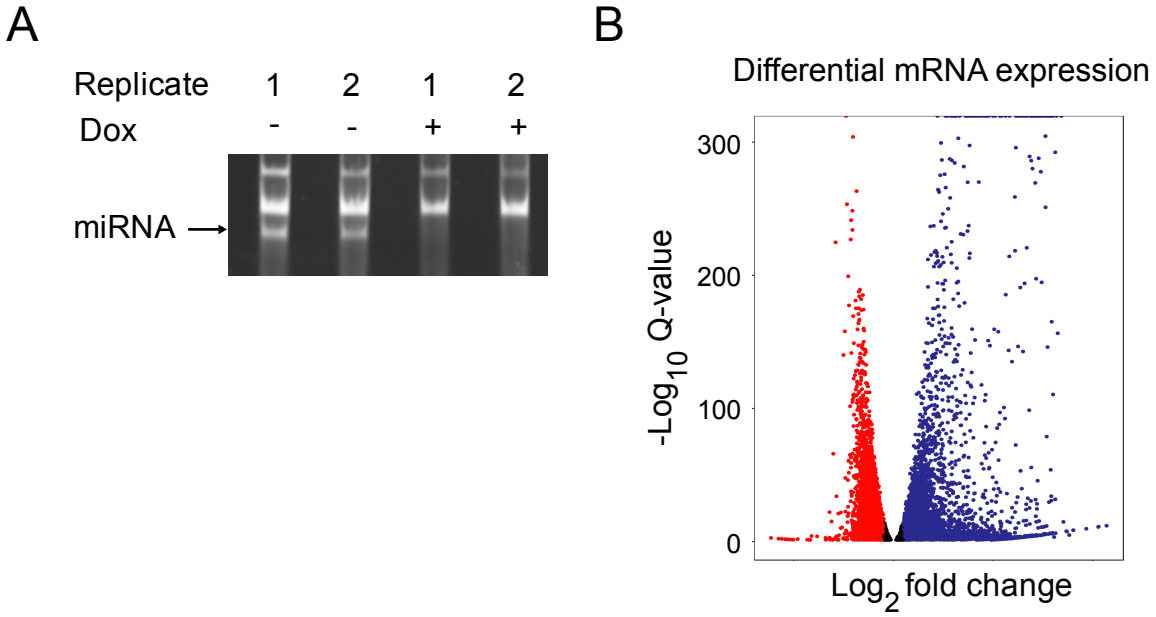


Supplemental Figure S1. AGO2 binding sites identified by iCLIP are enriched for binding sites of highly expressed miRNAs. (A) Correlation of miRNA enrichment in AGO2 iCLIP data (X-axis) with their expression (Y-axis). AGO2 iCLIP enriched miRNAs shown in Fig 1D (right) are indicated. (B) Enrichment for predicted mRNA targets of AGO2-interacting miRNAs among protein-coding genes interacting with AGO2.



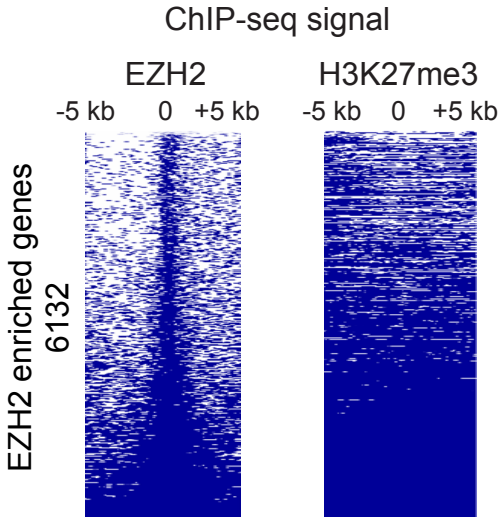
Supplemental Figure S2. miR-23a-3p directly represses PPIF expression. (A) Genome browser view showing AGO2 iCLIP peaks at the 3' UTR of PPIF. Green bars represent predicted miRNA binding sites with miR-23a-3p binding site highlighted in red. (B) Luciferase assays showing rescue of miRNA inhibition on treatment with miR-23a-3p inhibitor or negative control inhibitor for WT 3' UTR and mutant 3' UTR (seed deletion, Mut-1) transfected U87MG cells. (C) Changes in luciferase signal on deletion of miR-23a-3p binding site (Mut-1) or disruption of miRNA-PPIF interactions through base substitutions (Mut-2) in T98G cells.

Supplemental Figure S3



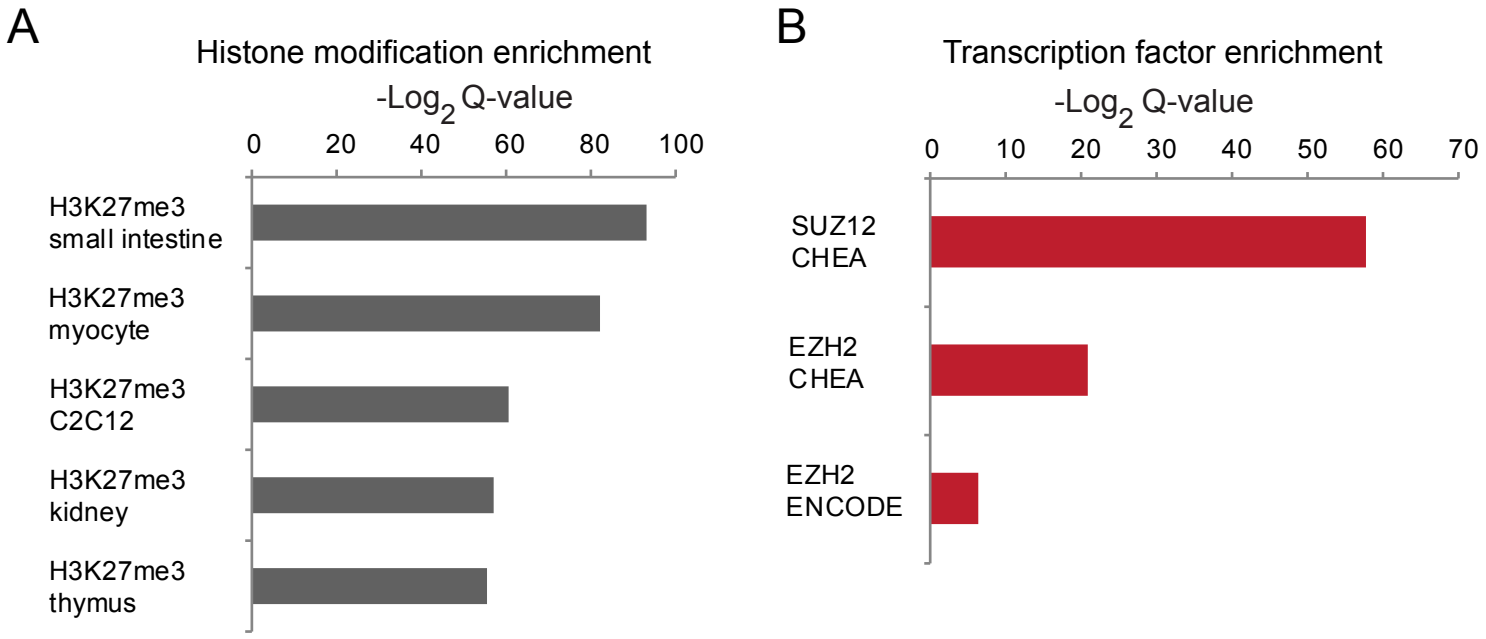
Supplemental Figure S3. Identification of miRNA-repressed genes by global depletion of miRNAs. (A) cDNA libraries prepared from miRNA depleted (+ Dox) and non-depleted (- Dox) T98G cells. (B) Volcano plot of differentially expressed protein-coding genes in response to VP55 induction.

Supplemental Figure S4



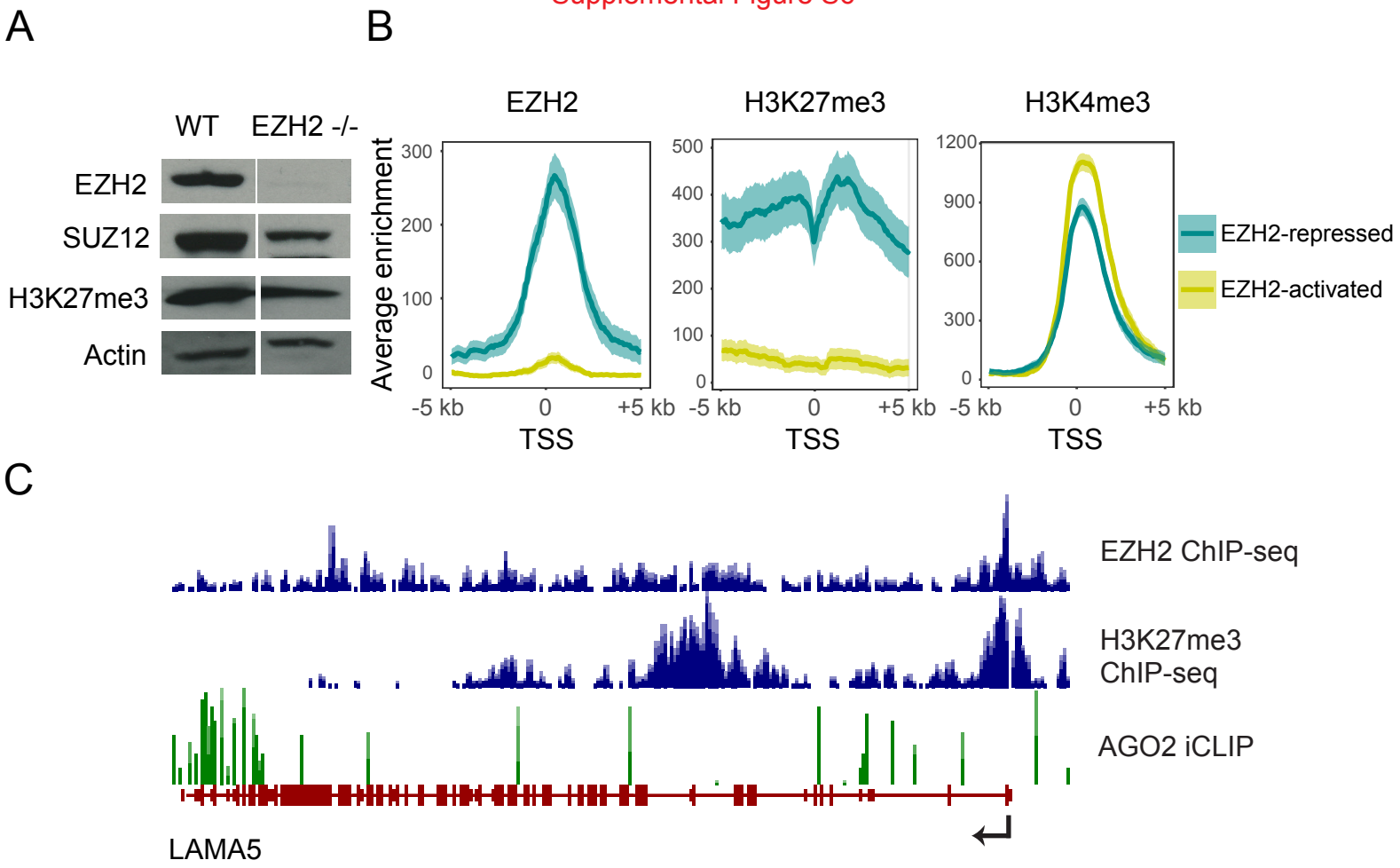
Supplemental Figure S4. EZH2 and H3K27me3 ChIP-seq enrichment scores for protein-coding genes containing at least one significant EZH2 ChIP peak. Each row represents a gene containing at least one EZH2 ChIP-seq peak and the columns are binned into 50 bp windows spanning 10 kb around the TSS.

Supplemental Figure S5



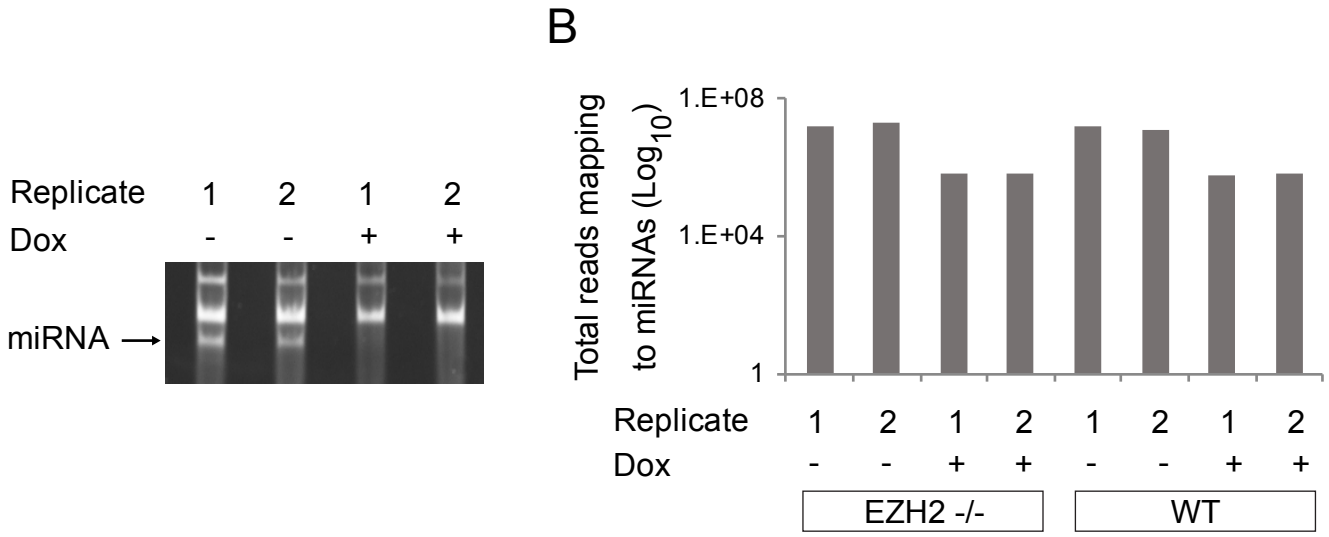
Supplemental Figure S5. miRNA-repressed genes in mESCs are enriched for H3K27me3 and PRC2. (A,B) Enrichr analysis showing enrichment for H3K27me3 (A) and SUZ12 (B) at genes up-regulated in miRNA-depleted mESCs (DICER knockout mESCs).

Supplemental Figure S6



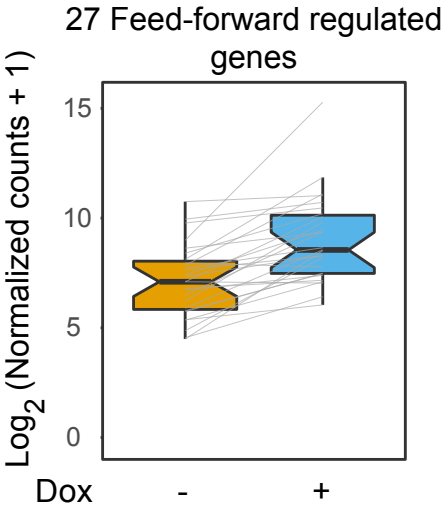
Supplemental Figure S6. Genes derepressed on loss of EZH2 are enriched for PRC2. (A) Immunoblots for EZH2, SUZ12 and H3K27me3 in WT and EZH2 -/- cells. (B) Normalized density of EZH2, H3K27me3 and H3K4me3 ChIP enrichment scores in the 10 kb region spanning the TSS of EZH2-regulated genes plotted in Fig. 3A. (C) Genome browser view of LAMA5, a gene enriched with EZH2 and H3K27me3 on chromatin, and whose transcript is also bound by AGO2.

Supplemental Figure S7



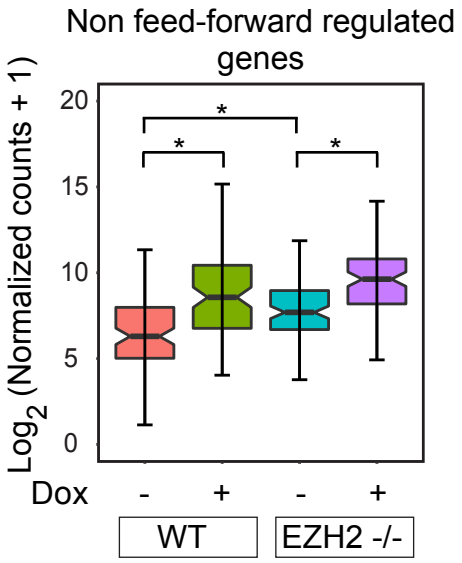
Supplemental Figure S7. Global miRNA depletion in EZH2 -/- cells is comparable to WT cells. (A) cDNA libraries prepared from EZH2 -/- cells with (+ Dox) and without depletion of miRNAs (- Dox). (B) Total miRNA counts (Log_{10} total reads mapping to miRNAs) in WT and EZH2 -/- cells with and without depletion of miRNAs.

Supplemental Figure S8



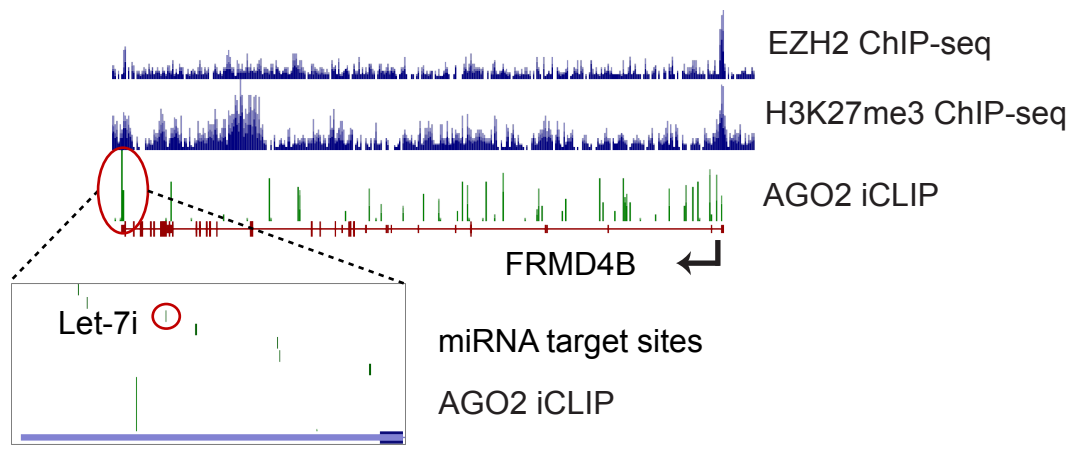
Supplemental Figure S8. Some feed-forward regulated genes show derepression in response to miRNA depletion in EZH2 -/- cells. Box plot comparing expression of a subset of feed-forward regulated genes (27 genes) in miRNA depleted and non-depleted EZH2 -/- cells.

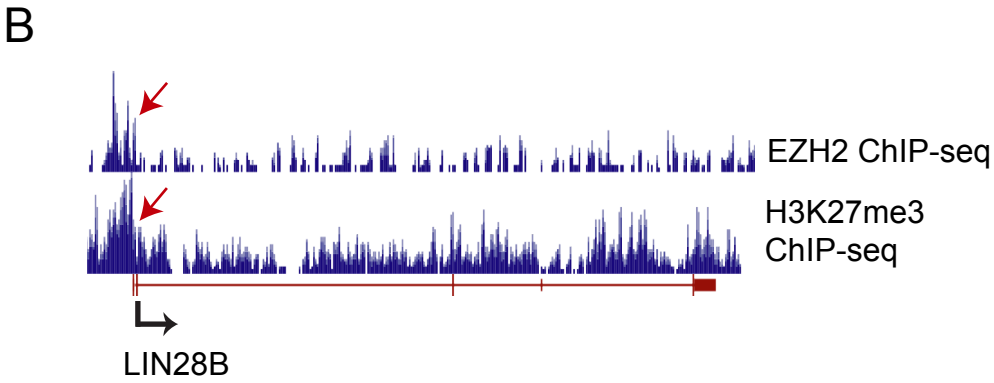
Supplemental Figure S9

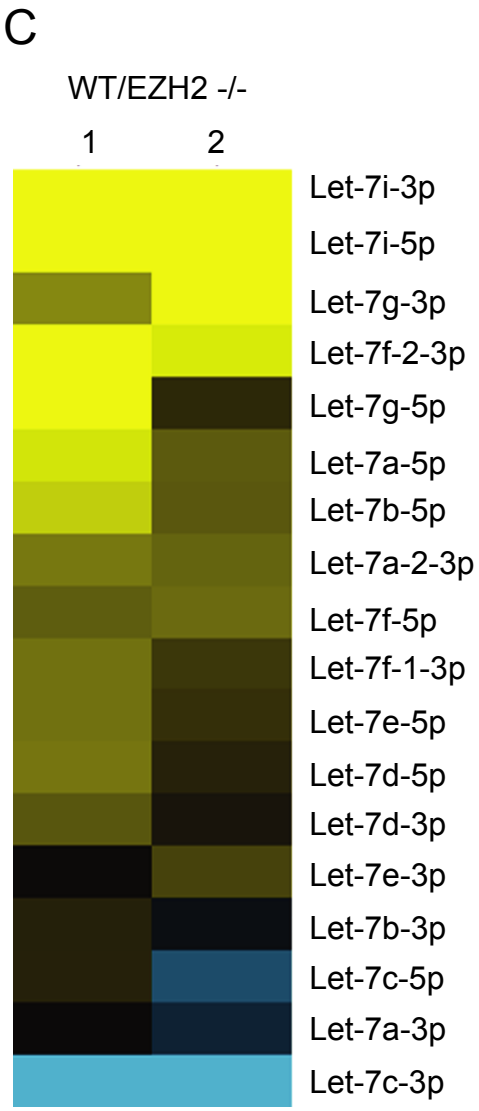


Supplemental Figure S9. Non feed-forward regulated genes show derepression in response to miRNA loss in EZH2 -/- cells and WT cells. Box plot comparing expression of non feed-forward regulated genes in miRNA depleted and non-depleted WT and EZH2 -/- cells.

Supplemental Figure S10

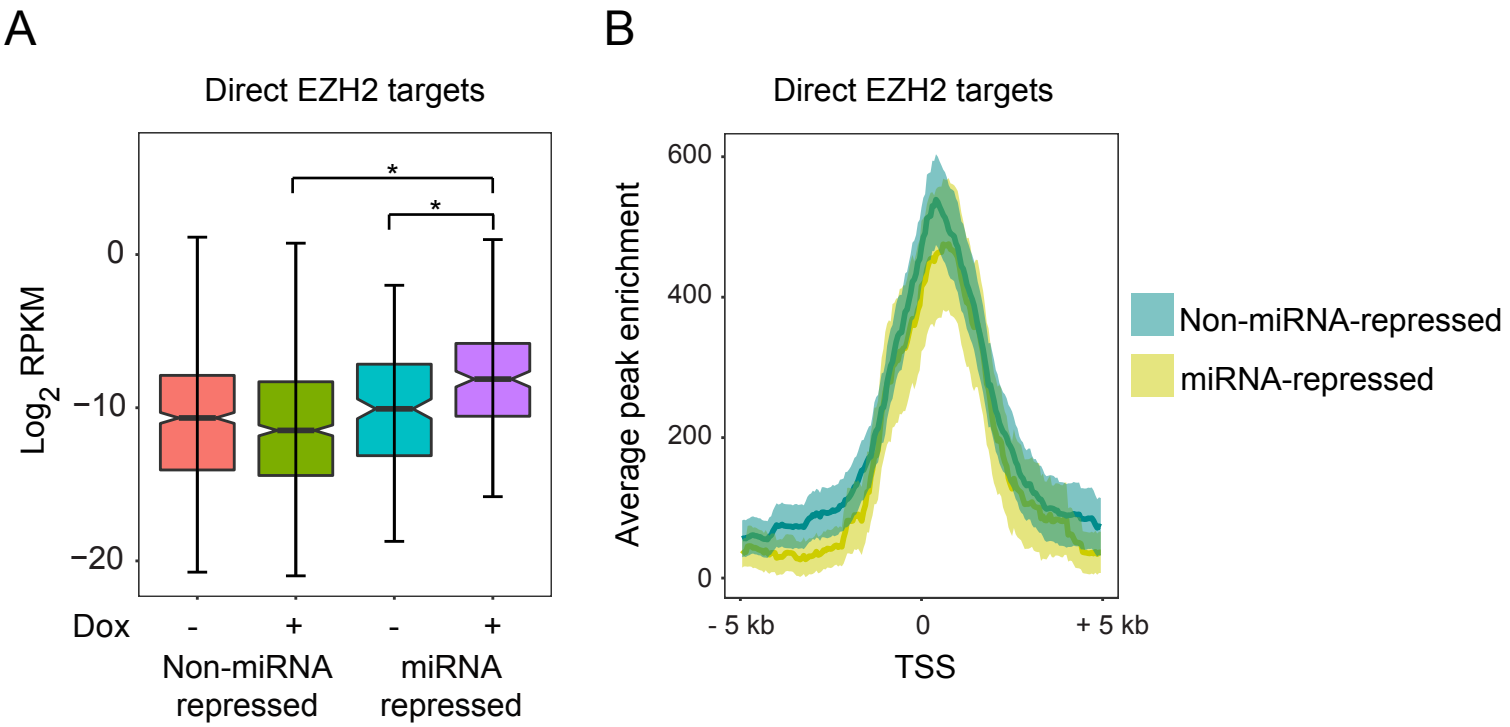






Supplemental Figure S10. EZH2 activates let-7 miRNAs by directly repressing LIN28B. (A) Genome browser view of FRMD4B showing tracks for H3K27me3 ChIP-seq, EZH2 ChIP-seq and AGO2 iCLIP highlighting the binding site for let-7i. (B) Genome browser view of LIN28B showing tracks for EZH2 and H3K27me3 ChIP-seq. (C) Heatmap showing expression change of all let-7 miRNAs in WT and EZH2 -/- cells. Rows are ranked by low to high $\log_2$ fold change (WT/EZH2 -/-).

Supplemental Figure S11



Supplemental Figure S11. Genes co-repressed by EZH2 and miRNAs are less tightly repressed by EZH2 than genes silenced by EZH2 alone. (A) Box plot showing average expression in Dox treated (miRNA depleted) and untreated WT cells for two sets of genes - PRC2-repressed genes that are not co-repressed by miRNAs (non-miRNA-repressed) and genes that are co-repressed by PRC2 and miRNAs (miRNA-repressed). (B) Normalized density of EZH2 ChIP enrichment scores in a 10 kb region spanning the TSS of PRC2 target genes that are either non-miRNA-repressed or miRNA-repressed.