HyperCas12a enables highly-multiplexed epigenome editing screens

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Abstract

Interactions between multiple genes or cis-regulatory elements (CREs) underlie a wide range of biological processes in both health and disease. High-throughput screens using dCas9 fused to epigenome editing domains have allowed researchers to assess the impact of activation or repression of both coding and non-coding genomic regions on a phenotype of interest, but assessment of genetic interactions between those elements has been limited to pairs. Here, we combine a hyper-efficient version of *Lachnospiraceae bacterium* dCas12a (dHyperLbCas12a) with RNA Polymerase II expression of long CRISPR RNA (crRNA) arrays to enable efficient highly-multiplexed epigenome editing. We demonstrate that this system is compatible with several activation and repression domains, including the P300 histone acetyltransferase domain and SIN3A interacting domain (SID). We also show that the dCas12a platform can perform simultaneous activation and repression using a single crRNA array via co-expression of multiple dCas12a orthologues. Lastly, demonstrate that the dCas12a system is highly effective for high-throughput screens. We use dHyperLbCas12a-KRAB and a ~19,000-member barcoded library of crRNA arrays containing six crRNAs each to dissect the independent and combinatorial contributions of CREs to the dose-dependent control of gene expression at a glucocorticoid-responsive locus. The tools and methods introduced here create new possibilities for highly multiplexed control of gene expression in a wide variety of biological systems.

Introduction

Many biological systems depend on coordinated activity between genetic elements. In mammalian systems, it is common that several cis-regulatory elements (CREs) control the expression of the same gene both at steady state and in response to environmental

conditions[1–10]. Recent work to experimentally test and validate thousands of CRE-gene pairs found between two and three CREs per gene, while predictive modeling of millions of CRE-promoter interactions estimates about four CREs per gene[11,12]. It is also common that coordinated expression of several genes controls aspects of cell differentiation and disease[13–28].

One way to evaluate the function of combinations of gene regulatory elements is to perturb their function *in vitro* or *in vivo*. Several technologies for genetic or epigenetic perturbation of regulatory elements have been developed based on bacterial clustered regularly interspaced short palindromic repeats (CRISPR) systems. Briefly, bacterial CRISPR systems use short antisense crispr RNAs (crRNAs) to target a Cas nuclease to specific ~20 bp DNA sequences[29–33]. Those systems have been adapted for gene activation and repression by fusing an epigenome-modifying domain to nuclease-inactivated “dead” dCas proteins[34,35]. Moreover, by delivering multiple crRNAs at once, it is possible to study interactions between CREs. For example, dCas9-based studies of pairwise epigenetic perturbations have revealed combinations of transcription factors that enhance neuronal differentiation[13]; long-distance synergistic interactions between enhancers controlling MYC expression[2]; synthetic lethality in the human genome[27]; and hierarchies in CRE interactions[19].

Scaling beyond pairwise or three-way combinations of sgRNAs is challenging, however, for several reasons. Cas9 requires an RNA molecule known as a trans-activating crRNA (tracrRNA) to bind the crRNA. To simplify studies using Cas9, the tracrRNA and crRNA are commonly expressed as a single ~100 bp molecule known as a single guide RNA or sgRNA[30,31]. However, repetitive sequences in multi-sgRNA plasmids, including the tracrRNA, can also lead to extensive plasmid recombination[36–39]. Creating further design constraints, each sgRNA must either be expressed from its own promoter, or expressed as part of a longer sgRNA array that is cleaved via separately expressed enzymes[40]. Together, those complexities severely limit highly multiplexed Cas9 genome- or epigenome-editing screens.

In contrast, several features of the Cas12a nuclease make it ideally suited for highly multiplexed manipulation of gene regulatory elements. Like Cas9, deoxyribonuclease-inactivated dCas12a can be fused with epigenetic effectors to activate or repress gene expression[25,41–44]. Unlike Cas9, however, a ~40 bp crRNA is sufficient to target Cas12a or dCas12a to sites in the human genome[32,33]. Cas12a also has an endoribonuclease activity that allows it to cleave individual crRNAs from a single pre-crRNA molecule containing a tandem array of many crRNAs[32]. Using those features, previous studies have targeted 20 genomic sites at once via a single pre-crRNA[44]. Several studies have also used that multiplexing capability in high-throughput genome-editing screens to identify synthetic lethal combinations of human genes; and in epigenome-editing screens to identify combinations of gene regulatory elements contributing to cell fitness[10,21,23–25,45].

Here, we introduce a suite of novel dCas12a epigenome editors for effective, highly-multiplexed gene activation and repression; and demonstrate that those effectors together with additional advances enable multiplexed high-throughput screens of combinatorial regulatory element activity. To do so, we systematically compared epigenome editing activity across a wide range of

existing and novel dCas12a epigenome effectors. We identify dCas12a effectors sensitive enough to alter regulatory element activity when lentivirally integrated into the genome. We also demonstrate that we can express complex pre-crRNAs that include selectable markers and up to 10 crRNAs from an RNA polymerase II promoter. Further, by combining crRNAs for different dCas12a proteins in a single pre-crRNA, we can simultaneously activate and repress gene expression. Together, those advances enable highly-multiplexed high-throughput epigenome editing screens, which we demonstrate by dissecting the cis-regulatory architecture for a dose-dependent gene expression response to glucocorticoids.

Results

dHyperLbCas12a and dEnAsCas12a outperform other dCas12a variants for epigenome editing

To optimize the dCas12a epigenome editing system for high-throughput screening, we first evaluated which Cas12a variants are most effective for epigenome editing. In addition to the wild-type Cas12a from *Acidaminococcus* sp. BV3L6 (As) and *Lachnospiraceae bacterium* (Lb), several engineered Cas12a versions with improved activity have been published[46–48]. We systematically compared the strength of gene activation and gene repression across five dCas12a variants: the wild type, enhanced, and ultra versions of dAsCas12a; and the wild type and hyper versions of dLbCas12a. For activation, we fused each dCas12a variant to the tripartite synthetic activator VPR (VP64-p65-Rta), while for repression we fused each dCas12a variant to a Krüppel-associated box (KRAB) domain. We cloned each of the resulting dCas12a effector proteins into the same plasmid backbone where they are expressed co-transcriptionally with a green fluorescent protein (EGFP). We then evaluated the effects on gene expression by co-transfecting each dCas12a effector into HEK293T cells along with a pre-crRNA array encoding three crRNAs targeting a gene of interest, or three non-targeting control crRNAs. Because Lb and AsCas12a have the same PAM sequence (TTTV), we used the same crRNA target sequences throughout, enabling better-controlled comparisons between As and Lb-derived Cas12a proteins. We then assessed the impact of each Cas12a variant on target gene expression 48 hours later using RT-qPCR **(Fig. 1a-b)**.

The dHyperLbCas12a had the strongest effects for both activation and repression, though dEnAsCas12a showed consistently strong activity as well. EnAsCas12a has the additional benefit of being able to recognize several non-canonical PAM sequences, with an estimated 7-fold increase in target sites in the human genome over wild type Lb and AsCas12a[46]. Our results also replicated previous studies showing that wild type AsCas12a has lower epigenome editing activity than wild type LbCas12a, but that the engineered dAsCas12a variants have comparable activity[41,46]. Due to claims that HyperLbCas12a most strongly outperforms other Cas12a variants under low crRNA conditions[48], we also tested the activators in a “low crRNA" condition where we transfected 1/10th the amount of pre-crRNA array as in **Fig. 1a**. Under those conditions, dHyperLbCas12a had the strongest gene activation as well **(Supp. Fig. 1a)**.

The dCas12 constructs we evaluated all have a C terminal nuclear localization signal (NLS). We also evaluated adding an additional NLS to the N-terminus of the dCas12a protein, but found no increase in epigenome editing **(Supp. Fig. 1b)**. Taken together, we focused on developing multiplexed high throughput screening using dHyperLbCas12a due to its high overall activity, and dEnAsCas12a due to its expanded targeting range.

Improved dCas12a pre-crRNA expression for long arrays via an RNA Pol II promoter

Next, we focused on optimizing the Cas12a array expression system. In bacteria, RNA polymerase expresses Cas12a pre-crRNA arrays without a 5' cap or poly-A tail; and synthetic mammalian systems recreate that structure using RNA Pol III. Expression with RNA Pol III limits the length of the pre-crRNA, however. For example, a recent study showed that U6-driven pre-crRNA expression drops off after ~4 crRNAs[23]. Because our goal is to enable highly-multiplexed screening involving arrays of >4 crRNAs, we expected that driving array expression with RNA Pol III would be insufficient and we would need to use a different expression system.

We hypothesized that an RNA pol II promoter would effectively drive longer arrays due to the higher processivity of RNA pol II compared to RNA pol III. To test this, we created pre-crRNA arrays containing 10 crRNAs wherein we placed three crRNAs targeting the *HBE1* promoter either at the front or back of the array. We then expressed the pre-crRNAs via the RNA pol III promoter U6 or the RNA pol II promoter CAG (**Fig. 1c)**. We then co-transfected the pre-crRNA arrays along with plasmids expressing variants of dCas12a-VPR or dCas12a-P300 (described below) into HEK293T cells. As negative controls, we used pre-crRNA arrays containing ten non-targeting crRNAs **(Fig. 1d-e)**. When we used the U6 promoter to express the pre-crRNA arrays, all dCas12a variants robustly activated *HBE1* expression when *HBE1*-targeting crRNAs were at the front of the array; but there was little or no *HBE1* activation for three of the four dCas12a epigenome activators when we moved the *HBE1*-targeting crRNAs to the back of the array. Expressing the same pre-crRNA arrays using the CAG promoter rescued *HBE1* activation from the back of the pre-crRNA array for all dCas12a variants tested. There was also no substantial difference in *HBE1* activation from the front or back of the pre-crRNA array. Together, those results indicate that RNA Pol II improves expression of long pre-crRNA arrays.

dCas12a-SID fusions enable multiplexed gene repression

To date, dCas12a-based repression studies have largely used the KRAB repressor domain[10,42,44,48]. Though KRAB is a potent repressor of transcription, there are potential advantages for repressing via other effector domains. First, KRAB is a potent repressor that recruits KAP1 and induces heterochromatin[49]. In some cases, more subtle repression may be more relevant to model disease. Second, there are also reports that heterochromatin formation can spread over long distances in some cases, causing potential off target effects. For example, targeting KRAB to an enhancer element can repress multiple enhancers or gene promoters nearby in some cases[50], but not always[51,52]. Third, there are cases when dCas12a-KRAB

fusions have little to no effect on gene expression, and alternative effectors may be able to repress those genes[10,44,53].

To develop additional dCas12a-based repressors, we created and tested dCas12a fusions to four copies of the SIN3A interacting domain (SID) derived from MAD1. The SID domain mediates gene repression through recruitment of SIN3A and the histone deacetylases HDAC1 and HDAC2[54]. Previous studies have also shown that SID fusion proteins can repress enhancers and promoters[4,55], and we sought here to extend those findings to dCas12a fusions.

We first compared fusions of KRAB, 3xKRAB, 4xSID, and both 3xKRAB and 4xSID to dLbCas12a or dEnAsCas12a. We co-transfected plasmids encoding those fusion proteins and a pre-crRNA containing three crRNAs targeting the *VEGFA* or *RAB5A* promoter into HEK293T cells. We then evaluated changes in *VEGFA* and *RAB5A* expression relative to cells expressing the same fusion protein but a control pre-crRNA array targeting an unrelated gene (**Supp. Fig 2a-b)**. The C-terminal fusions of KRAB and 4xSID had the strongest overall repressive effect for both dLbCas12a and EnAsCas12a, demonstrating the dCas12a-4xSID fusions can repress gene expression.

We further evaluated dCas12a-4xSID repression using multiple crRNAs targeted to the promoter of individual genes. We cloned dEnAsCas12a and dHyperLbCas12a repressor fusions into a plasmid backbone with a stronger promoter and a strong NLS[48] **(Fig. 2a)**. We then tested the efficacy of these repressors in HEK293T cells using a pre-crRNA containing three crRNAs targeting *VEGFA* or *RAB11A* **(Fig. 2b)**. All repressors tested were able to induce significant and robust gene repression, with dHyperLbCas12a repressor fusions achieving >50% repression for both gene targets ($p < 0.005$, FDR-adjusted Student's T-test) **(Fig. 2c-d, and Fig. 1)**.

Next, we tested multiplexed repression by targeting several genes simultaneously. To do so, we co-transfected the dCas12a-based repressors with a pre-crRNA array targeting four different genes into HEK293Ts **(Fig. 2e)**. All repressors significantly repressed all four target genes ($p < 0.05$ for EnAs repressors, $p < 0.005$ for HyperLb repressors, FDR-adjusted Student's T-test), including *PIK3C3* which is only targeted by a single crRNA. The dCas12a-4xSID fusions were as effective as dCas12a-KRAB fusions at repressing gene expression **(Fig. 2f-g)**. Taken together, these data demonstrate that dCas12a-SID repressors are highly effective and can serve as an alternative to dCas12a-KRAB.

Lentivirally delivered dHyperCas12a-KRAB enables strong gene repression

High-throughput, multiplexed screens require stably expressed dCas12a fusions, typically achieved via lentiviral delivery. A challenge is that previous studies have found that lentivirally-delivered dCas12a-KRAB fusions are hypoactive[10]. We hypothesized that dHyperLbCas12a effectors, which have the strongest activity under low crRNA expression (**Supp Fig. 1a**), can overcome that limitation.

To test that hypothesis, we created a HepG2 hepatocellular carcinoma cell line that stably expressed dHyperLbCas12a-KRAB. We then lentivirally transduced a construct expressing a pre-crRNA containing four crRNAs targeting the promoter of hexokinase domain containing 1 (HKDC1), a gene involved in maternal glucose homeostasis[56–58]. In doing so, we repressed *HKDC1* expression by 85%. That amount of repression was comparable to the ~75% reduction in *HKDC1* transcript we observed in *HKDC1* knockout cell lines **(Supp. Fig 2c).** From those results, we conclude that lentivirally-delivered dHyperLbCas12a-KRAB is highly effective at repressing the expression of individual genes.

We further generalized that outcome to multiplexed repression in an additional cell line. We created A549 lung adenocarcinoma cell lines that stably expressed dHyperLbCas12a-KRAB or dHyperbLbCas12a-4xSID. We then transduced the cell lines with a construct expressing a pre-crRNA with crRNAs targeting the promoter of *MYC*, *PIK3C*, *RAB11A*, and *VEGFA*. In that system, dHyperLbCas12a-KRAB significantly repressed all four target genes, while dHyperbLbCas12a-4xSID repressed two ($p < 0.05$, FDR-adjusted Student's T-test). For dHyperLbCas12a-KRAB, repression ranged from only ~10% repression for *MYC* to 55-60% repression for *VEGFA* and *RAB11A*. Meanwhile, dHyperLbCas12a-4xSID was unable to repress *MYC* or *VEGFA*, but achieved ~15% repression of *PIK3C3* and 35% repression of *RAB11A* **(Supp. Fig 2d)**. Because *MYC* and *PIK3C3* are both essential genes in A549 cells, it is possible that the weaker repression of those target genes resulted from effects on cell fitness[59]. In support of that possibility, we used flow-cytometry to sort A549 repressor cell lines transfected with the multiplexed pre-crRNA plasmid, and found that dCas12a repressor expression was several-fold lower in array-expressing cells than in a bulk transfected population (**Supp. Fig 2e)**. Taken together, these results demonstrate that dHyperLbCas12a repressors are suitable for use in multiplexed high-throughput screens. Also, as a step towards low multiplicity of infection screens, we demonstrate repression when pre-crRNA constructs are transduced and expressed from the genome at a copy number that is much lower than with transient transfection.

dCas12a-P300 fusions robustly activate gene expression

Gene activation using dCas12a has most frequently been shown using fusions to the VPR activation domain[41,42,46,48]. While VPR is a potent activator of transcription, it has mostly been used to target gene promoters, not enhancer elements, and has been shown to be toxic in certain contexts[25,60–62]. Fusions of dCas9 to the histone acetyltransferase domain from P300 have been shown to be highly effective for targeting the non-coding genome, and a previous study showed that dCas9-P300 leads to stronger activation than dCas9-VPR when targeting a gene enhancer and promoter simultaneously[60,63]. dLbCas12a, but not dAsCas12a, has been shown to activate gene expression from both enhancers and promoters using single crRNAs[43]. We also tested activation using dAsCas12a-P300 and dLbCas12a-P300 fusions at two genomic loci using single crRNAs **(Supplementary Fig. 3a-b)**. We found that dLbCas12a-P300 was able to activate both target genes, while dAsCas12a-P300 was only able to activate one, consistent with previous reports that LbCas12a is a stronger epigenome editor than AsCas12a, and our dCas12- VPR and KRAB comparisons **(Fig. 1a-b)**[41]. From this we conclude that the

dLbCas12a-P300 fusions more strongly and more robustly activate gene expression than dAsCas12a-P300 fusions.

Above, we show that fusing KRAB and VPR to other variants of dCas12a can enhance repression or activation. Based on that result, we hypothesize that dEnAsCas12a-P300 and dHyperLbCas12a-P300 will also improve the efficacy of gene activation over fusions to wild type Cas12a proteins. Specifically, we created dEnAsCas12a-P300 and dHyperLbCas12a-P300 fusions in a plasmid containing a strong promoter and a NLS[48] **(Fig. 3a)**. We then created pre-crRNA arrays targeting the HBE1 promoter, the *MYOD1* promoter, or a *MYOD1* enhancer ~5 kb upstream of the *MYOD1* promoter[63,64]. We co-transfected plasmids expressing the dCas12a activators with plasmids expressing the pre-crRNA arrays into HEK293T cells. **(Fig. 3b)**. All four fusion proteins modestly but significantly increased target gene expression ($p < 0.005$, FDR-adjusted Student's T-test). dEnAsCas12a-P300 activation strength ranged from 1.2- to 18.2-fold, while dHyperLbCas12a activation strength ranged from 2.5- to 16.7-fold over non-targeting control. Contrary to our expectations, dCas12a-VPR led to stronger *MYOD1* activation from the distal enhancer than dCas12a-P300 for both orthologues **(Fig. 3c-d)**.

We also evaluated whether all three crRNAs were needed, and whether there were additive or epistatic interactions between the crRNAs as reported previously[41,42,65,66]. We designed three crRNAs to target either the *HBE1* or *MYOD1* promoter and created arrays containing all one-, two-, and three-way combinations of those crRNAs. We then co-transfected the pre-crRNA plasmids into HEK293T cells along with a dCas12a activator and assessed gene activation (**Supp Fig. 3c-f**). Generally, for dCas12a-VPR fusion proteins, multiple crRNAs increased activation strength. At both loci tested, the strongest pre-crRNA contained more than one crRNA, and combinations of three crRNAs were at least as strong as the best single crRNA. In contrast, for P300, we frequently observed reduced activation when we delivered multiple crRNAs, including examples where the combinations of crRNAs had weaker effects than the best single crRNA. Previous studies have also shown mixed effects from using multiple Cas9 gRNAs or Cas12a crRNAs, suggesting potential steric hindrance between the P300 fusions[43,65].

dCas12a-P300 fusions enable multiplexed gene activation

We next evaluated whether dCas12a activators are also capable of highly multiplexed gene activation. To do so, we co-transfected dCas12a activators with a plasmid expressing a pre-crRNA of ten crRNAs from an RNA Pol II promoter into HEK293T cells. The crRNAs consisted of one crRNA targeting the *AR* promoter, three crRNAs targeting the *HBE1* promoter, and six crRNAs targeting either the promoter or a distal regulatory region of *MYOD1* **(Fig. 4a)**. In all but one case, the dCas12a fusions significantly increased expression of all three target genes ($p < 0.01$, FDR-adjusted Student's T-test, dHyperLbCas12a-VPR activation of *AR* was not significant) **(Fig. 4b-c)**. The range of activation varied among genes and between activators. For example, activation of *HBE1* was stronger with VPR fusions (up to ~3200-fold) than with P300 fusions (up to 5-fold); whereas activation of *MYOD1* was consistently ~16-fold for both P300 and VPR. Meanwhile, activation of AR was weaker overall (1.2-2.0-fold). That range of *AR* activation was the same order of magnitude as observed in previous studies[41]. Those results

generalize both to stably expressed dCas12a activators, and to the A549 cell model with comparable effects on gene expression **(Supp Fig. 3g)**. Both *MYOD1* and *HBE1* were significantly upregulated ($p < 0.005$, FDR-adjusted Student's T-test), with effects ranging from 6-fold to ~2500-fold; whereas there was no significant effect on *AR* expression. Together, these results demonstrate that dCas12a activators are capable of multiplexed activation of gene expression in multiple cell types.

Multiplexed activation and repression within a single cell using dCas12a effectors

The use of epigenome editors from two different Cas12a orthologues created an opportunity for simultaneous gene activation and repression within a single cell. Since Cas12a will process crRNAs from a transcript containing other elements, and each Cas12a orthologue specifically recognizes its own direct repeat sequence, we hypothesized that a hybrid array containing both As and Lb Cas12a crRNAs could enable programmed activation and repression from a single array. To test that possibility, we created a hybrid pre-crRNA array containing four AsCas12a crRNAs targeting the *HBE1* or *MYOD1* promoters and four LbCas12a crRNAs targeting the *RAB11A* or *MYC* promoters, separated by a non-targeting crRNA (**Fig. 5a)**. We validated the hybrid pre-crRNA array with single dCas12 effectors by co-transfecting with either dHyperLbCas12a-KRAB or dEnAsCas12-VPR into HEK293T cells. Doing so increased expression of *HBE1* and *MYOD1* with dEnAsCas12-VPR, and decreased expression of *MYC* and *RAB11A* with dHyperLbCas12a-KRAB **(Supp. Fig 4a-b)**.

We then evaluated simultaneous activation and repression by co-transfecting a dEnAsCas12 activator, a dHyperLbCas12a repressor, and hybrid pre-crRNA arrays into HEK293T cells. We evaluated two combinations of activators and repressors: dEnAsCas12a-VPR for activation with dHyperLbCas12a-KRAB for repression; and dEnAsCas12a-P300 for activation with dHyperLbCas12a-4xSID for repression. For both pairs of effectors, we observed significant and simultaneous activation and repression of all the target genes **Fig. 5b-c** ($p < 0.05$, FDR-adjusted Student's T-test). From this, we demonstrate that hybrid pre-crRNA arrays can simultaneously activate and repress gene expression within the same cell.

High-throughput multiplexed epigenome editing screens via dHyperLbCas12a-KRAB

The above findings create the foundation for high-throughput combinatorial epigenome editing screens with the dCas12a platform. To demonstrate that possibility, we performed a combinatorial screen of two enhancers that previously were hypothesized to control activation of Period 1 (PER1) gene expression in response to glucocorticoids. Glucocorticoids are steroid hormones that have a major role in peripheral circadian rhythms throughout the body[67]. *PER1* is a key gene in that regulation. In A549 cells, *PER1* stands out because its expression is uniquely sensitive to very low concentrations of glucocorticoids[8]. The mechanism by which such low concentrations of glucocorticoids activate *PER1* expression remains unresolved. Based on

ChIP-seq and reporter assays studies, there are two promoter-proximal glucocorticoid-responsive regulatory elements, denoted here as Enhancer A and Enhancer B, that may cause glucocorticoid-dependent *PER1* activation. Furthermore, Enhancer A can control reporter gene expression at very low glucocorticoid concentrations, suggesting it may be responsible for the *PER1* gene expression response at those low concentrations[8] **(Fig. 6a)**.

We first definitively established that Enhancer A and Enhancer B together control the PER1 glucocorticoid response. To do so, we transduced A549 cells stably expressing dHyperLbCas12a-KRAB with either a pre-crRNA array targeting both Enhancer A and Enhancer B, or a non-targeting pre-crRNA array. We then selected cells for pre-crRNA expression, and treated them for three hours with either 100 nM dexamethasone (Dex) or ethanol as a vehicle control. With non-targeting crRNAs, *PER1* expression increased nearly 11-fold with dex treatment; while with crRNAs targeting Enhancer A and Enhancer B, *PER1* expression increased by only ~1.3-fold in the dex condition ($p = 8.8 \times 10^{-6}$, Student's T-Test) **(Fig. 6b)**. Together, these results indicate that Enhancer A and Enhancer B together account for all or nearly all of the *PER1* glucocorticoid response.

Enhancer A and Enhancer B were also sensitive to specific chromatin modifications. The *PER1* response was not significantly altered when we repeated the experiments in A549 cells expressing dHyperLbCas12a-4xSID instead of dHyperLbCas12a-KRAB ($p = 0.45$, Student's T-Test). That finding suggests the *PER1* glucocorticoid response is differentially sensitive to KAP1-mediated vs Sin3a-mediated repression; and that the dCas12a repressors described here can identify such differential sensitivities. Additionally, activating the enhancers with VPR or P300 in the absence of glucocorticoids did not significantly increase *PER1* expression ($p = 0.68$, $p = 0.20$, Student's T-Test). **(Supp. Fig. 4c)**, indicating that glucocorticoid receptor binding and co-factor recruitment may be necessary for *PER1* activation via those enhancers.

Next, to dissect the independent and combinatorial contributions of Enhancer A and Enhancer B, we designed a library of pre-crRNA arrays to target dCas12a-KRAB to the enhancers alone or in combination **(Fig. 6c)**. Overall, our design was to deliver pre-crRNAs containing six crRNAs, where the first three crRNAs target Enhancer B, and the second three target Enhancer A. To target the enhancers together, we used on-target crRNAs in all six positions. To target Enhancer A or Enhancer B individually, we used non-targeting crRNAs in the first three or second three positions, respectively. In total, we designed nine on-target crRNAs per enhancer, and assembled DNA sequences for all 84 unique triples thereof **(Fig. 6c, Supp. Fig. 5a)**. We also designed ten sets of three non-targeting crRNAs to target enhancers individually.

To construct the screening library, we used a nested cloning strategy to simultaneously assemble the pre-crRNA array and a series of 8 bp barcodes that encode the contents of the array **(Supp. Fig 5)**[68,69]. In the first round of cloning, we inserted the crRNAs targeting Enhancer B or non-targeting crRNAs **(Supp. Fig 5b)**. In the second round of cloning, we inserted the crRNAs targeting Enhancer A or non-targeting crRNAs **(Supp. Fig 5c)**. That resulted in a library of 8,836 unique pre-crRNA arrays composed of 7,056 pre-crRNA arrays targeting both enhancers, 1,680 pre-crRNA arrays targeting the enhancers individually, and 100 pre-crRNA

arrays targeting neither enhancer **(Fig. 6c-d, Supp. Fig 5d)**. In parallel, we separately constructed a control library of 10,000 non-targeting six-crRNA arrays and three pre-crRNAs targeting the *PER1* promoter **(Supp. Fig 6)**. When constructing the targeting and control libraries, we also enabled sorting crRNA-expressing cells by co-transcribing a selectable marker with the pre-crRNA[44] **(Fig. 6d, Supp. Fig 5)** and high-throughput sequencing of the barcode by including TruSeq adapter sequences flanking the embedded barcode **(Supp. Fig 5)**. We confirmed the library construction via sequencing on an Illumina Nextseq instrument. We recovered all on-target pre-crRNA arrays, and 9,890 of the 10,000 negative control pre-crRNA arrays. The missing negative control pre-crRNA arrays were explained by missing or low-abundant crRNAs during DNA synthesis. Aside from those dropouts, the pre-crRNA arrays were evenly represented in the assay library **(Fig. 6 e-f)**.

To assay the pre-crRNA libraries, we lentivirally transduced them into A549 cells stably expressing dHyperLbCas12a-KRAB **(Fig. 7a)**. Ten days post-transduction, we treated crRNA-expressing cells with either low (200 pM) or high (100 nM) concentrations of the synthetic glucocorticoid dexamethasone, or with ethanol to control for solvent effects. After three hours, we collected cells with the highest and lowest 12% *PER1* expression[70] **(Supp. Fig. 7a)**. Finally, we estimated the abundance of each array in the sorted cell populations via high-throughput sequencing of the barcodes. We also confirmed that pre-crRNA arrays remained intact and correctly associated to the corresponding barcode throughout the screen, consistent with findings from other Cas12a studies[71] (**Supp. Fig 8**, Supplementary text).

Overall, we found that Enhancer A controls the *PER1* response to low glucocorticoid concentrations, and that both Enhancers A and B together control the *PER1* response at high glucocorticoid concentrations. Specifically, at 200 pM dexamethasone, pre-crRNA arrays targeting Enhancer A were ~2.6-fold enriched in low-*PER1*-expressing cells, and depleted by 2.9-fold in high-*PER1*-expressing cells. In contrast, pre-crRNA arrays targeting only Enhancer B were 1.7-fold enriched in low-*PER1*-expressing cells and depleted by 1.4-fold in high-PER1-expressing cells **(Fig. 7c)**. At 100 nM dexamethasone, pre-crRNA arrays that target both Enhancer A and Enhancer B were ~5.4-fold enriched in low-*PER1*-expressing cells and 3.7-fold depleted in high-*PER1*-expressing cells; and pre-crRNA arrays targeting only Enhancer A or only Enhancer B had intermediate effects (3.9-fold and 3.0-fold enriched, and 2.7- and 2.4-fold depleted, respectively) **(Fig. 7d)**. Finally, in the ethanol treatment condition, the on-target pre-crRNA arrays were only weakly depleted from high-*PER1*-expressing cells (up to 1.4-fold) **(Fig. 7b)**. Together, these results reveal glucocorticoid-dose-dependent enhancer activity controlling *PER1* expression.

Discussion

We systematically evaluated and improved technologies for dCas12-based epigenome editing. First, we consistently evaluated a wide range of dCas12a-based epigenome effectors delivered both transiently and lentivirally. Through that effort, we identified a number of more effective epigenome effectors, and demonstrated that the effectors work when lentivirally integrated into the genome. Second, we demonstrated that expressing pre-crRNAs using RNA polymerase II allows for longer and more complex multiplexing than using RNA polymerase III. Finally, combining those advances, we present what is to the best of our knowledge the first dCas12a CRISPRi screen, and the first dCas12a screen on changes in gene expression. Those screens involve simultaneous delivery of six crRNAs, demonstrating the potential for highly multiplexed CRISPRi screens on human gene expression.

A key challenge in epigenome editing screens is that effectors and crRNAs typically must be delivered at low copy number. Previous dCas12a-based CRISPRi studies have required delivery of high levels of dCas12a and pre-crRNA into HEK293T cells[42,44]; and others have found similar results for dCas12a-based gene activation[25]. Those limitations can be overcome by using a nicking-biased version of AsCas12a to improve repression. While effective, doing so also introduces insertions of deletions in up to ~7% of target sites[10]. Alternatively, co-expressed nanobodies have been used to activate promoters genome-wide; but doing so increases complexity by adding components to the screening system[25]. By instead basing our screen on dHyperLbCas12a, which we and others have found to be more effective under low copy number conditions[48], we were able to screen directly using a dCas12a-based epigenome effector. Further, we expect other dHyperLbCas12a epigenome editing fusions can be used in similarly designed screens. Finally, by reading out on gene expression directly using HCR[70,72] rather than on cell proliferation as in previous Cas12a-based epigenome screening studies, we discover new mechanisms controlling the expression of individual genes.

A key enabling feature for our screens is the creation of barcoded pre-crRNA libraries using a nested cloning strategy[68,69]. Up to four crRNAs and a barcode sequence can be added to the pre-crRNA during each round of cloning, allowing for the generation of large combinatorial libraries from small and easy to synthesize oligonucleotide pools. For example, the 10,000 member non-targeting library in this study was generated from 200 oligonucleotides that were 235 nt each. The end result is a library of uniquely barcoded pre-crRNAs containing all possible combinations of crRNA sets added during each round of cloning. The barcode allows for screening with pre-crRNAs that exceed the length of high-throughput short-read sequencing methods. Together, those advances make the screening method flexible and applicable to a wide range of highly-multiplexed combinatorial studies of gene expression.

We also develop and validate a number of dCas12a-based technologies that can be used for future highly-multiplexed epigenome editing studies. We introduce a suite of newly-validated dCas12a epigenome editors that expand the available options for multiplexed targeting of the non-coding genome. We selected two epigenome-modifying domains, P300 and SID, that had been used successfully to modulate gene expression from both promoters and enhancers when

fused to dCas9 or other DNA-binding proteins[4,43,55,63]. We successfully expand these results by creating dCas12a-P300 and dCas12a-4xSID fusions for both As and LbdCas12a variants, and demonstrate highly-multiplexed repression and activation with pre-crRNAs of up to ten crRNAs, including when dCas12a effectors are stably expressed in A549 cells.

Many aspects of health and disease involve both increases and decreases in gene expression. Modeling those systems via simultaneous activation of some genes and repression of others has been previously demonstrated using dCas9 orthologs and a dual sgRNA expression vector[13,18,19]. Here, we demonstrate an alternative approach using a hybrid dCas12a pre-crRNA to simultaneously activate two genes and repress two genes. That strategy can be expanded in several ways. For example, it is likely that other CRISPR proteins that process their own pre-crRNAs, such as Cas13, could be added[73,74]. The approach could also be used for high-throughput multiplexed screens that combine activation and repression. Together, those advances have the potential to greatly improve modeling of biological systems that involve activation and repression.

The results of this study also point to areas for future mechanistic investigation. We and others have found that using multiple gRNAs or crRNAs to target a genomic element generally often leads to stronger activation or repression for dCas9 or dCas12a fusions to KRAB and VPR, but that for P300 adding additional gRNAs or crRNAs can lead to weaker activation, even in cases where the same combination of crRNAs leads to stronger activation for dCas12a-VPR fusions[41–43,65,66]. Therefore, there may be rules for effective multiplexed activation with P300 that have not yet been discovered. Additionally, whether all dHyperLbCas12a epigenome editors are suitable for high-throughput screening and the upper limit for pre-crRNA length in a high-throughput screening setting have yet to be determined.

Finally, though the experimental technologies now exist for highly multiplexed epigenome editing screens, the potential scale of such studies will demand corresponding statistical methods for experimental design and interpretation. Though our screen focused on two enhancers controlling glucocorticoid dependent *PER1* expression, we demonstrated high activity of pre-crRNAs containing six crRNAs that could be used to target combinations of six regulatory elements. The scale of combinatorial libraries that can be generated with six crRNAs can already exceed practical limitations in the number of cells or sequencing required. For example, there are 10^{16} unique combinations of six transcription factors, far exceeding the number of cells that could be reasonably cultured; and a screen of one million three-way combinations of transcription factors would only cover less than 0.2% of the possible combinations. Meanwhile, the pre-crRNA libraries could readily be expanded to include more crRNAs due to the high processivity of Pol II. For those reasons, highly multiplexed combinatorial screens will necessarily rely on and generate very sparse observations relative to the number of possible combinations, and statistical methods to prioritize informative combinations and interpret effects at that sparsity will be needed to realize the full potential of highly multiplexed epigenome editing screens.

Figure Legends

Figure 1. Optimization of the dCas12a system for multiplexed epigenome editing

(a) Comparison of dCas12a repressors. Five different Cas12a variants were fused to the transcriptional repressor KRAB. Each dCas12a-KRAB construct was co-transfected into HEK293T cells with either a pre-crRNA of three crRNAs targeting the *RAB11A* promoter or a control pre-crRNA containing three non-targeting crRNAs. After two days, cells were sorted on Cas12a expression using a GFP marker, mRNA was harvested and relative *RAB11A* expression levels were quantified using RT-qPCR (two or more independent experiments per transfection condition) **(b)** Comparison of dCas12a activators. Five different Cas12a variants were fused to the transcriptional activator VPR. Each dCas12a-VPR construct was co-transfected into HEK293T cells with either a pre-crRNA of three crRNAs targeting the *HBE1* promoter or a control pre-crRNA containing three non-targeting crRNAs. After 2 days, mRNA was harvested and relative *HBE1* expression levels were quantified using RT-qPCR (n = 3 independent experiments per transfection condition) **(c)** Constructs used to assess different promoters for pre-crRNAs of length ten. Pre-crRNAs of 10 crRNAs, driven by CAG or U6, were designed to contain three crRNAs targeting the *HBE1* promoter in either position 1-3 (Front of array) or 8-10 (Back of array). These constructs were then co-transfected into HEK293T cells with either **(d)** dCas12a-VPR or **(e)** dCas12a-P300. After 2 days, mRNA was harvested and relative *HBE1* expression levels were quantified using RT-qPCR (two or more independent experiments per transfection condition)

Figure 2. Multiplexed gene repression by dCas12a-KRAB and dCas12a-4xSID

(a) dCas12a repressor constructs **(b)** Experimental design for testing multiplexed repression of a single gene by dCas12a-KRAB and dCas12a-4xSID constructs. Repressors were co-transfected into HEK293T cells along with a pre-crRNA of three crRNAs targeting a gene promoter of interest or a control pre-crRNA containing three non-targeting crRNAs. Two days post-transfection, dCas12a-positive cells were sorted on EGFP expression, RNA was harvested, and gene expression changes were assessed via RT-qPCR**. (C)** Repression of the *RAB11A* and *VEGFA* promoters by dEnAsCas12a- KRAB and 4xSID fusions (n = 3 independent experiments per transfection condition) **(d)** Repression of the *RAB11A* and *VEGFA* promoters by dHyperLbCas12a KRAB and 4xSID fusions (at least two independent experiments per transfection condition) **(e)** Experimental design for testing highly multiplexed gene repression by dCas12a repressors. A pre-crRNA of ten crRNAs targeting four genes: *VEGFA*, *MYC*, *RAB11A*, and *PIK3C3* was designed to test highly multiplexed repression. HEK293T cells were co-transfected with dCas12a repressors and either this array or a control pre-crRNA containing ten non-targeting crRNAs and relative gene expression was determined as in **(b). (f)** Repression of four target genes by dEnAsCas12a KRAB and 4xSID fusions using a pre-crRNA of length ten (n = 3 independent experiments per transfection condition) **(g)** Repression of four target genes by dHyperLbCas12a KRAB and 4xSID fusions using a a pre-crRNA of length ten (n = 3 independent experiments per transfection condition)

Figure 3. Activation of single genes using dCas12a

(a) dCas12a activator constructs **(b)** Experimental design for testing multiplexed repression by dCas12a-VPR and P300 constructs. Activator constructs were co-transfected into HEK293T cells along with a pre-crRNA of three crRNAs targeting a gene of interest or a control pre-crRNA of three non-targeting crRNAs. Two days post-transfection, dCas12a-expressing cells were sorted on EGFP, RNA was harvested, and gene expression changes were assessed via RT-qPCR**. (c,d)** Activation of the *HBE1* promoter, *MYOD1* promoter, or *MYOD1* distal regulatory region (DRR) by **(c)** dEnAsCas12a- VPR and P300 fusions (at least two independent experiments per transfection condition) or **(d),** dHyperLbCas12a-VPR and P300 fusions (at least two independent experiments per transfection condition)

Figure 4. Activation of multiple genes using dCas12a

(a) Experimental design for testing highly multiplexed gene activation by dCas12a activators. A pre-crRNA containing ten crRNAs targeting three genes: *AR*, *MYOD1*, and *HBE1*, was designed to test highly multiplexed activation. HEK293T cells were co-transfected with dCas12a activators and either this pre-crRNA or a control pre-crRNA of ten non-targeting crRNAs and gene expression was determined as in **Fig. 3. (b,c)** Activation of three target genes using the multiplexed pre-crRNA by **(b)** dEnAsCas12a-VPR and P300 fusions and **(C)** dHyperLbCas12a- VPR and P300 fusions (n = 3 independent experiments per transfection condition)

Figure 5. Programmed, simultaneous activation and repression within a single cell

(**a)** Experimental design for simultaneous activation and repression. A hybrid pre-crRNA composed of both Lb and As Cas12a crRNAs is co-transfected with a dEnAsCas12a activator and a dHyperLbCas12a repressor in order to achieve programmed activation and repression within a single cell. The hybrid pre-crRNA or a control pre-crRNA of ten non-targeting crRNAs were co-transfected into HEK293T cells with a dEnAsCas12a activator and a dHyperLbCas12a repressor. Cells were sorted and changes in target gene expression were assessed as in **Fig 2-4. (b,c)** Simultaneous activation and repression by **(b)** dEnAsCas12a-VPR and dHyperLbCas12a-KRAB or **(c)** dEnAsCas12a-P300 and dHyperLbCas12a-4xSID (n = 3 independent experiments per transfection condition).

Figure 6. Construction of a crRNA library to target two *PER1* enhancers

(a) two enhancers, labeled A and B, control the glucocorticoid-responsiveness of the gene *PER1*. These sites are bound by the glucocorticoid receptor after treatment with the synthetic glucocorticoid dexamethasone **(b)** dHyperLbCas12a-KRAB is capable of dampening the *PER1* glucocorticoid response through dual targeting of the enhancers in (**a**). A549 cells stably expressing dHyperLbCas12a-KRAB and either a pre-crRNA of four crRNAs targeting the *PER1* enhancers (2 crRNAs per enhancer) or a control pre-crRNA of four non-targeting crRNAs were created. These cell lines were treated with either 100nM dexamethasone or vehicle for three hours before RNA was harvested and changes in *PER1* expression were determined using RT-qPCR. **(c)** Schematic showing makeup of the 8,836 member *PER1*-targeting pre-crRNA library. Pre-crRNAs of six crRNAs were created to target each of the *PER1* enhancers either individually or in combination **(d)** schematic showing the completed library array constructs. Two sets of 3 crRNAs were cloned into a CAG expression vector to create dual-targeting pre-crRNAs of 6 crRNAs with a bipartite barcode flanked by TruSeq adapter sequences. The crRNAs are also located on the same transcript as the mCherry-P2A-puroR selection marker. **(e,f)** Density plots showing the distribution of crRNA counts for each library. The distribution of pre-crRNA barcode counts for **(e)** the 10,003 member non-targeting library. **(f)** the 8,836 member *PER1*-targeting library

Figure 7. *PER1* Screen Results

(a) Timeline of the *PER1* screen. A549 cells stably expressing dHyperLbCas12a-KRAB were transduced with either the library of *PER1*-targeting arrays (n = 8,836) or the library of non-targeting arrays and promoter-targeting arrays (n=10,003). They were then selected with puromycin, after which the cell populations for the two different libraries were combined. Cells were treated with either an ethanol control (vehicle), 0.2nM dexamethasone (low Dex), or 100nM dexamethasone (high Dex) for three hours before being harvested for HCR. Cells were then sorted into high and low bins (top and bottom 12%) based on *PER1* expression. gDNA was recovered and sequenced and array abundance in high and low bins was compared to input. **(b,c,d)** Enrichment in pre-crRNA barcode abundance vs. input for **(b)** vehicle **(c)** 0.2nM Dex and **(d)** 100nM Dex. Log_2 fold change is an average of enrichment in the high and low *PER1*-expressing populations

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Contributions

T.E.R. conceived, funded, and supervised the study. T.E.R. and S.M.M. designed and planned experiments. M.C.H. cloned LbCas12a pre-crRNA expression plasmids used in **Figs.2c-d, 3c-d, and Supp. Fig.3c-f**, and completed the dLbCas12a activation experiments in **Supp. Fig. 3c-f**. S.M.M. completed all other experiments and analyzed resulting data. T.E.R. and S.M.M. wrote the manuscript, with input from M.C.H.

Methods

Plasmid generation

Unless otherwise noted, cloning was performed using PCR of desired insert fragments with Q5 (New England Biolabs (NEB)), followed by Gibson Assembly using NEBuilder Hifi 2x Master Mix (NEB) to add inserts into the desired digested plasmid backbone. Cloning reactions were

transformed into chemically competent NEB Stable E. Coli (NEB), purified using the Zyppy Plasmid Miniprep Kit (Zymo), and validated using Sanger sequencing (Azenta). All Cas12a ORFs and epigenome editing domain sequences can be found in **Supplemental Information.** All key plasmids from this study will be posted on Addgene.

First-generation dCas12a effector fusions were cloned into the FUGW plasmid backbone. FUGW-dLbCas12a was a gift from Dr. Charles Gersbach, and dLbCas12a sequence was originally derived from pY109 (Addgene 847740). dAsCas12a and dEnAsCas12a were derived from Addgene plasmids (114078 and 107943, respectively)[46]. Mutations to make dAsCas12a Ultra were introduced into the dAsCas12a plasmid. A gene fragment (Twist) with the dHyperLbCas12a ORF was synthesized and cloned into the same FUGW backbone. Effector fusions were generated using Gibson assembly or by standard subcloning methods. The 4xSID sequence was derived from Addgene 106399[4] and the 3xKRAB sequence was taken from Addgene 128132[44]. Versions of each dCas12a effector were made with either a T2A-EGFP or T2A-puroR using subcloning. These constructs were used in **Fig. 1a-b, Supp. Fig 1, Supp. Fig 2a-b, and Supp. Fig 3a-f**.

The FUGW vector was then modified using Gibson assembly and subcloning to create a second generation of dCas12a effector fusions. The hUbc promoter was replaced with the EF1A promoter. The nucleoplasmin NLS on the C-terminus of dEnAsCas12a was exchanged for two copies of the c-Myc NLS. Lastly, the T2A-puroR was replaced by T2A-BlasticidinR. These dCas12a effectors were used in **Fig. 1c-d, Fig. 2-7, Supp. Fig 2c-e, Supp. Fig 3g, Supp. Fig 4, and Supp. Fig 7.**

For expression of Cas12a pre-crRNA arrays using U6, pRDA_052 (Addgene 136474)[45] was used for AsCas12a and an FUGW plasmid was used for LbCas12a. These constructs were used in **Fig. 1a-b, Fig.2c-d, Fig. 3c-d, Supp. Fig 1, Supp. Fig 2a-b, and Supp. Fig 3a-f**. The FUGW LbCas12a crRNA expression vector was modified for U6 expression of either As or LbCas12a crRNAs by inserting an Esp3I cloning site flanked by two direct repeat sequences just downstream of the U6 promoter. These plasmids also included an hUbc promoter to express mCherry-P2A-PuroR for selection. These plasmids were used in **Fig. 1c-d**. A pHAGE plasmid was modified using Gibson assembly for CAG expression of either As or LbCas12a crRNAs. First, the promoter was changed from EF1A to CAG. Next, an mCherry-P2A-puroR ORF, followed by the MALAT1 triplex sequence used in a previous study[44], followed by two direct repeat sequences flanking an Esp3I cloning site were inserted into the vector downstream of the CAG promoter. Finally, a kanamycin resistance cassette was inserted between the direct repeat sequences in order to limit recombination. This plasmid was used in **Fig. 1c-d, Fig. 2f-g, Fig. 4-6, Supp. 2c-e, Supp. 3g, Supp. Fig. 4.** The sequence of this transcript can be found in **Supplemental Information**.

Cloning of crRNAs into expression vectors was performed using one-pot golden gate cloning **(Fig. 1a-b, Fig.2c-d, Fig. 3c-d, Fig. 6, Supp. Fig 1, Supp. Fig 2a-c, Supp. Fig 3a-f, and Supp. Fig. 4c)**. All crRNA spacer sequences used in this study can be found in **Supplemental Table 1**. Briefly, pairs of oligos were annealed, then phosphorylated using T4 Polynucleotide Kinase

(NEB) in a 50 µL reaction. Next, 40 fmol of plasmid backbone and 2 µL of each annealed oligo pair were added to a one-pot golden gate reaction with Esp3I and T4 DNA Ligase (NEB) as has been done in previous studies[73,75]. Long pre-crRNA arrays in **Fig. 1c-d, Fig. 2f-g, Fig. 4-6, Supp. 2d-e, Supp. 3g, and Supp. Fig. 4a-b** were constructed using PCR followed by golden gate cloning. Briefly, the crRNA array was created by using multiple rounds of low cycle number PCR. Primers added one crRNA to each end of the growing array with each round of PCR. The last round of PCR also added Esp3I sites for Golden Gate cloning. This amplicon was then cloned into the plasmid backbone using one pot Golden Gate cloning with 40 fmol of plasmid and 120 fmol of PCR product.

PER1 and Non-Targeting crRNA library generation

First, the CAG crRNA expression vector described above was modified to have the 3' TruSeq adapter sequence downstream of the Esp3I cloning site instead of a flanking direct repeat sequence. This was done with Gibson Assembly. This sequence can be found in **Supplemental Information**. Spacer sequences using in the *PER1* and non-targeting libraries can be found in **Supplemental Table 3**, while oligos ordered for cloning these libraries can be found in **Supplemental Table 4**.

Next, nine unique crRNAs were designed to target each *PER1* enhancer using CRISPick. From those nine crRNAs 84 (9 choose 3) unique sets of three crRNAs were created to target each enhancer. Using those sets of crRNAs, a pool of 188 oligos, composed of two different groups was designed (Twist). Group 1 was composed of oligos containing the 84 sets of three enhancer B-targeting crRNAs plus 10 sets of three non-targeting crRNAs, as well as a nested cloning site and a unique 8nt barcode sequence **(Supp. Fig 5a)**. Group 2 was composed of oligos containing the 84 sets of three enhancer A-targeting crRNAs plus 10 sets of three non-targeting crRNAs, as well as the 5' TruSeq adapter sequence for NGS and a unique 8nt barcode sequence. Barcode sequences were designed with a Hamming Distance of 3. Group 1 and group 2 oligos were amplified from the pool using separate PCR primer pairs **(Supp. Table 2)**. Then, Golden Gate cloning was used to insert the group 1 amplicons into the plasmid backbone. The plasmid was digested with Esp3I (NEB), while the PCR amplicon was digested with BsaI (NEB). These were purified using the DNA Clean and Concentrator Kit (Zymo). These DNA fragments were then input into 8 ligation reactions using 120fmol of PCR product and 40 fmol of digested plasmid. Ligated products were pooled and concentrated using the DNA Clean and Concentrator kit before being electroporated into Endura chemically competent cells (Lucigen). The 94-member library was purified using the Midi Plus kit (Qiagen). Group 2 amplicons were then cloned into the 94-member library using 8x one pot Golden Gate cloning with Esp3I as described in plasmid generation methods. This library was electroporated and purified as before, resulting in a final library with six crRNAs per array and 8,836 total crRNA arrays.

The non-targeting library was created by designing 50 non-targeting control crRNAs as well as three promoter-targeting crRNAs using CRISPick. To create a 10,000 member library 200 sets

of 3 crRNAs were randomly chosen from the 50 non-targeting crRNAs and broken up into 100 sets for group 1 and 100 sets for group 2. Three sets of three promoter-targeting crRNAs were also included with group 1. The non-targeting library was then cloned the same way as the PER1 enhancer-targeting library. The three promoter-targeting crRNAs did not contain a nested cloning site and were therefore unchanged during the second round of cloning.

Cell Culture

All cell lines were obtained from ATCC. Cell lines were authenticated using STR profiling through the Duke Cell Culture and DNA Analysis facility. Mycoplasma testing was performed by Eurofins**.** All cell lines were grown in humidified incubators at 37 °C and 5% CO_2. Cells were cultured using the following media:

HEK293T: DMEM (Gibco) + 10% FBS + 1% penicillin-streptomycin
A549: F12K (Gibco) + 10% FBS + 1% penicillin-streptomycin
HepG2: EMEM (ATCC) + 10% FBS + 1% penicillin-streptomycin

Transfection

For experiments performed in 24-well plates **(Figs 1a, 2-5, Supp. 3a-b, Supp. 2e)** , cells were co-transfected with 400 ng of dCas12a effector plasmid and 250 ng array plasmid 24 hours after seeding using Lipofectamine 3000. For the experiments in **Figure 5**, cells were co-transfected with 250 ng of each dCas12a effector plasmid, and 250 ng of hybrid crRNA, or 125ng of each control array (As and Lb Cas12a crRNAs). 48 hours post-transfection, cells were either harvested for flow cytometry **(Figs 1a, 2-5, Supp. 2e)**, or RNA was extracted directly from bulk transfected cells **(Supp. Fig 3a-b)**

For experiments performed in 96-well plates **(Figs. 1b, 1d-e, 6b, Supp. 1, Supp. 2a-d, Supp. 3c-g, Supp. 4c)** cells were reverse transfected with 80 ng of dCas12a effector plasmid and 50 ng array plasmid using Lipofectamine 3000. For the low crRNA condition in **Supp. Fig 1A**, cells were transfected with 5 ng of array plasmid. RNA was harvested from cells 48 hours post-transfection.

RT-qPCR

For all experiments except those specifically listed below, RNA extraction and cDNA synthesis were completed using the two step Cells to Ct Kit (Thermo). 2 µL of cDNA was used in each PCR reaction. Multiplexed qPCR was performed using TaqMan Gene Expression Master Mix (Thermo), a FAM-labeled Taqman assay (Thermo) for the appropriate target gene, and a

VIC-labeled, primer-limited endogenous control assay (Thermo) for ACTB. qPCR was performed on a StepOne Plus thermal cycler under the following conditions:

95 °C for 10 min
40x
  95 °C for 15 seconds
  60 °C for 1 minute

In instances where gene targets failed to amplify due to low/no expression, a Ct value of 40 was used.

For experiments in **Supp. Fig 3a-b**, qPCR was performed as above except that RNA was extracted using the RNeasy mini kit (Qiagen). cDNA was then created using the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences) using 1 µg of RNA. 1 µL of cDNA was used for qPCR.

For experiments to test dCas12a effector expression **(Supp. Fig 2e)**, the Cells to Ct kit was used as above and 2 µL of cDNA was used in the qPCR reaction. qPCR was performed using SYBR Green (Thermo) and primers to detect the Cas12a transcript or primers to detect ACTB. Primer sequences can be found in **Supp. Table 2**. Thermal cycling conditions were as follows:

95 °C for 10 min
40x
  95 °C for 15 seconds
  60 °C for 1 minute

Stable cell line generation

In order to create cell lines that stably express a dCas12a effector, lentivirus was created by the Duke Viral Vectors Core using standard reagents (psPAX2 and MD2.G). A549 and HepG2 cells were transduced with lentivirus at 1x concentration in media containing 10 µg/mL polybrene (Thermo). Media was changed 24 hours post-transduction. Cells were selected with 2.5 µg/mL Blasticidin-S-HCl. Once selection and expansion was complete, cell lines were validated by transduction or transfection of array constructs.

In order to create the stable crRNA array-expressing cell lines in Fig 6b, Supp. Fig 2c-d, Supp. Fig 3g, and Supp. Fig 4c and the Cas9 knockout cell lines in Supp. Fig 2c, we created lentivirus using standard reagents. Briefly, 90% confluent HEK293T cells in a 6 well plate were transfected with 600 ng psPax2, 450 ng of the lentiviral vector of interest, and 150 ng of MD2.G using Lipofectamine 3000. Media was changed 24 hours post-transfection, and supernatant was harvested 72 hours post-transfection. A549 and HepG2 cells were transduced with lentivirus at 1x concentration in media containing 10 µg/mL polybrene Media was changed 24 hours post-transduction. HepG2 cells were selected with 0.75 µg/mL puromycin, while A549 cells were

selected with 1.0 µg/mL puromycin. Once selection and expansion was complete, cell lines were validated by RT-qPCR.

Flow Cytometry

For studies involving transfected cells, live cells were harvested from a 24 well plate 48 hours post-transfection and resuspended in 200 µL MACS Buffer (DPBS + 0.5% BSA + 2 mM EDTA). Cells were then sorted on a SONY SH800 sorter using a 100 µm chip. Populations were gated on FSC/SSC for single cells, and then gated for GFP positive cells, a marker of Cas12a expression. Between 15,000 and 50,000 cells were sorted per sample, after which we proceeded with RNA extraction using the Cells to Ct kit.

For the dHyperLbCas12a-KRAB screen, flow cytometry was performed by the flow cytometry core at the Duke Cancer Institute using an MoFlo Astrios EQ High Speed Sorter (Beckman Coulter). Cells were first gated on single cells using FSC/SSC. Next cells were gated on compensated mCherry, a marker of crRNA expression, and compensated FITC-488, a marker of PER1 transcripts by HCR. The top and bottom 12% of FITC-expressing cells were then sorted.

PER1 CRISPRi Screen

First, lentivirus was created for the PER1-Targeting and Non-Targeting crRNA libraries. For each library, two T-75s containing 90% confluent HEK293T cells were transfected with 4 µg of psPAX2, 3 µg of array plasmid, and 2 µg of MD2.G using 27 µL of PEI (1 mg/mL). Media was changed 24 hours post-transfection, and supernatant was harvested 72 hours post-transfection. For each library, 24 mL of supernatant was concentrated 10x using Lenti-X concentrator (Takara Bio) as directed.

Next, for each library $1x10^8$ cells in 500 cm^2 dishes were transduced at low MOI using the concentrated lentivirus in media containing 10 µg/mL polybrene to achieve 1,000x library coverage. After 48 hours, cells were passaged 1:4 into media containing 0.75 µg/mL puromycin. Cells were passaged two more times (5 more days) to ensure that selection was complete. In order to ensure good library coverage, at each passage, cells from all plates per library were pooled and the number of cells passaged was at least 2,000x library size. After 3 passages in puromycin (7 days post-transduction), cells from each of the two libraries were pooled in equal numbers and co-cultured. Ten days post-transduction, cells were treated for 3 hours with 0.1% ethanol (vehicle), 200 pM dexamethasone, or 100 nM dexamethasone, with $2x10^8$ cells per condition. $1.4x10^7$ cells were taken from each condition, mixed, and pelleted in order to create a bulk sample to use as a comparison for crRNA enrichment. Additionally, $5x10^6$ A549 dHyperLbCas12a-KRAB cells (untransduced) were harvested for use as flow cytometry controls.

Cells were harvested and HCR Flow-FISH was performed[70]. PER1 transcripts were detected using a custom set of 20 probe pairs containing the B3 amplifier. Probe was used at 2x concentration compared to the standard protocol. Amplification was performed for 22 hours using the B3-488 amplifier. Flow cytometry controls were created as follows: negative control cells were taken from A549 cells that expressed dCas12a-KRAB, but were not transduced with a crRNA library. They were hybridized with probe, but set aside after the probe wash step. mCherry single color controls were taken from cells transduced with the PER1 and Non-targeting libraries. They were hybridized with probe, but set aside after the probe wash step. FITC-488 single color controls were taken from A549 cells that expressed dCas12a-KRAB, but were not transduced with a crRNA library. These cells went through the whole HCR protocol and had labeled PER1 transcripts. All cells were resuspended in MACS buffer and sorted as described above.

NGS Library Prep and Sequencing

For the *PER1* screen, genomic DNA was extracted from bulk-transduced cells that did not undergo HCR using the Puregene Cell Kit (Qiagen). Meanwhile, genomic DNA from cells sorted into high and low *PER1* expression bins was extracted using the FFPE miniprep kit (Zymo). Genomic DNA purified using the FFPE miniprep kit was then cleaned using Axiprep Magnetic Beads (Axygen) at a ratio of 0.9x in order to remove any small DNA fragments. Genomic DNA was then analyzed on a TapeStation 4200 in order to confirm that DNA was high quality and average fragment size was large (> 20 kb).

Next, crRNAs were amplified from genomic DNA (primer sequences can be found in **(Supp. Table 2)** using Q5 2x Master Mix (NEB) in 100 µL reactions with the following thermal cycling parameters:

98 °C for 45 seconds

27x
  98 °C for 15 seconds
  60 °C for 20 seconds
  72 °C for 1 minute and 15 seconds

72°C for 5 minutes

We performed 18 PCR reactions for each sorted cell sample and 96 PCR reactions for the bulk unsorted sample. Axiprep magnetic beads were used at a 0.9x ratio to clean and size select 200 µL of PCR product in order to remove any adapter dimer. PCR products were quantified using a qPCR standard curve (KAPA ABI Prism qPCR Master Mix). Preparation of libraries from plasmid DNA used the same PCR conditions except with 12 cycles and 25 PCR reactions per plasmid library. In both cases, the number of cycles of PCR was determined empirically by first performing a qPCR using Q5 2x Master Mix and 1x EvaGreen (Thermo), and choosing the

number of cycles that gave about one half of the maximum signal. Libraries were run using a 600 cycle paired-end kit NextSeq 2000 at a concentration of 950 pM. Custom primers were used for sequencing (Read 1, Index 1, Index 2, and Read 2, **Supp. Table 2**)

Sequencing Data Processing

FastQ files were generated using BCLConvert in BaseSpace. To extract barcode sequences, reads containing the barcode were first trimmed using Cutadapt, aligned to a custom reference of barcode sequences using Bowtie2, and counts for each barcode were generated using Samtools with the following parameters:

```
cutadapt -a AGATCGGAAGAGCGTCGTGTAGGGAAAGAatctacacttagtagaaatt -m20
--discard-untrimmed
bowtie2 -x new.primers.BC.only -p 12 --very-sensitive --nofw
samtools view -bS Input.umi.sam > Input.umi.bam
samtools sort Input.umi.bam -o Input.umi_Sorted.bam
samtools index Input.umi_Sorted.bam
samtools idxstats Input.umi_Sorted.bam > Input.umi.idxstats.txt
```

Only reads with a perfect match to a barcode sequence were considered. MaGeck was then run, using control normalization to the non-targeting crRNAs, on the counts table to quantify barcode enrichment in high and low bins compared to bulk unsorted cells. High and low for each condition were analyzed separately. We calculated an average fold change for each barcode in each condition as ($\log_2$ fold-change$_{High}$ - $\log_2$ fold-change$_{low}$)/2. To calculate an overall fold change for each crRNA array in each drug condition, we took the average of the -$\log_2$ fold change for low bin vs. bulk population and the log2 fold change high bin vs. bulk population, meaning that a negative fold change indicates an array is enriched in the low bin and depleted from the high bin. Data for each bin can be found in **Supp. Fig. 7b-d**, and raw read counts and $\log_2$ fold changes for each pre-crRNA can be found in **Supp. Table 5-6**.

To assess whether barcode sequences were paired with the correct crRNA array sequences, barcode sequences were extracted from each read using UMI-tools. Reads were then trimmed using Cutadapt, and aligned to a custom crRNA array reference in a paired fashion using Bowtie2. Samtools was used to create a sorted bam file, and then UMI-tools was used to group reads by barcode sequence. The specific parameters used were

```
umi_tools extract --extract-method=regex
--bc-pattern='^(?P<umi_1>.{8})(?P<discard_2>TCGG)(?P<umi_2>.{8})'
cutadapt -g ^NNNNNNNatcctggtattggtctgcgaaatttctactaagtgtagat -m70
--discard-untrimmed
bowtie2 -x new.primers.array -p 12 --very-sensitive
samtools view -bS Input.umi.sam > Input.umi.bam
```

```
samtools sort  Input.umi.bam  -o Input.umi_Sorted.bam
samtools index  Input.umi_Sorted.bam
samtools idxstats Input.umi_Sorted.bam > Input.umi.idxstats.txt
umi_tools       group       -I       Input.umi_Sorted.bam       --paired
--group-out=Input.umi_counts.txt         --output-bam                -S
Input.umi_grouped.bam
```

For each barcode sequence, the number of reads associated with the correct array was determined and the percentage of reads with the correct array was calculated as 100 x (# of reads for correct array/ # total reads)

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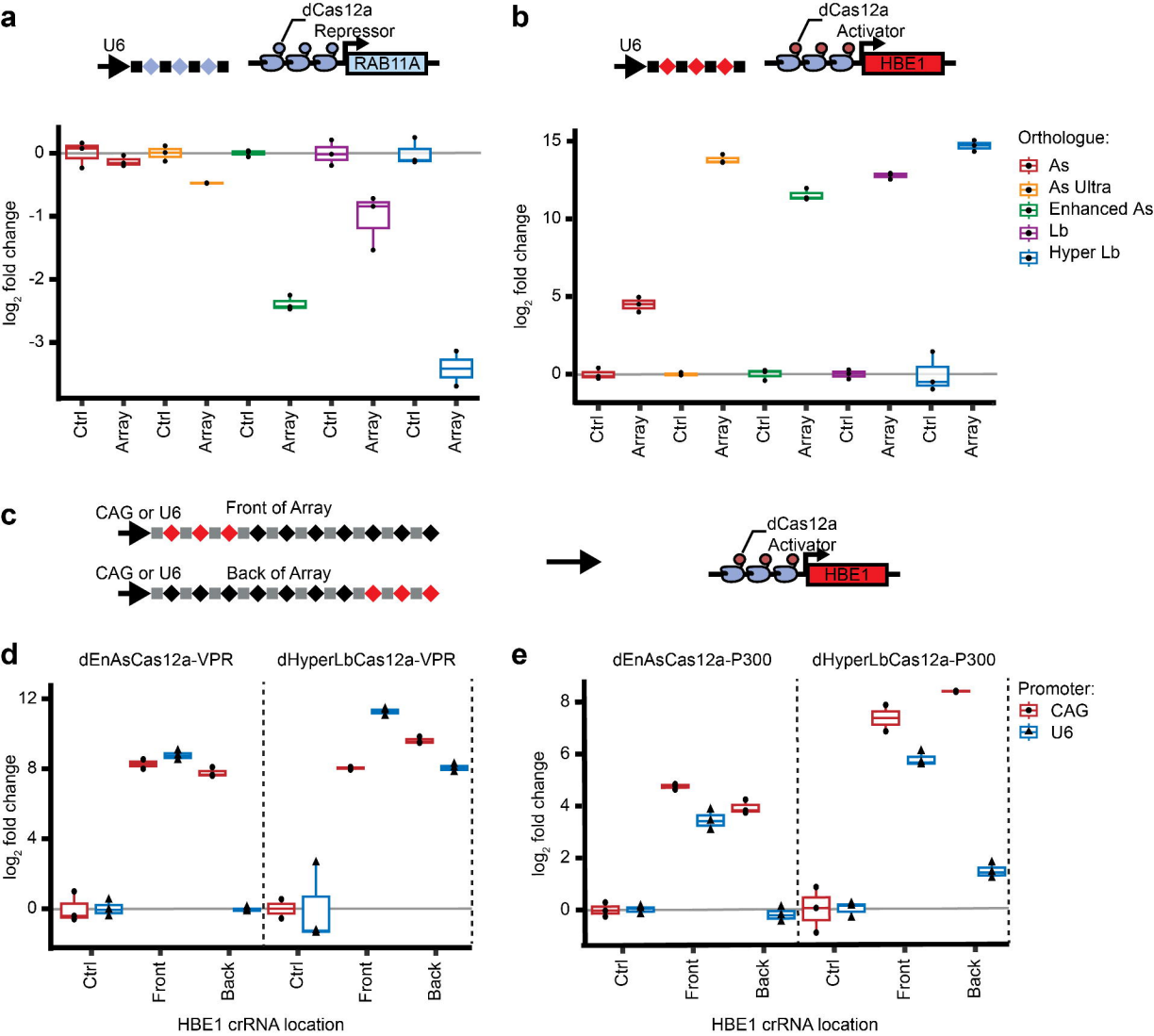
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Repressor
RAB11A
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-1
-2
-3
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Array
b
U6
dCas12a
Activator
HBE1
log2 fold change
15
10
5
0
Orthologue:
As
As Ultra
Enhanced As
Lb
Hyper Lb
c
CAG or U6
Front of Array
CAG or U6
Back of Array
dCas12a
Activator
HBE1
d
dEnAsCas12a-VPR
dHyperLbCas12a-VPR
log2 fold change
12
8
4
0
Ctrl
Front
Back
HBE1 crRNA location
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2
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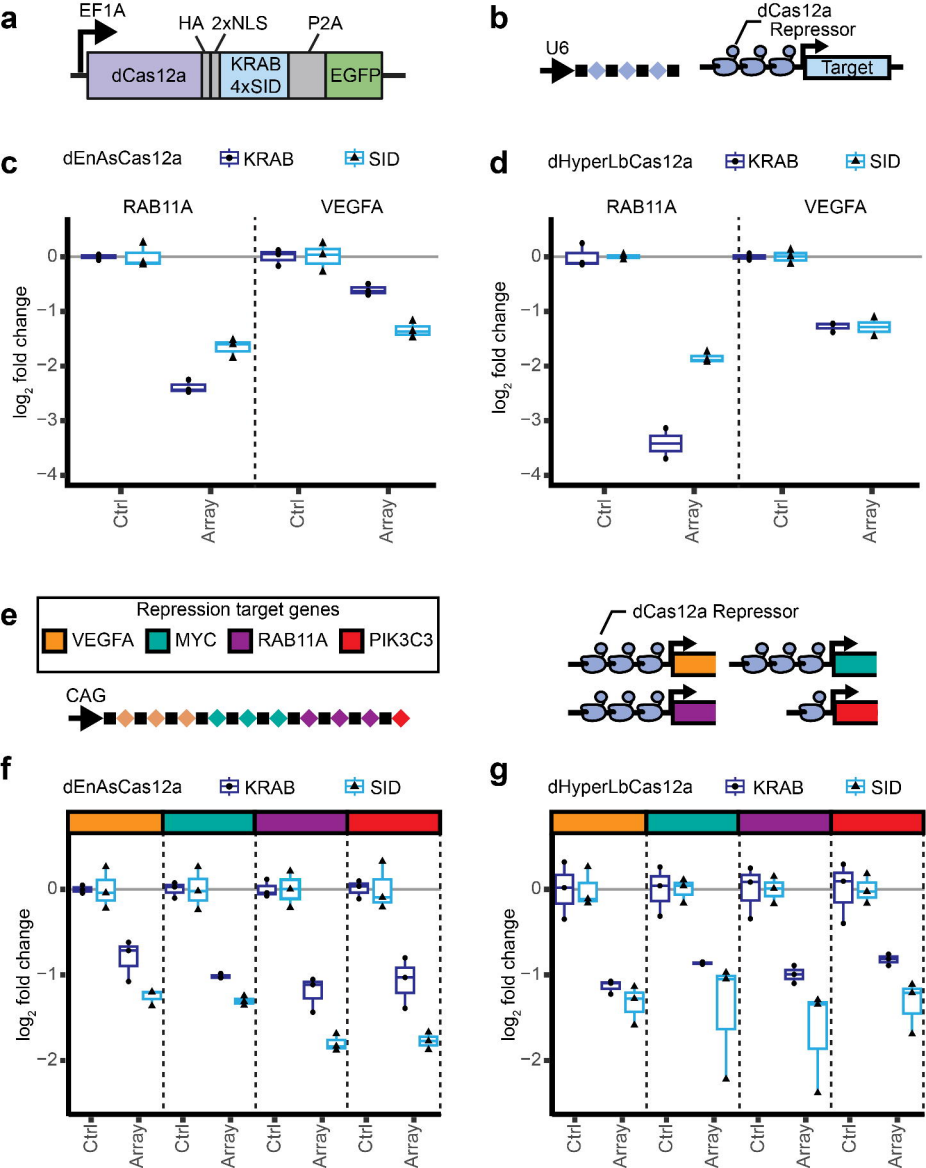
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KRAB
SID
RAB11A
VEGFA
log2 fold change
0
−1
−2
−3
−4
Ctrl
Array
Ctrl
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d
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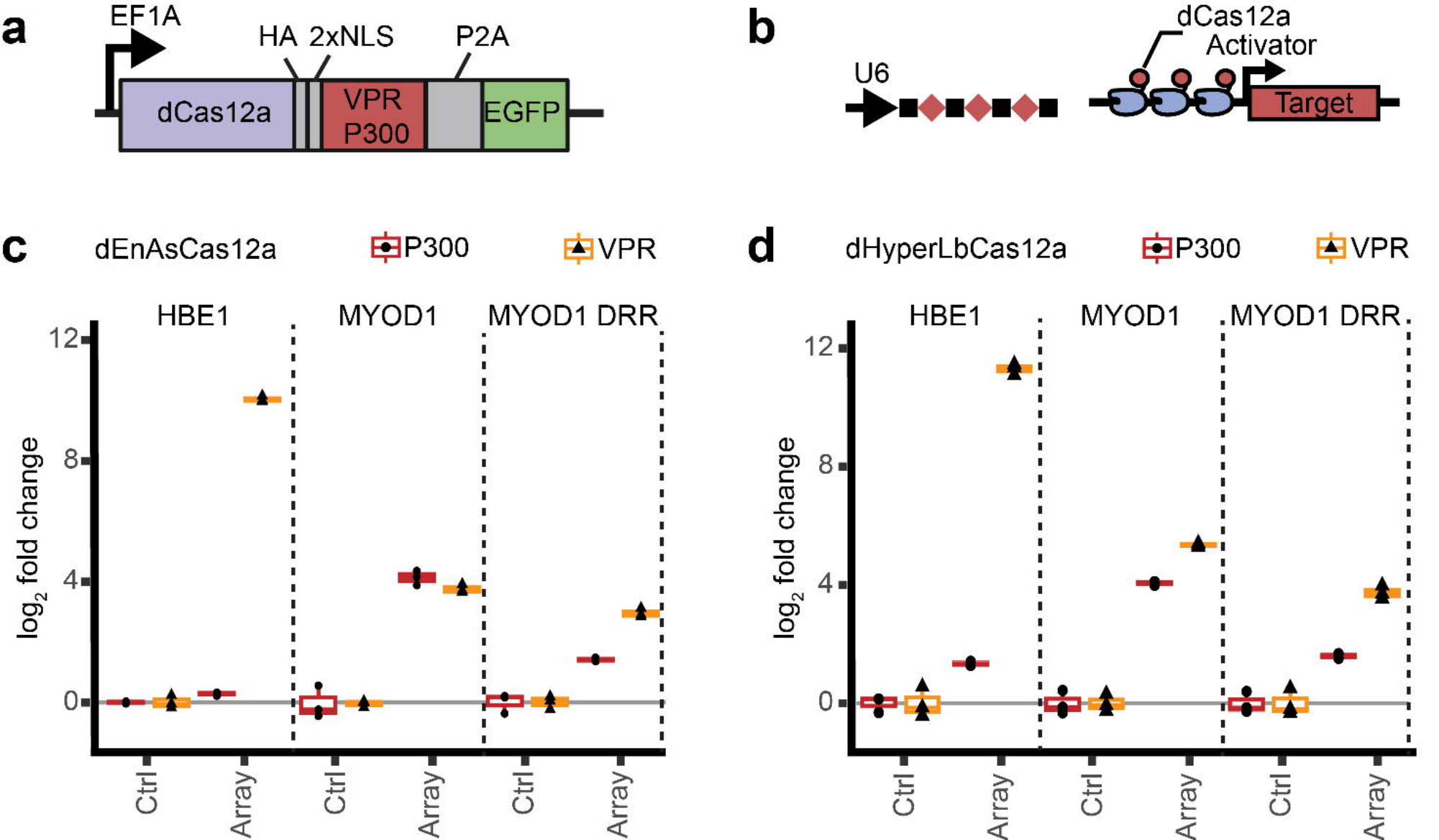
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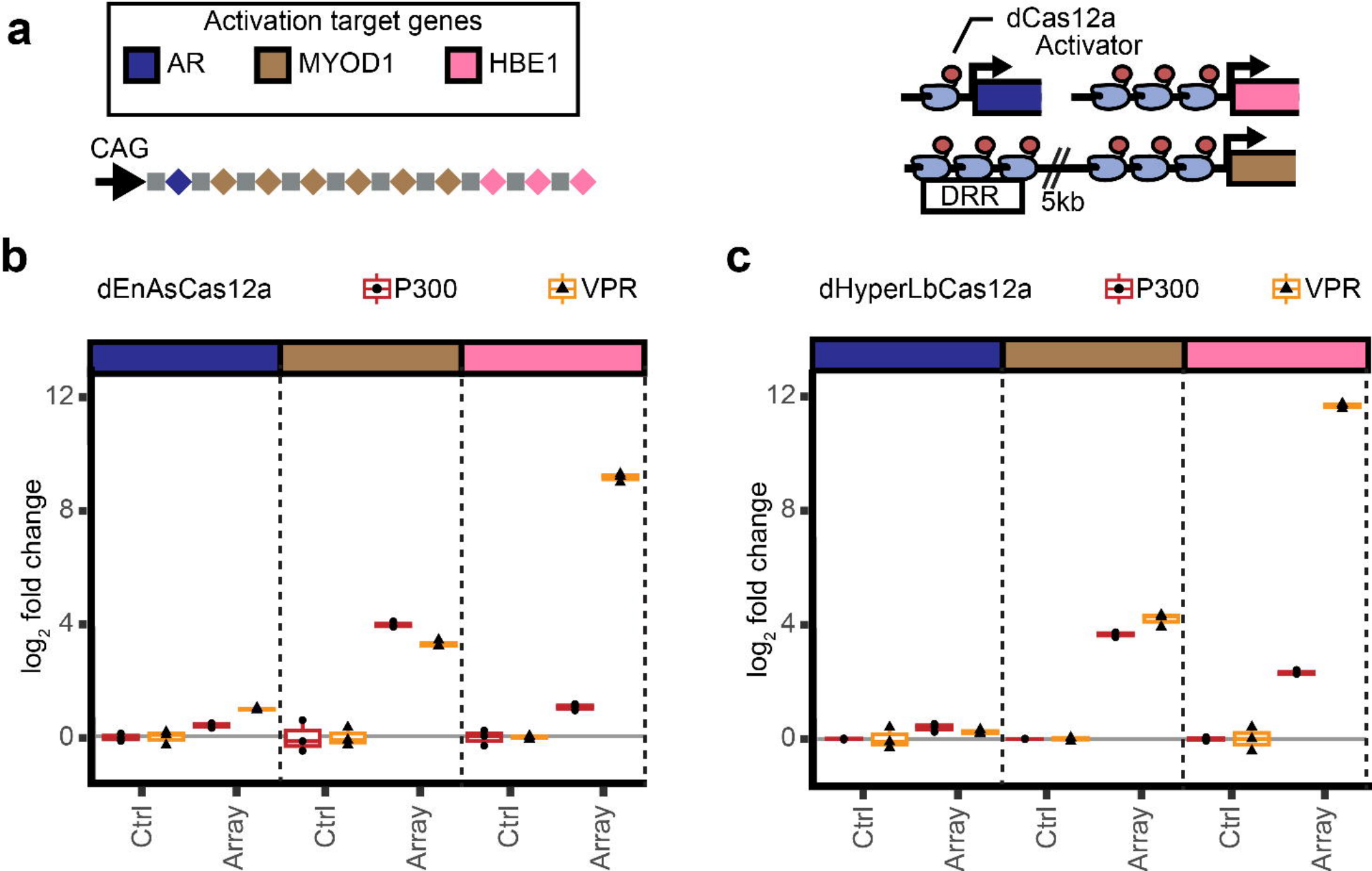
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Ctrl
Array
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Array
Ctrl
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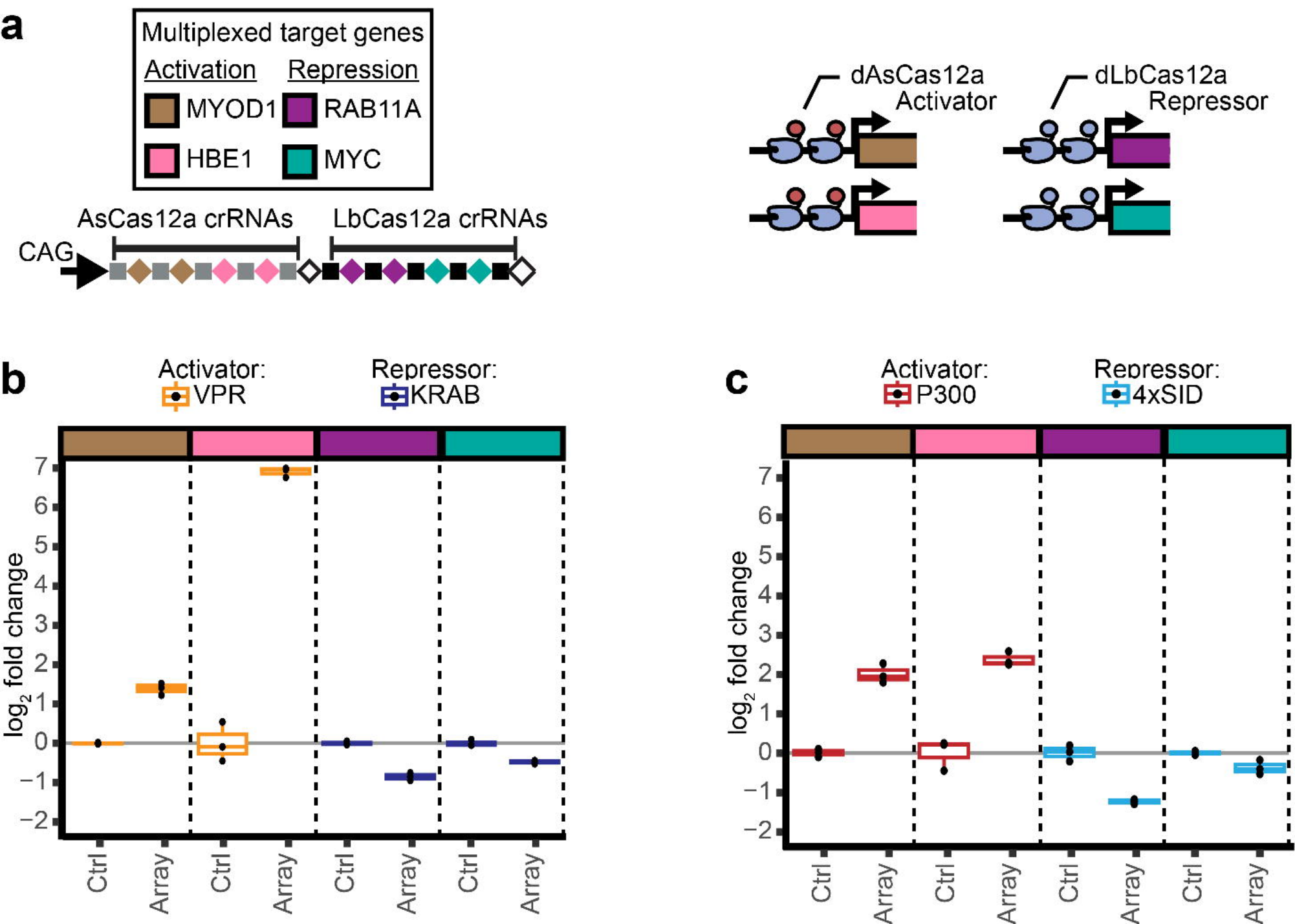
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Multiplexed target genes
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Repression
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RAB11A
HBE1
MYC
AsCas12a crRNAs
LbCas12a crRNAs
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dAsCas12a
Activator
dLbCas12a
Repressor
b
Activator:
VPR
Repressor:
KRAB
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Ctrl
Array
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P300
Repressor:
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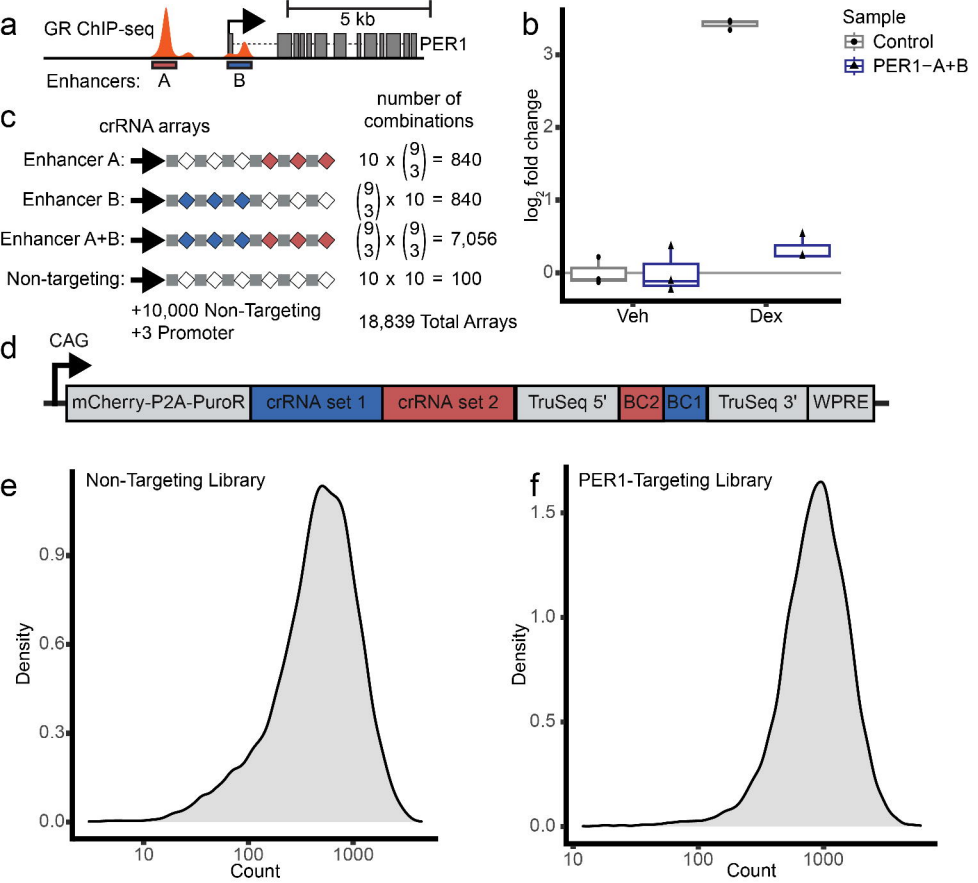
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GR ChIP-seq
5 kb
PER1
Enhancers:
A
B
b
Sample
Control
PER1−A+B
log2 fold change
0
1
2
3
Veh
Dex
c
crRNA arrays
number of combinations
Enhancer A:
10 x (9 3) = 840
Enhancer B:
(9 3) x 10 = 840
Enhancer A+B:
(9 3) x (9 3) = 7,056
Non-targeting:
10 x 10 = 100
+10,000 Non-Targeting
+3 Promoter
18,839 Total Arrays
d
CAG
mCherry-P2A-PuroR
crRNA set 1
crRNA set 2
TruSeq 5'
BC2
BC1
TruSeq 3'
WPRE
e
Non-Targeting Library
Density
0.0
0.3
0.6
0.9
10
100
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Count
f
PER1-Targeting Library
Density
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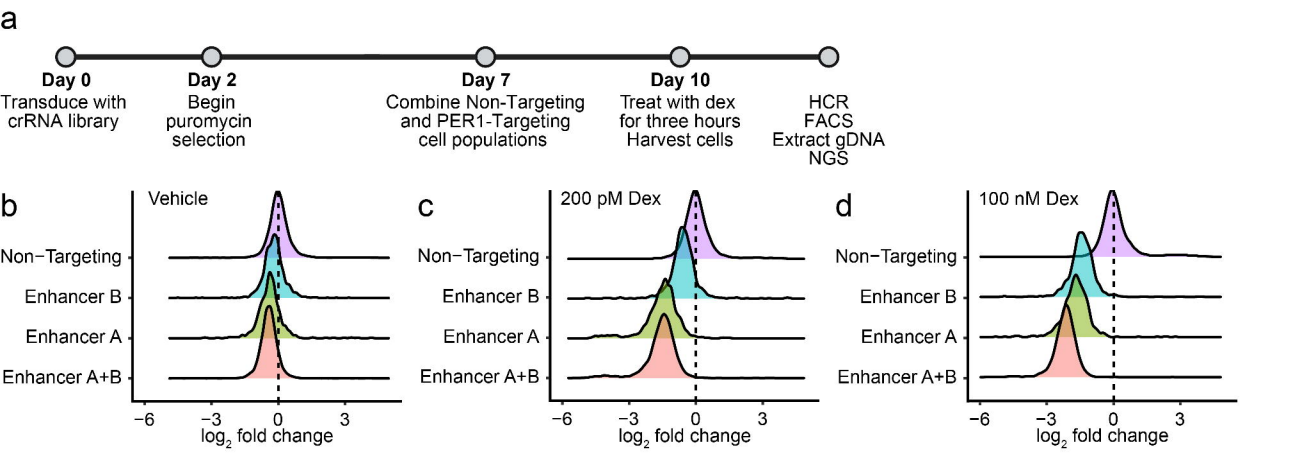
0.5
1.0
1.5
10
100
1000
Count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Day 0
Transduce with crRNA library
Day 2
Begin puromycin selection
Day 7
Combine Non-Targeting and PER1-Targeting cell populations
Day 10
Treat with dex for three hours
Harvest cells
HCR
FACS
Extract gDNA
NGS
b
Vehicle
c
200 pM Dex
d
100 nM Dex
Non−Targeting
Enhancer B
Enhancer A
Enhancer A+B
−6
−3
0
3
log2 fold change