Published in final edited form as:

Methods. 2017 May 15; 121-122: 118–129. doi:10.1016/j.ymeth.2017.03.008.

Functional interrogation of non-coding DNA through CRISPR genome editing

Matthew C. Canver¹, Daniel E. Bauer^{1,2,3,*}, and Stuart H. Orkin^{1,2,3,4,*}

¹Harvard Medical School, Boston, MA 02115

²Division of Hematology/Oncology, Boston Children's Hospital, Boston, MA 02115

³Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115

⁴Howard Hughes Medical Institute, Boston, MA 02115

Abstract

Methodologies to interrogate non-coding regions have lagged behind coding regions despite comprising the vast majority of the genome. However, the rapid evolution of clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing has provided a multitude of novel techniques for laboratory investigation including significant contributions to the toolbox for studying non-coding DNA. CRISPR-mediated loss-of-function strategies rely on direct disruption of the underlying sequence or repression of transcription without modifying the targeted DNA sequence. CRISPR-mediated gain-of-function approaches similarly benefit from methods to alter the targeted sequence through integration of customized sequence into the genome as well as methods to activate transcription. Here we review CRISPR-based loss- and gain-of-function techniques for the interrogation of non-coding DNA.

1. INTRODUCTION

The human genome consists of approximately 3.3 billion base pairs with only 1% protein coding sequence [1–3]. The remaining 99% of the non-coding genome potentially harbors function. However, unlike the coding genome it lacks mRNA and protein production as functional intermediaries. Decades of research has sought to determine the rules to predict and identify functional elements within the non-coding genome including evolutionary, biochemical/epigenetic, and genetic approaches [2,4]. The understanding of the non-coding genome has been further hampered by a paucity of methods to definitively demonstrate

*Correspondence should be addressed to S.H.O. (Stuart_Orkin@dfci.harvard.edu) or D.E.B. (Daniel.Bauer@childrens.harvard.edu). Corresponding Address: Boston Children's Hospital, 300 Longwood Avenue, Boston, MA 02115.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

AUTHOR CONTRIBUTIONS

M.C.C. reviewed the literature. M.C.C., D.E.B., and S.H.O. wrote the manuscript.

CONFLICT OF INTEREST DISCLOSURES

M.C.C., D.E.B., and S.H.O. declare no competing financial interests.

function with reliance on such methods as correlative epigenetic marks [5], heterologous reporter assays including massively parallel reporter assays [6–8], and evolutionary conservation [2,9].

The development of genome editing technologies including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) offered new methods to identify functional non-coding sequences through loss- and gain-of-function genetic studies [10,11]. The rapid emergence of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease system as a facile genome editing platform has revolutionized genome editing approaches to study the non-coding genome [12–19]. The CRISPR/Cas9 nuclease system was originally discovered as a prokaryotic adaptive defense system that has been repurposed for eukaryotic genome editing [20]. The CRISPR/Cas9 platform requires an RNA molecule to guide the Cas9 nuclease for site-specific cleavage upstream of a genomic protospacer adjacent motif (PAM) sequence [12,13]. The endogenous tracrRNA and crRNA have been synthetically fused to create a single chimeric guide RNA (sgRNA) to facilitate genome editing experiments. CRISPR-based editing originally relied on *S. pyogenes* Cas9 (SpCas9) with an NGG-restricted PAM sequence. However, the CRISPR toolbox has been expanded to include a wide-range of PAM sequences with many validated for eukaryotic genome editing including engineered *S. pyogenes* and *S. aureus* Cas9's to have altered PAM specificities [12,13,21–27]. Notably, the Cpf1 and C2c1 offer alternative CRISPR nucleases to Cas9 as well as uniquely offer site-specific cleavage downstream of PAM sequences [28–30].

CRISPR-based genome editing to interrogate non-coding sequences relies on the endogenous DNA repair pathways that are engaged following double strand break (DSB) induction by a CRISPR-associated nuclease. These repair pathways principally include non-homologous end joining (NHEJ) and homology-directed repair (HDR). The HDR pathway can be exploited by supplying an extrachromosomal repair template to allow for the insertion of customized sequence as opposed to using the sister chromatid as the template for repair [31].

CRISPR-based genome editing systems have offered a host of strategies to study non-coding genomes through both loss-of-function and gain-of-function approaches. In particular, enhancers have been a large focus of study via genome editing [32]. Here we review CRISPR genome editing methodologies for the interrogation of the non-coding genome (Table 1).

2. LOSS OF FUNCTION STUDIES

CRISPR based loss-of-function studies focus on two different strategies that are distinguished by the requirement to disrupt the underlying sequences. The first strategy employs insertion/deletion (indel) induction following genomic cleavage to disrupt putative regulatory sequences. Loss-of-function with indel production relies on indel-prone NHEJ repair with a typical deletion spectrum of 1–10 base pairs [12,13,33–35]. The second strategy relies on interference or repression of the underlying sequence without altering the sequence itself. Interference approaches rely on an sgRNA to guide a catalytically inactive

Cas9 (dCas9) to mediate locus-specific transcriptional repression through steric hindrance of biological processes such as blocking RNA polymerase binding/progression or interfering with the binding of transcription factors [36–39]. Repression strategies extend this concept by fusing a repressive effector domain such as the Krüppel-associated box (KRAB) [36,40], Lys-specific histone demethylase 1 (LSD1) [41], or mSin3 interaction domains [42] to facilitate epigenetic-based repression of the underlying sequence.

2.1 LOSS OF FUNCTION APPROACHES WITH DNA SEQUENCE MODIFICATION

2.1.1 Individual sgRNA targeting—Individual sgRNA targeting can be utilized to identify functional non-coding sequences. Given the relatively narrow indel spectrum of an sgRNA, this approach typically requires additional insight to enhance the pre-experiment probability of successfully identifying functional sequences (Fig. 1a). For example, one study focused on transcription factor binding footprints to identify putative sites to target within the +58 DNase hypersensitive site (DHS) within the enhancer of *BCL11A*, a known repressor of fetal hemoglobin [43]. Specifically, this study identified 8 potential transcription factor binding sites within the 162 base pair DHS [43,44]. This study used ZFNs and TALENs to target all 8 candidate motifs and identified enhancer function in 1/8 sites. Using a functional genomic footprinting approach for analysis at nucleotide resolution, the region of enhancer function was further reduced to a GATA motif [43]. While this study utilized ZFNs and TALENs to engender DSBs, this strategy could be utilized to inform similar CRISPR-based targeting. Another example involved utilizing patient samples to assist with sgRNA targeting. Overlapping mutations within multiple patient samples were identified in an intergenic region 7.5 kilobases from the transcriptional start site of *TAL1* that led to the creation of an oncogenic superenhancer [45]. These mutations suggested a narrow site to target via genome editing to study the activity of an aberrant super-enhancer element. Finally, other examples have involved targeting CTCF binding sites within insulators separating topologically associated domains (TADs). These studies have demonstrated that disrupting these CTCF binding sites with indels results in aberrant enhancer activity, changes in gene expression, and disruption of genomic topology [46,47].

Individual sgRNA targeting with Cas9 is also possible *in vivo* [48,49]. Similarly, *in vivo* targeting using the Cpf1 nuclease has also been demonstrated by both microinjection and ribonucleoprotein electroporation in mice [50,51]. New strategies for using Cpf1 may allow for multiplex targeting using a single construct. This approach has been used for the simultaneous editing of four targets *in vitro* and three *in vivo* [52].

Individual sgRNA targeting is useful for a small number of specific, narrow target(s) often informed by epigenetic marks or transcription factor binding motifs. However, it becomes laborious with multiple targets or to evaluate large regions since large deletions represent a low frequency event following individual sgRNA targeting [12,13,33–35]. The recent identification of regulatory elements lacking typical chromatin signatures highlights the potential shortcomings of targeting only a few predicted functional sequences based on incomplete current knowledge and emphasizes the potential benefits of high-throughput approaches for studying non-coding DNA [53].

2.1.2 Dual sgRNA targeting for targeted genomic deletion, inversion, and translocations

—The usage of pairs of sgRNAs, or dual sgRNAs, to produce simultaneous DSBs have a large number of applications including induction of targeted genomic deletions, inversion, and more complex genomic rearrangements such as translocations (Fig. 1b) [54]. Two sgRNAs have been shown to reliably engender large genomic deletions *in vitro*, including greater than 1 megabase [33,55–59]. Deletions have also been engendered *in vivo* [60–62]. The use of targeted deletion has been used for a wide variety of applications to study non-coding elements. Notably, targeted deletion has been used to delete composite enhancers as well as enhancer substituents to identify functional sequence within large enhancer regions with characteristic epigenetic marks [63–65]. This strategy has also been used to generate genomic inversions with inverted sequences of greater than 1 megabase [33,55,66–69]. For example, inversion of CTCF binding sites induced dysregulation of genomic topology and alteration of enhancer-promoter contacts, which suggest that enhancers containing CTCF binding sites may not display the orientation independence traditionally ascribed to enhancer elements [70]. Similarly, inversions have validated *in situ* enhancer element orientation independence, which had been originally described using heterologous reporter constructs [9,63].

A dual sgRNA strategy has been successfully applied to *in vivo* applications, including mice [66,69,71–75], zebrafish [67], and *C. elegans* [76]. One study utilized a dual sgRNA approach to engender a 1.2 kilobase deletion and inversion of an insulator regulatory element ~12 kilobases upstream of the murine *tyrosinase* gene, an enzyme necessary for melanin synthesis. CRISPR reads were delivered through microinjection of CRISPR/Cas9 reagents into fertilized mouse eggs. Using coat pigmentation as a readout, this study confirmed the functionality of this regulatory element as both deletion and inversion of the element resulted in a reduction in both *Tyr* expression and coat pigmentation [69]. Another study deleted individual DHS and a composite enhancer in mouse embryonic stem cells to ultimately generate germ-line transmitting chimeric mice to examine the role of the enhancer and its substituent components during development [77]. Another notable study disrupted boundary elements through deletions, inversions, and duplications to evaluate the effect on embryologic development. The resulting changes in enhancer-promoter interactions resulted in limb malformations [78]. Lastly, utilization of two sgRNA at different chromosomal loci can result in translocations both *in vitro* and *in vivo* [79–82], which can allow for chromosomal rearrangements resulting in aberrant enhancer/super-enhancer recruitment [83,84].

The dual sgRNA approach for targeted deletion is useful for interrogation of large regions at low resolution. It is also useful for creating precise deletion events, although indels may be observed at deletion junctions [33]. Dual sgRNA approaches are well suited for generating complex genomic rearrangements such as inversions and translocations. However, the dual sgRNA approach for targeted deletions, inversions, and translocations has been hampered by low throughput, efficiency, and resolution. However, recent strategies have been developed for the cloning of pooled libraries consisting of dual sgRNA constructs to allow for pooled deletion/inversion screens [85,86]. This strategy was applied to 51 long non-coding RNAs (lncRNAs) to identify lncRNAs with roles in cancer cell proliferation [87]. These screens

may serve as a reliable method to increase the throughput of targeted deletion strategies. However, a challenge may be that alternative repair outcomes such as small indels at each of the dual sgRNA cleavage sites may limit signal strength in pooled screens, where enrichment phenotypes may be easier to detect than dropout phenotypes.

2.1.3 Tiling sgRNAs/Saturating mutagenesis—Pooled CRISPR screening has become widely available and allows for high-throughput and/or multi-loci studies [88–90]. In general, most pooled CRISPR screens have focused on gene knockout for *in vitro* and *in vivo* applications [86,91–113]. However, pooled screens targeting non-coding DNA have increased [63,87,114–117]. Notably, a recently developed technique of tiling sgRNAs (“saturating mutagenesis”) has provided a methodology to circumvent the low-throughput, efficiency, and resolution of targeted deletion to interrogate regulatory DNA (Fig. 1c). This approach relies on the same indel spectrum of utilizing an sgRNA of 1–10 base pairs [12,13,33–35]. Saturating mutagenesis becomes high-throughput and high-resolution by using every sgRNA within a given region in a pooled screening format, which allows for saturating a region with indels to identify functional sequence. The resolution is a function of the number of sgRNA available within a given region. This methodology led to functional fine-mapping of 3.9 kilobases of DHS sequence within the 12 kilobase *BCL11A* enhancer using a 533 sgRNA library [63]. This study utilized distances between adjacent genomic cleavages between sgRNA within the library as a measure of degree of saturation. For the enhancer-targeted library, there was a median of 4 base pairs between adjacent genomic cleavages and 18 base pairs at 90th percentile. Another study utilized 18,315 sgRNA to mutagenize ~715 kilobases of noncoding sequence adjacent to three genes to identify regions involved in resistance to pharmacologic inhibitors by disrupting transcription factor binding and long-range interactions [115]. Yet another group performed saturating mutagenesis of 174 cis regulatory elements including DHS, CTCF sites, and predicted enhancers within a one megabase TAD containing the gene *POUF51*. This work led to the identification of so-called temp enhancers, whereby the phenotypic effect of enhancer disruption is lost after several rounds of cell division [116]. A final study tiled sgRNA across binding motifs. Specifically, the study utilized 1,116 sgRNA to target p53 binding motifs within 685 genomic loci to identify enhancers required for oncogene-induced senescence. The same study also used 97 sgRNA tiling binding motifs within 73 enhancers for ER α , an estrogen-activated transcription factor implicated in breast cancer. This approach allowed for identification of novel enhancer elements within both regions [118]. Lastly, a similar strategy was used to tile sgRNAs across regulatory elements with delivery of library sgRNA via HDR to investigate four loci tagged by a fluorescent marker to aid detection of indel perturbations [53].

Enhanced resolution of tiling sgRNA/saturating mutagenesis can be achieved by design of a CRISPR library based on an alternative PAM sequence and a corresponding variant Cas9 (eg, a GC-rich PAM for a GC-rich region or an AT-rich PAM for an AT-rich region). Alternatively, resolution can be enhanced by using multiple nucleases in combination with unique PAM sequences for the same region [119]. One notable study utilized the *S. pyogenes* NGG-restricted Cas9 and the *S. pyogenes* variant NGA-restricted Cas9 to perform saturating mutagenesis of all DHS within the *HBS1L-MYB* intergenic region [119]. In this

study, the usage of NGG-restricted sgRNA alone (n=2,166 sgRNA) led to a median and 90th percentile gap between adjacent genomic cleavages of 5 and 22.5 base pairs, respectively. The usage of NGA-restricted sgRNA targeting the same region (n=2,564 sgRNA) led to a median and 90th percentile between adjacent genomic cleavages of 6 and 18 base pairs, respectively. The NGG-and NGA-restricted combination library led to the highest resolution with a total of 4,690 sgRNA resulting in a median of 3 base pairs and 90th percentile of 11 base pairs between adjacent genomic cleavages [119]. With the continual identification of novel Cas9 species and other nucleases with unique PAM sequences [12,13,21–29], the ability to enhance saturating mutagenesis resolution with combination of nucleases will also continue to improve. Lastly, recent work has also highlighted the importance of taking variants into account during saturating mutagenesis library design for genetically implicated regions marked by common genetic variation. These variant aware libraries help minimize attenuation of sgRNA activity by variants in the cells used for study as well as aide in the detection of SNP-marked functional sequence [119].

Tiling sgRNA/saturating mutagenesis provides a high-throughput, high resolution methodology to functionally dissect non-coding DNA. This technique has been validated for *in vitro* applications large regions and multi-loci studies. While most published studies have utilized NGG-restricted Cas9 [63,114–116], recent work using the NGA-restricted Cas9 variant and Cpf1 have demonstrated that other species can be used for pooled CRISPR screening [119,120]. One potential pitfall encountered with all sgRNA within a given region is the potential for inclusion of guides with significant off-target potential, which can confound results. To this end, multiple studies have identified potential false positive hits due to targeting of repetitive DNA [119,121,122] including an example when performing saturating mutagenesis of non-coding DNA sequences [119]. Therefore, when performing saturating mutagenesis, it is essential to perform off-target analysis for all sgRNA in the library especially in regions involving repetitive sequences. In addition to off-target analysis, recent work has demonstrated that switching to a modified/adapted tracrRNA sequence can also improve the reliability of data obtained from dropout CRISPR screens and increases the efficacy of library sgRNA [40,123].

2.2 LOSS OF FUNCTION APPROACHES WITHOUT DNA SEQUENCE MODIFICATION

The initial strategy for studying non-coding DNA without disrupting the underlying sequences relied on CRISPR interference (CRISPRi) to sterically hinder the binding of transcription factors or interference with transcriptional initiation or elongation [36–39]. This strategy led to a ~2-fold reduction in gene expression [37]. This technique was enhanced by dCas9 fusion to chromatin modifiers [36,40]. In one study, dCas9 fusion with KRAB domain (dCas9-KRAB) derived from Kox1 resulted in improved gene repression to 5–15 fold repression with repression correlating with the levels of sgRNA expression (Fig. 1d). Notably, this approach also was successfully used to repress transcription via enhancer targeting [36]. While the dCas9-only effect was improved through modification of the sgRNA stem loop and hairpin structures, it still resulted in inferior gene repression as compared to the dCas9-KRAB fusion [40].

Another example of using dCas9-KRAB to interrogate noncoding DNA involved targeting the HS2 enhancer within the locus control region (LCR) of the (β -globin locus. This led to a reduction in gene expression specific to globin genes and induction of H3K9me3 at HS2 without affecting the other four DHS within the LCR. There was also a decrease in enhancer/promoter accessibility as determined by DNase-sequencing at both HS2 as well as several globin gene promoters [124]. A similar approach was utilized to identify novel distal enhancer elements using a 98,000 sgRNA library of all sgRNA within a 1.29 megabase region flanking the *GATA1* and *MYC* genes, which led to the identification of nine distal enhancer elements [125]. Finally, dCas9-KRAB has been used for genome wide screens for gene targets and long non-coding RNAs, thus demonstrating proof-of-principle for high-throughput, large-scale repression screens [126,127].

Given the role of the histone demethylase LSD1 in enhancer repression, a dCas9-LSD1 fusion was targeted to a known enhancer of *Oct4*, a regulator of embryonic stem cell state, which resulted in downregulation of *Oct4* expression (Fig. 1d). The dCas9-LSD1 fusion was then used to evaluate eight candidate enhancers for a role in regulating embryonic stem cell state ultimately leading to validation of four sites. One validated enhancer region was ~10 kilobases upstream of *Tbx3*, a gene with a known role in the maintenance of pluripotency. Targeting the enhancer region with dCas9-LSD1 resulted in a reduction of *Tbx3* at both the RNA and protein level. Notably, there were no changes in enhancer-promoter interaction frequencies. However, there was 33 to 54-fold reduction in H3K27ac and a 6–8 fold reduction of H3K4me2 at the sgRNA binding site without any associated changes in repressive marks including H3K27me3 and H3K9me3. Surprisingly, upon investigation of the *Tbx3* promoter, there was a reduction of H3K27Ac and elevation of H3K27me3 and H3K9me3 when targeting the enhancer with dCas9-KRAB; however, the effect at the promoter was not observed using dCas9-LSD1 [41].

This approach can be used for similar applications as the indel-forming approaches including individual targeting and large-scale screening. However, CRISPRi techniques may be advantageous for investigations where disruption of an enhancer or other regulatory DNA may be cell lethal. The resolution of using dCas9-KRAB or dCas9-LSD1 for saturating mutagenesis as comparing to catalytically active Cas9 has yet to be explored.

Another notable potential drawback to the CRISPRi approach is that the effect may be transient such that the chromatin may revert to its original state after the CRISPRi machinery is no longer present. However, recent reports have suggested that at least in certain cases the CRISPRi effect may be extremely durable, lasting for weeks to months, although the duration of epigenetic memory may depend on a number of variables including the specific locus, cellular context, and combinations of repressors [128].

3. GAIN OF FUNCTION STRATEGIES

Similar to CRISPR/Cas9 methods for loss-of function study, CRISPR/Cas9 based gain-of-function studies focus on two different strategies that are distinguished by the requirement to modify the underlying sequences. The first exploits the endogenous HDR pathway to stimulate repair from an extrachromosomal template to insert customized sequence into the

genome. The second strategy relies on activation of the underlying sequence without direct modification. This approach relies on a catalytically inactive Cas9 (dCas9) coupled to transcriptional activation domains [129,130].

3.1 GAIN OF FUNCTION APPROACHES WITH DNA SEQUENCE MODIFICATION

HDR has been an extremely useful tool to study sequence function, but is hampered by low efficiency [131–133]. However, induction of DSBs has been shown to greatly stimulate HDR [134,135]. Inducing HDR is further complicated by cell-cycle regulation such that HDR only occurs during the G2 and late S phase while absent during the G1 phase [136–139]. A variety of strategies have been developed to increase the efficiency of HDR, which have included inhibition of NHEJ machinery, cell cycle synchronization, and optimization of CRISPR and repair template reagents [136,137,139–146]. Despite these technical challenges, HDR has been used for a variety of applications for non-coding DNA including the insertion/knock-in of variants, large fragments of DNA, and reporters at endogenous loci (Fig. 1e) [147–150]. One notable study used single cell labeling of proteins via HDR within neuronal progenitors to allow for imaging of endogenous proteins [151]. HDR has also been successful *in vivo* for the creation of customized knock in mice [49,152–155].

Recent work demonstrated ~50% of successful HDR alleles to correct a pathogenic SNP using delivery of CRISPR/Cas9 as ribonucleoproteins and adeno-associated virus (AAV) delivery of the repair template. Furthermore, this approach was able to achieve HDR-mediated SNP correction in repopulating hematopoietic stem cells, a quiescent cell type traditionally resistant to HDR [146]. In a similar fashion, one study created four different SNP knock-in mice to examine the effect of these SNPs that had previously been implicated in human infertility [156] while another study created knock-in of two disease risk-associated SNPs implicated for lipid levels both *in vitro* and *in vivo* [157]. Finally, HDR approaches have also been used to engender genomic deletions as an alternative to dual sgRNA with NHEJ repair as discussed above. In one study, a *rex* site was deleted at a TAD boundary through HDR replacement with a *NcoI* restriction site, which facilitated identification of the desired mutation [158].

One longstanding technical challenge has been identification of causal single nucleotide polymorphisms (SNPs) and their target genes that have been implicated by genome-wide association studies in human traits and disease [159]. One study proposed a four step pipeline for the identification of causal SNPs through (1) fine-mapping of putative causal variants in conjunction with (2) epigenetic profiling. The study identified rs339331 as a candidate causal SNP that was suggested to be located within a regulatory element controlling *RFX6* expression. Then the authors used (3) epigenetic editing to demonstrate that targeting the rs339331 region with TALE-LSD1 fusion reduced *RFX6* expression, a finding consistent with enhancer function as discussed above. Finally, rs339331 was inserted into cell lines via (4) TALE-mediated HDR to evaluate its functional consequences [160]. This pipeline provides a systematic approach to evaluating of genetically implicated variants marking regulatory DNA.

Recent reports have suggested an alternative approach to the stimulated HDR following nuclease cleavage paradigm to insert/alter sequence. Specifically, two studies fused cytidine

deaminase enzymes to dCas9 for site-specific C to T or G to A substitutions in an ~5 base pair window in the absence of nuclease-mediated cleavage [161,162]. To further this technique, other groups used a variant dCas9-AID fusion that was capable of creating C and G substitutions to the three other bases at appreciable frequencies (Fig. 1f). This system was used to identify previously reported and novel mutations associated with resistance to the tyrosine kinase inhibitor imatinib [163]. Finally, another group used dCas9-AID with its nuclear export signal deleted in conjunction with a sgRNA with two MS2 activation domains to generate significant levels of genomic diversity to drive directed evolution [164].

Saturation mutagenesis, distinct from the tiling sgRNA/saturating mutagenesis discussed above, is an HDR-based technique for comprehensive mutagenesis of narrow regions. To be explicit, we are using the term “saturating mutagenesis” to describe design of all possible sgRNAs for a given nuclease within a given target sequence, and the term “saturation mutagenesis” to describe introduction of all possible sequence variants within a given target sequence. Saturation mutagenesis has been used for a 6 base pair region within exon 18 of the *BRCA1* gene to generate all possible single nucleotide variants via HDR from a library of single nucleotide substituted templates to examine the effects of each substitution on transcript levels [165]. This approach provides a comprehensive interrogation of a given region. While this approach has only been demonstrated in coding sequence, this could be used for non-coding regions as evaluation of transcription factor binding motifs. While this strategy benefits from its comprehensive approach to a region, it is limited in its throughput and requirement for narrow regions. Lastly, another recent report demonstrated the use of HDR to integrate a library of 12,000 175 base pair donors with 100 base pair variable regions into heterochromatin to confirm computationally predicted sequences associated with chromatin accessibility *in situ* [166].

Several studies have attempted to circumvent the HDR requirement for the insertion of exogenous sequence into the genome. One study achieved sequence knock-in of a fluorescent marker from a repair template by exploiting microhomology-mediated end joining (MMEJ), a repair process present during G1 and early S phases that relies on nearby microhomologous sequences of 2–25 bp in length (Fig. 1e). This approach was also successful *in vivo* in both silkworms and frogs [167]. It had been previously shown that simultaneous ZFN-mediated cleavage of both a genomic locus and a plasmid containing the same cleavage recognition site led to integration of the plasmid (Fig. 1g) [168]. Using a similar strategy, others demonstrated homology-independent integration in zebrafish with simultaneous CRISPR/Cas9-mediated DSB induction at a genomic locus with concurrent plasmid linearization [169,170]. Finally, recent work showed that NHEJ-mediated targeted integration approach involving concurrent genomic locus and plasmid cleavage outperformed HDR and MMEJ integration, could be used in both dividing and non-dividing cells, and demonstrated its use *in vivo* [171].

3.2 GAIN OF FUNCTION APPROACHES WITHOUT DNA SEQUENCE MODIFICATION

The initial strategies for gain-of-function studying non-coding DNA without modifying the underlying sequences have relied on CRISPR activation (CRISPRa) to augment transcription. One initial study created a fusion of VP64 activation domain to the C-terminus

of Cas9, which led to transcriptional activation (Fig. 1h). In this study, the 3' end of the sgRNA was also modified with two copies of MS2 bacteriophage coat protein-binding aptamers to allow for sgRNA recruitment of activation domains [130]. Taken together, these two modifications allowed for CRISPR-mediated gene activation. Other studies have used alternative activation domains such as VP160 [172].

This approach was furthered in a subsequent study that modified Cas9 to include MS2 protein-interacting RNA aptamers on the sgRNA's tetraloop and stem loop 2, which outperformed dCas9-VP64 fusion 3–5 fold individually and a 12-fold increase when both aptamers were included. The combination of these modifications with dCas9-VP64 further increased activation of gene expression [173]. Notably, the MS2 aptamer modification on the tetraloop and stem loop 2 were more effective than the 3' end fusion of MS2 [130,173]. In this same study, dCas9 was modified to add additional activation domains including VP64, the NF- κ B trans-activating subunit p65, and the activation domain from human heat shock factor 1 (HSF1). Collectively, the optimal system for transcriptional activation was demonstrated to include sgRNAs with MS2 aptamer modifications on the tetraloop and stem loop 2, dCas9-VP64, and the addition of activators MS2-p65-HSF1. This system was successfully used at both individual loci and for genome scale activation screening [173,174].

A recent study applied the CRISPRa strategy to non-coding regions by using the dCas9-VP64 system for an unbiased screen to identify functional enhancer sequences by tiling sgRNA across a >100 kilobase genomic region genetically implicated to play a role in autoimmunity. This approach led to the identification of multiple enhancer elements responsible to CRISPR activation [175]. Finally, another approach to transcriptional activation is the fusion of dCas9 to the catalytic core of the human acetyltransferase p300 to allow for the catalysis of H3K27Ac at target loci (Fig. 1h). dCas9-p300 outperformed dCas9-VP64 to increase gene expression when targeting multiple promoters. Notably, targeting of the HS2 enhancer within the (β -globin locus by dCas9-p300 led to increased expression of globin genes at levels higher than dCas9-VP64. The authors further showed that activation correlated with increased H3K27Ac at both promoters and enhancer regions [176].

4. GENERAL CONSIDERATIONS

There are many general considerations that are applicable to both loss-of-function and gain-of-function approaches to studying non-coding DNA. While these considerations are beyond the scope of this review, we will briefly highlight several important issues. For example, several studies have investigated methods to predict/enhance sgRNA activity at both the individual sgRNA level [94,98,177–188] and for large-scale pooled screening [94,189]. In addition, many methods have been developed to predict/detect off target cleavages [35,178,190–193]. Other methods to minimize off-targets have been developed including the use of truncated sgRNAs, dimeric RNA-guided FokI nucleases, and high-fidelity Cas9 mutants [34,54,194–197]. It is important to maximize sgRNA activity and minimize off-target cleavages for all CRISPR-based as allowed by the experimental design. Recent work has provided further insight to the targeting specificity of Cpf1 [120,198,199].

Methodologies continue to emerge that allow for increased spatiotemporal control of genome editing experiments, such as chemical- and light-inducible Cas9 [200–202] as well as identification of naturally occurring inhibitors of Cas9 that may be exploited as an on/off switch [203]. Furthermore, combining CRISPR pooled screens with RNA-sequencing at the single cell level will aid both loss-of-function and gain-of-function experiments [204–206]. Finally, systems for simultaneous repression and activation of gene expression through the use of scaffold RNAs may usher in an era where combinatorial loss- and gain-of-function studies are possible to dissect complex transcriptional networks [207].

5. CONCLUSIONS

The study of regulatory DNA has been plagued by a paucity of methods to interrogate non-coding sequences in their native chromatin, chromosomal, and cellular context. CRISPR-based technologies continue to emerge at a rapid pace, which offer new methodologies to study non-coding DNA for laboratory experiments and therapeutic applications [43,63,208–210]. CRISPR-based strategies allow for efficient loss- and gain-of-function studies, which includes approaches with and without modification of the targeted DNA sequence. Advancements have continued to improve throughput, efficiency, and specificity of CRISPR-based methodologies. CRISPR technologies will continue to unmask and further understanding of the non-coding genome.

Acknowledgments

M.C.C. is supported by a National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Award (F30DK103359-01A1). D.E.B. is supported by NIDDK (K08DK093705, R03DK109232), NHLBI (DP2OD022716), Burroughs Wellcome Fund, Doris Duke Charitable Foundation Innovations in Clinical Research Award, ASH Scholar Award, Charles H. Hood Foundation Child Health Research Award, and Cooley's Anemia Foundation fellowship. S.H.O. is supported by an award from the NHLBI award (P01HL032262) and an award from the NIDDK (P30DK049216, Center of Excellence in Molecular Hematology).

References

1. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P, McKernan K, Meldrim J, Mesirov JP, Miranda C, Morris W, Naylor J, Raymond C, Rosetti M, Santos R, Sheridan A, Sougnez C, Stange-Thomann N, Stojanovic N, Subramanian A, Wyman D, Rogers J, Sulston J, Ainscough R, Beck S, Bentley D, Burton J, Clee C, Carter N, Coulson A, Deadman R, Deloukas P, Dunham A, Dunham I, Durbin R, French L, Grafham D, Gregory S, Hubbard T, Humphray S, Hunt A, Jones M, Lloyd C, McMurray A, Matthews L, Mercer S, Milne S, Mullikin JC, Mungall A, Plumb R, Ross M, Shownkeen R, Sims S, Waterston RH, Wilson RK, Hillier LW, McPherson JD, Marra MA, Mardis ER, Fulton LA, Chinwalla AT, Pepin KH, Gish WR, Chissole SL, Wendl MC, Delehaunty KD, Miner TL, Delehaunty A, Kramer JB, Cook LL, Fulton RS, Johnson DL, Minx PJ, Clifton SW, Hawkins T, Branscomb E, Predki P, Richardson P, Wenning S, Slezak T, Doggett N, Cheng JF, Olsen A, Lucas S, Elkin C, Uberbacher E, Frazier M, Gibbs RA, Muzny DM, Scherer SE, Bouck JB, Sodergren EJ, Worley KC, Rives CM, Gorrell JH, Metzker ML, Naylor SL, Kucherlapati RS, Nelson DL, Weinstock GM, Sakaki Y, Fujiyama A, Hattori M, Yada T, Toyoda A, Itoh T, Kawagoe C, Watanabe H, Totoki Y, Taylor T, Weissenbach J, Heilig R, Saurin W, Artiguenave F, Brottier P, Bruls T, Pelletier E, Robert C, Wincker P, Smith DR, Doucette-Stamm L, Rubenfield M, Weinstock K, Lee HM, Dubois J, Rosenthal A, Platzer M, Nyakatura G, Taudien S, Rump A, Yang H, Yu J, Wang J, Huang G, Gu J, Hood L, Rowen L, Madan A, Qin S, Davis RW, Federspiel NA, Abola AP, Proctor MJ, Myers RM, Schmutz J, Dickson M, Grimwood J, Cox DR, Olson MV, Kaul R, Raymond C, Shimizu N,

Kawasaki K, Minoshima S, Evans GA, Athanasiou M, Schultz R, Roe BA, Chen F, Pan H, Ramser J, Lehrach H, Reinhardt R, McCombie WR, de la Bastide M, Dedhia N, Blocker H, Hornischer K, Nordsiek G, Agarwala R, Aravind L, Bailey JA, Bateman A, Batzoglu S, Birney E, Bork P, Brown DG, Burge CB, Cerutti L, Chen HC, Church D, Clamp M, Copley RR, Doerks T, Eddy SR, Eichler EE, Furey TS, Galagan J, Gilbert JG, Harmon C, Hayashizaki Y, Haussler D, Hermjakob H, Hokamp K, Jang W, Johnson LS, Jones TA, Kasif S, Kasprzyk A, Kennedy S, Kent WJ, Kitts P, Koonin EV, Korf I, Kulp D, Lancet D, Lowe TM, McLysaght A, Mikkelsen T, Moran JV, Mulder N, Pollara VJ, Ponting CP, Schuler G, Schultz J, Slater G, Smit AF, Stupka E, Szustakowski J, Thierry-Mieg D, Thierry-Mieg J, Wagner L, Wallis J, Wheeler R, Williams A, Wolf YI, Wolfe KH, Yang SP, Yeh RF, Collins F, Guyer MS, Peterson J, Felsenfeld A, Wetterstrand KA, Patrinos A, Morgan MJ, de Jong P, Catanese JJ, Osoegawa K, Shizuya H, Choi S, Chen YJ, C. International Human Genome Sequencing. Initial sequencing and analysis of the human genome. *Nature*. 2001; 409:860–921. [PubMed: 11237011]

2. Kellis M, Wold B, Snyder MP, Bernstein BE, Kundaje A, Marinov GK, Ward LD, Birney E, Crawford GE, Dekker J, Dunham I, Elnitski LL, Farnham PJ, Feingold EA, Gerstein M, Giddings MC, Gilbert DM, Gingeras TR, Green ED, Guigo R, Hubbard T, Kent J, Lieb JD, Myers RM, Pazin MJ, Ren B, Stamatoyannopoulos JA, Weng Z, White KP, Hardison RC. Defining functional DNA elements in the human genome. *Proc Natl Acad Sci U S A*. 2014; 111:6131–6138. [PubMed: 24753594]
3. Chi K. The dark side of the human genome. *Nature*. 2016; 538:275–277. [PubMed: 27734873]
4. Dunham I, Kundaje A, Aldred SF, Collins PJ, Davis Ca, Doyle F, Epstein CB, Fietze S, Harrow J, Kaul R, Khatun J, Lajoie BR, Landt SG, Lee B-K, Pauli F, Rosenbloom KR, Sabo P, Safi A, Sanyal A, Shores N, Simon JM, Song L, Trinklein ND, Altshuler RC, Birney E, Brown JB, Cheng C, Djebali S, Dong X, Dunham I, Ernst J, Furey TS, Gerstein M, Giardine B, Greven M, Hardison RC, Harris RS, Herrero J, Hoffman MM, Iyer S, Kellis M, Khatun J, Kheradpour P, Kundaje A, Lassmann T, Li Q, Lin X, Marinov GK, Merkel A, Mortazavi A, Parker SCJ, Reddy TE, Rozowsky J, Schlesinger F, Thurman RE, Wang J, Ward LD, Whitfield TW, Wilder SP, Wu W, Xi HS, Yip KY, Zhuang J, Bernstein BE, Birney E, Dunham I, Green ED, Gunter C, Snyder M, Pazin MJ, Lowdon RF, Dillon LaL, Adams LB, Kelly CJ, Zhang J, Wexler JR, Green ED, Good PJ, Feingold Ea, Bernstein BE, Birney E, Crawford GE, Dekker J, Elnitski L, Farnham PJ, Gerstein M, Giddings MC, Gingeras TR, Green ED, Guigó R, Hardison RC, Hubbard TJ, Kellis M, Kent WJ, Lieb JD, Margulies EH, Myers RM, Snyder M, Stamatoyannopoulos Ja, Tenenbaum Sa, Weng Z, White KP, Wold B, Khatun J, Yu Y, Wrobel J, Risk Ba, Gunawardena HP, Kuiper HC, Maier CW, Xie L, Chen X, Giddings MC, Bernstein BE, Epstein CB, Shores N, Ernst J, Kheradpour P, Mikkelsen TS, Gillespie S, Goren A, Ram O, Zhang X, Wang L, Issner R, Coyne MJ, Durham T, Ku M, Truong T, Ward LD, Altshuler RC, Eaton ML, Kellis M, Djebali S, Davis Ca, Merkel A, Dobin A, Lassmann T, Mortazavi A, Tanzer A, Lagarde J, Lin W, Schlesinger F, Xue C, Marinov GK, Khatun J, Williams Ba, Zaleski C, Rozowsky J, Röder M, Kokocinski F, Abdelhamid RF, Alioto T, Antoshechkin I, Baer MT, Batut P, Bell I, Bell K, Chakraborty S, Chen X, Chrast J, Curado J, Derrien T, Drenkow J, Dumais E, Dumais J, Dutttagupta R, Fastuca M, Fejes-Toth K, Ferreira P, Foissac S, Fullwood MJ, Gao H, Gonzalez D, Gordon A, Gunawardena HP, Howald C, Jha S, Johnson R, Kapranov P, King B, Kingswood C, Li G, Luo OJ, Park E, Preall JB, Presaud K, Ribeca P, Risk Ba, Robyr D, Ruan X, Sammeth M, Sandhu KS, Schaeffer L, See L-H, Shahab A, Skancke J, Suzuki AM, Takahashi H, Tilgner H, Trout D, Walters N, Wang H, Wrobel J, Yu Y, Hayashizaki Y, Harrow J, Gerstein M, Hubbard TJ, Reymond A, Antonarakis SE, Hannon GJ, Giddings MC, Ruan Y, Wold B, Carninci P, Guigó R, Gingeras TR, Rosenbloom KR, Sloan Ca, Learned K, Malladi VS, Wong MC, Barber GP, Cline MS, Dreszer TR, Heitner SG, Karolchik D, Kent WJ, Kirkup VM, Meyer LR, Long JC, Maddren M, Raney BJ, Furey TS, Song L, Grassegger LL, Giresi PG, Lee B-K, Battenhouse A, Sheffield NC, Simon JM, Showers Ka, Safi A, London D, Bhinge Aa, Shestak C, Schaner MR, Kim SKi, Zhang ZZ, Mieczkowski Pa, Mieczkowska JO, Liu Z, McDaniell RM, Ni Y, Rashid NU, Kim MJ, Adar S, Zhang Z, Wang T, Winter D, Keefe D, Birney E, Iyer VR, Lieb JD, Crawford GE, Li G, Sandhu KS, Zheng M, Wang P, Luo OJ, Shahab A, Fullwood MJ, Ruan X, Ruan Y, Myers RM, Pauli F, Williams Ba, Gertz J, Marinov GK, Reddy TE, Vielmetter J, Partridge E, Trout D, Varley KE, Gasper C, Bansal A, Pepke S, Jain P, Amrhein H, Bowling KM, Anaya M, Cross MK, King B, Muratet Ma, Antoshechkin I, Newberry KM, McCue K, Nesmith AS, Fisher-Aylor KI, Pusey B, DeSalvo G, Parker SL, Balasubramanian S, Davis NS, Meadows SK, Eggleston T, Gunter C, Newberry JS, Levy SE, Absher DM, Mortazavi A, Wong WH, Wold B,

Blow MJ, Visel A, Pennachio La, Elnitski L, Margulies EH, Parker SCJ, Petrykowska HM, Abyzov A, Aken B, Barrell D, Barson G, Berry A, Bignell A, Boychenko V, Bussotti G, Chrast J, Davidson C, Derrien T, Despacio-Reyes G, Diekhans M, Ezkurdia I, Frankish A, Gilbert J, Gonzalez JM, Griffiths E, Harte R, Hendrix Da, Howald C, Hunt T, Jungreis I, Kay M, Khurana E, Kokocinski F, Leng J, Lin MF, Loveland J, Lu Z, Manthavadi D, Mariotti M, Mudge J, Mukherjee G, Notredame C, Pei B, Rodriguez JM, Saunders G, Sboner A, Searle S, Sisu C, Snow C, Steward C, Tanzer A, Tapanari E, Tress ML, van Baren MJ, Walters N, Washietl S, Wilming L, Zadissa A, Zhang Z, Brent M, Haussler D, Kellis M, Valencia A, Gerstein M, Reymond A, Guigo R, Harrow J, Hubbard TJ, Landt SG, Fietze S, Abyzov A, Addleman N, Alexander RP, Auerbach RK, Balasubramanian S, Bettinger K, Bhardwaj N, Boyle AP, Cao AR, Cayting P, Charos A, Cheng Y, Cheng C, Eastman C, Euskirchen G, Fleming JD, Grubert F, Habegger L, Hariharan M, Harmanci A, Iyengar S, Jin VX, Karczewski KJ, Kasowski M, Lacroute P, Lam H, Lamarre-Vincent N, Leng J, Lian J, Lindahl-Allen M, Min R, Miotto B, Monahan H, Moqtaderi Z, Mu XJ, O'Geen H, Ouyang Z, Patacsil D, Pei B, Raha D, Ramirez L, Reed B, Rozowsky J, Sboner A, Shi M, Sisu C, Slifer T, Witt H, Wu L, Xu X, Yan K-K, Yang X, Yip KY, Zhang Z, Struhl K, Weissman SM, Gerstein M, Farnham PJ, Snyder M, Tenenbaum Sa, Penalva LO, Doyle F, Karmakar S, Landt SG, Bhanvadia RR, Choudhury A, Domanus M, Ma L, Moran J, Patacsil D, Slifer T, Victorsen A, Yang X, Snyder M, White KP, Auer T, Centanin L, Eichenlaub M, Gruhl F, Heermann S, Hoeckendorf B, Inoue D, Kellner T, Kirchmaier S, Mueller C, Reinhardt R, Schertel L, Schneider S, Sinn R, Wittbrodt B, Wittbrodt J, Weng Z, Whitfield TW, Wang J, Collins PJ, Aldred SF, Trinklein ND, Partridge EC, Myers RM, Dekker J, Jain G, Lajoie BR, Sanyal A, Balasundaram G, Bates DL, Byron R, Canfield TK, Diegel MJ, Dunn D, Ebersol AK, Frum T, Garg K, Gist E, Hansen RS, Boatman L, Haugen E, Humbert R, Jain G, Johnson AK, Johnson EM, Kutayavin TV, Lajoie BR, Lee K, Lotakis D, Maurano MT, Neph SJ, Neri FV, Nguyen ED, Qu H, Reynolds AP, Roach V, Rynes E, Sabo P, Sanchez ME, Sandstrom RS, Sanyal A, Shafer AO, Stergachis AB, Thomas S, Thurman RE, Vernot B, Vierstra J, Vong S, Wang H, Weaver Ma, Yan Y, Zhang M, Akey JM, Bender M, Dorschner MO, Groudine M, MacCoss MJ, Navas P, Stamatoyannopoulos G, Kaul R, Dekker J, Stamatoyannopoulos Ja, Dunham I, Beal K, Brazma A, Flicek P, Herrero J, Johnson N, Keefe D, Lusk M, Luscombe NM, Sobral D, Vaquerizas JM, Wilder SP, Batzoglou S, Sidow A, Hussami N, Kyriazopoulou-Panagiotopoulou S, Libbrecht MW, Schaub Ma, Kundaje A, Hardison RC, Miller W, Giardine B, Harris RS, Wu W, Bickel PJ, Banfai B, Boley NP, Brown JB, Huang H, Li Q, Li JJ, Noble WS, Bilmes Ja, Buske OJ, Hoffman MM, Sahu AD, Kharchenko PV, Park PJ, Baker D, Taylor J, Weng Z, Iyer S, Dong X, Greven M, Lin X, Wang J, Xi HS, Zhuang J, Gerstein M, Alexander RP, Balasubramanian S, Cheng C, Harmanci A, Loehovsky L, Min R, Mu XJ, Rozowsky J, Yan K-K, Yip KY, Birney E. An integrated encyclopedia of DNA elements in the human genome. *Nature*. 2012; 489:57–74. [PubMed: 22955616]

5. Haussler M, Joly JS. When needles look like hay: How to find tissue-specific enhancers in model organism genomes. *Dev Biol*. 2011; 350:239–254. [PubMed: 21130761]
6. Patwardhan RP, Hiatt JB, Witten DM, Kim MJ, Smith RP, May D, Lee C, Andrie JM, Lee S-I, Cooper GM, Ahituv N, Pennachio La, Shendure J. Massively parallel functional dissection of mammalian enhancers in vivo. *Nat Biotechnol*. 2012; 30:265–270. [PubMed: 22371081]
7. Melnikov A, Murugan A, Zhang X, Tesileanu T, Wang L, Rogov P, Feizi S, Gnirke A, Callan CG, Kinney JB, Kellis M, Lander ES, Mikkelsen TS. Systematic dissection and optimization of inducible enhancers in human cells using a massively parallel reporter assay. *Nat Biotechnol*. 2012; 30:271–277. [PubMed: 22371084]
8. Ulirsch JC, Nandakumar SK, Wang L, Do R, Mikkelsen TS, Sankaran VG. Systematic Functional Dissection of Common Genetic Variation Affecting Red Blood Cell Traits. *Cell*. 2016; 165:1530–1545. [PubMed: 27259154]
9. Banerji J, Rusconi S, Schaffner W. Expression of a (β -globin gene is enhanced by remote SV40 DNA sequences. *Cell*. 1981; 27:299–308. [PubMed: 6277502]
10. Joung JK, Sander JD. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol*. 2013; 14:49–55. [PubMed: 23169466]
11. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet*. 2010; 11:636–46. [PubMed: 20717154]

12. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013; 339:819–23. [PubMed: 23287718]
13. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. *Science*. 2013; 339:823–6. [PubMed: 23287722]
14. Hsu PD, Lander ES, Zhang F. Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell*. 2014; 157:1262–1278. [PubMed: 24906146]
15. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol*. 2014; 32:347–55. [PubMed: 24584096]
16. Nowak CM, Lawson S, Zerez M, Bleris L. Guide RNA engineering for versatile Cas9 functionality. *Nucleic Acids Res*. 2016:gkw908.
17. Wang X, Huang X, Fang X, Zhang Y, Wang W. CRISPR-Cas9 System as a Versatile Tool for Genome Engineering in Human Cells. *Mol Ther Acids*. 2016; 5:e388.
18. Komor AC, Badran AH, Liu DR. CRISPR-Based Technologies for the Manipulation of Eukaryotic Genomes. *Cell*. 2017:1–17.
19. Barrangou R, Doudna JA. Applications of CRISPR technologies in research and beyond. *Nat Biotechnol*. 2016; 34:933–941. [PubMed: 27606440]
20. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. CRISPR provides acquired resistance against viruses in prokaryotes. *Science*. 2007; 315:1709–1712. [PubMed: 17379808]
21. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales AP, Li Z, Peterson RT, Yeh JR, Aryee MJ, Joung JK. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature*. 2015; 523:481–485. [PubMed: 26098369]
22. Anders C, Niewoehner O, Duerst A, Jinek M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature*. 2014; 513:569–73. [PubMed: 25079318]
23. Hirano H, Gootenberg JS, Horii T, Abudayyeh OO, Kimura M, Hsu PD, Nakane T, Ishitani R, Hatada I, Zhang F, Nishimasu H, Nureki O. Structure and Engineering of Francisella novicida Cas9. *Cell*. 2016; 164:950–961. [PubMed: 26875867]
24. Kleinstiver BP, Prew MS, Tsai SQ, Nguyen NT, Topkar VV, Zheng Z, Joung JK. Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition. *Nat Biotechnol*. 2015; 33:1293–1298. [PubMed: 26524662]
25. Esvelt KM, Mali P, Braff JL, Moosburner M, Yaung SJ, Church GM. Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nat Methods*. 2013; 10:1116–21. [PubMed: 24076762]
26. Karvelis T, Gasiunas G, Young J, Bigelyte G, Silanskas A, Cigan M, Siksnys V. Rapid characterization of CRISPR-Cas9 protospacer adjacent motif sequence elements. *Genome Biol*. 2015; 16:253. [PubMed: 26585795]
27. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F. In vivo genome editing using Staphylococcus aureus Cas9. *Nature*. 2015; 520:186–91. [PubMed: 25830891]
28. Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, Minakhin L, Joung J, Konermann S, Severinov K, Zhang F, Koonin EV. Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. *Mol Cell*. 2015; 60:385–397. [PubMed: 26593719]
29. Zetsche B, Gootenberg JS, Abudayyeh OO, Regev A, Koonin EV, Zhang F, Pam T, Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV, Zhang F. Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. *Cell*. 2015; 163:759–71. [PubMed: 26422227]
30. Yang H, Gao P, Rajashankar KR, Patel DJ. PAM-Dependent Target DNA Recognition and Cleavage by C2c1 CRISPR-Cas Endonuclease. *Cell*. 2016; 167:1814–1828.e12. [PubMed: 27984729]
31. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*. 2013; 8:2281–308. [PubMed: 24157548]
32. Lopes R, Korkmaz G, Agami R. Applying CRISPR-Cas9 tools to identify and characterize transcriptional enhancers. *Nat Rev Mol Cell Biol*. 2016; 17:597–604. [PubMed: 27381243]

33. Canver MC, Bauer DE, Dass A, Yien YY, Chung J, Masuda T, Maeda T, Paw BH, Orkin SH. Characterization of Genomic Deletion Efficiency Mediated by Clusted Regularly Interspaced Palindromic Repeats (CRISPR)/Cas9 Nuclease System in Mammalian Cells. *J Biol Chem*. 2014; 289:21312–21324. [PubMed: 24907273]
34. Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell*. 2013; 154:1380–9. [PubMed: 23992846]
35. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol*. 2013; 31:827–32. [PubMed: 23873081]
36. Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*. 2013; 154:442–51. [PubMed: 23849981]
37. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA. Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression. *Cell*. 2013; 152:1173–1183. [PubMed: 23452860]
38. Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS, Qi LS. CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat Protoc*. 2013; 8:2180–96. [PubMed: 24136345]
39. Gao X, Tsang JCH, Gaba F, Wu D, Lu L, Liu P. Comparison of TALE designer transcription factors and the CRISPR/dCas9 in regulation of gene expression by targeting enhancers. *Nucleic Acids Res*. 2014; 42
40. Chen B, Gilbert LA, Cimini Ba, Schnitzbauer J, Zhang W, Li G-W, Park J, Blackburn EH, Weissman JS, Qi LS, Huang B. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell*. 2013; 155:1479–91. [PubMed: 24360272]
41. Kearns NA, Pham H, Tabak B, Genga RM, Silverstein NJ, Garber M, Maehr R. Functional annotation of native enhancers with a Cas9-histone demethylase fusion. *Nat Methods*. 2015; 12:401–403. [PubMed: 25775043]
42. Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott Da, Church GM, Zhang F. Optical control of mammalian endogenous transcription and epigenetic states. *Nature*. 2013; 500:472–6. [PubMed: 23877069]
43. Vierstra J, Reik A, Chang K-H, Stehling-Sun S, Zhou Y, Hinkley SJ, Paschon DE, Zhang L, Psatha N, Bendana YR, O'Neil CM, Song AH, Mich AK, Liu P-Q, Lee G, Bauer DE, Holmes MC, Orkin SH, Papayannopoulou T, Stamatoyannopoulos G, Rebar EJ, Gregory PD, Urnov FD, Stamatoyannopoulos Ja. Functional footprinting of regulatory DNA. *Nat Methods*. 2015; 12:927–930. [PubMed: 26322838]
44. Neph S, Vierstra J, Stergachis AB, Reynolds AP, Haugen E, Vernot B, Thurman RE, John S, Sandstrom R, Johnson AK, Maurano MT, Humbert R, Rynes E, Wang H, Vong S, Lee K, Bates D, Diegel M, Roach V, Dunn D, Neri J, Schafer A, Hansen RS, Kutayavin T, Giste E, Weaver M, Canfield T, Sabo P, Zhang M, Balasundaram G, Byron R, MacCoss MJ, Akey JM, Bender MA, Groudine M, Kaul R, Stamatoyannopoulos JA. An expansive human regulatory lexicon encoded in transcription factor footprints. *Nature*. 2012; 489:83–90. [PubMed: 22955618]
45. Mansour MR, Abraham BJ, Anders L, Berezovskaya A, Gutierrez A, Durbin AD, Etchin J, Lawton L, Sallan SE, Silverman LB, Loh ML, Hunger SP, Sanda T, Young RA, Look AT. An oncogenic super-enhancer formed through somatic mutation of a noncoding intergenic element. *Science*. 2014; 12:1373–1377.
46. Flavahan WA, Drier Y, Liao BB, Gillespie SM, Venteicher AS, Stemmer-Rachamimov AO, Suva ML, Bernstein BE. Insulator dysfunction and oncogene activation in IDH mutant gliomas. *Nature*. 2016; 529:110–114. [PubMed: 26700815]
47. Narendra V, Rocha PP, An D, Raviram R, Skok JA, Mazzoni EO, Reinberg D. CTCF establishes discrete functional chromatin domains at the Hox clusters during differentiation. *Science*. 2015; 347:1017–21. [PubMed: 25722416]
48. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*. 2013; 153:910–8. [PubMed: 23643243]

49. Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell*. 2013; 154:1370–9. [PubMed: 23992847]
50. Kim Y, Cheong SA, Lee JG, Lee SW, Lee MS, Baek IJ, Sung YH. Generation of knockout mice by Cpf1-mediated gene targeting. *Nat Biotechnol*. 2016; 34:808–810. [PubMed: 27272387]
51. Hur JK, Kim K, Been KW, Baek G, Ye S, Hur JW, Ryu SM, Lee YS, Kim JS. Targeted mutagenesis in mice by electroporation of Cpf1 ribonucleoproteins. *Nat Biotechnol*. 2016; 34:807–808. [PubMed: 27272385]
52. Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, DeGennaro EM, Winblad N, Choudhury SR, Abudayyeh OO, Gootenberg JS, Wu WY, Scott DA, Severinov K, van der Oost J, Zhang F. Multiplex gene editing by CRISPR—Cpf1 using a single crRNA array. *Nat Biotechnol*. 2016
53. Rajagopal N, Srinivasan S, Kooshesh K, Guo Y, Edwards MD, Banerjee B, Syed T, Emons BJM, Gifford DK, Sherwood RI. High-throughput mapping of regulatory DNA. *Nat Biotechnol*. 2016; 34:167–174. [PubMed: 26807528]
54. Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol*. 2014; 32:279–284. [PubMed: 24463574]
55. Bauer DE, Canver MC, Orkin SH. Generation of Genomic Deletions in Mammalian Cell Lines via CRISPR/Cas9. *J Vis Exp*. 2015; 95:1–9.
56. Essletzbichler P, Konopka T, Santoro F, Chen D, Gapp BV, Kralovics R, Brummelkamp TR, Nijman SMB, Burckstummer T. Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line. *Genome Res*. 2014; 24:2059–65. [PubMed: 25373145]
57. Wettstein R, Bodak M, Ciaudo C. Generation of a Knockout Mouse Embryonic Stem Cell Line Using a Paired CRISPR/Cas9 Genome Engineering Tool. *Methods Mol Biol*. 2015
58. Aparicio-Prat E, Arnan C, Sala I, Bosch N, Guigo R, Johnson R. DECKO: Single-oligo, dual-CRISPR deletion of genomic elements including long non-coding RNAs. *BMC Genomics*. 2015; 16:846. [PubMed: 26493208]
59. Wolfs JM, Hamilton TA, Lant JT, Laforet M, Zhang J, Salemi LM, Gloor GB, Schild-Poulter C, Edgell DR. Biasing genome-editing events toward precise length deletions with an RNA-guided TevCas9 dual nuclease. *Proc Natl Acad Sci*. 2016
60. Tabebordbar M, Zhu K, Cheng JKW, Chew WL, Widrick JJ, Yan WX, Maesner C, Wu EY, Xiao R, Ran FA, Cong L, Zhang F, Vandenberghe LH, Church GM, Wagers AJ. In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science*. 2016; 351:407–11. [PubMed: 26721686]
61. Nelson C, Hakim C, Ousterout D, Thakore P, Moreb E, Castellanos Rivera R, Madhavan S, Pan X, Ran F, Yan W, Asokan A, Zhang F, Duan D, Gersbach C. In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science*. 2016; 351:403–7. [PubMed: 26721684]
62. Long C, Amoasii L, Mireault AA, Mcanally JR, Li H, Sanchez-Ortiz E, Bhattacharyya S, Shelton JM, Bassel-Duby R, Olson EN. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science*. 2016; 351:400–3. [PubMed: 26721683]
63. Canver MC, Smith EC, Sher F, Pinello L, Sanjana NE, Shalem O, Chen DD, Schupp PG, Vinjamur DS, Garcia SP, Luc S, Kurita R, Nakamura Y, Fujiwara Y, Maeda T, Yuan GC, Zhang F, Orkin SH, Bauer DE. BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. *Nature*. 2015; 527:192–7. [PubMed: 26375006]
64. Hnisz D, Schuijers J, Lin CY, Weintraub AS, Abraham BJ, Lee TI, Bradner JE, Young RA. Convergence of Developmental and Oncogenic Signaling Pathways at Transcriptional Super-Enhancers. *Mol Cell*. 2015; 58:362–370. [PubMed: 25801169]
65. Zhang X, Choi PS, Francis JM, Imielinski M, Watanabe H, Cherniack AD, Meyerson M. Identification of focally amplified lineage-specific super-enhancers in human epithelial cancers. *Nat Genet*. 2015; 48:1–8.
66. Andrey G, Kraft K, Geuer S, Will AJ, Chan WL, Paliou C, Borschiwer M. Deletions, Inversions, Duplications: Engineering of Structural Variants using CRISPR/Cas in Mice. *Cell Rep*. 2015; 10:833–839.

67. Xiao A, Wang Z, Hu Y, Wu Y, Luo Z, Yang Z, Zu Y, Li W, Huang P, Tong X, Zhu Z, Lin S, Zhang B. Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic Acids Res.* 2013; 41:e141. [PubMed: 23748566]
68. Li J, Shou J, Guo Y, Tang Y, Wu Y, Jia Z, Zhai Y, Chen Z, Xu Q, Wu Q. Efficient inversions and duplications of mammalian regulatory DNA elements and gene clusters by CRISPR/Cas9. *J Mol Cell Biol.* 2015; 7:284–98. [PubMed: 25757625]
69. Seruggia D, Fernandez A, Cantero M, Pelczar P, Montoliu L. Functional validation of mouse tyrosinase non-coding regulatory DNA elements by CRISPR-Cas9-mediated mutagenesis. *Nucleic Acids Res.* 2015; 43:4855–4867. [PubMed: 25897126]
70. Guo Y, Xu Q, Canzio D, Shou J, Li J, Gorkin DU, Jung I, Wu H, Zhai Y, Tang Y, Lu Y, Wu Y, Jia Z, Li W, Zhang MQ, Ren B, Krainer AR, Maniatis T, Wu Q. CRISPR Inversion of CTCF Sites Alters Genome Topology and Enhancer/Promoter Function. *Cell.* 2015; 162:900–910. [PubMed: 26276636]
71. Zhang L, Jia R, Palange NJ, Satheka AC, Togo J, An Y, Humphrey M, Ban L, Ji Y, Jin H, Feng X, Zheng Y. Large Genomic Fragment Deletions and Insertions in Mouse Using CRISPR/Cas9. *PLoS One.* 2015; 10:e0120396. [PubMed: 25803037]
72. Wang L, Shao Y, Guan Y, Li L, Wu L, Chen F, Liu M, Chen H, Ma Y, Ma X, Liu M, Li D. Large genomic fragment deletion and functional gene cassette knock-in via Cas9 protein mediated genome editing in one-cell rodent embryos. *Sci Rep.* 2015; 5:17517. [PubMed: 26620761]
73. Brandl C, Ortiz O, Röttig B, Wefers B, Wurst W, Kühn R. Creation of targeted genomic deletions using TALEN or CRISPR/Cas nuclease pairs in one-cell mouse embryos. *FEBS Open Bio.* 2015; 5:26–35.
74. Zhou J, Wang J, Shen B, Chen L, Su Y, Yang J, Zhang W, Tian X, Huang X. Dual sgRNAs facilitate CRISPR/Cas9-mediated mouse genome targeting. *FEBS J.* 2014; 281:1717–1725. [PubMed: 24494965]
75. Li Y, Park AI, Mou H, Colpan C, Bizhanova A, Akama-Garren E, Joshi N, Hendrickson Ea, Feldser D, Yin H, Anderson DG, Jacks T, Weng Z, Xue W. A versatile reporter system for CRISPR-mediated chromosomal rearrangements. *Genome Biol.* 2015; 16:111. [PubMed: 26018130]
76. Chen X, Xu F, Zhu C, Ji J, Zhou X, Feng X, Guang S. Dual sgRNA-directed gene knockout using CRISPR/Cas9 technology in *Caenorhabditis elegans*. *Sci Rep.* 2014; 4:7581. [PubMed: 25531445]
77. Smith EC, Luc S, Croney DM, Woodworth MB, Greig LC, Fujiwara Y, Nguyen M, Sher F, Macklis JD, Bauer DE, Orkin SH. Strict in vivo specificity of the Bcl11a erythroid enhancer. *Blood.* 2016; 128:1–4. [PubMed: 27389536]
78. Lupiáñez DG, Kraft K, Heinrich V, Krawitz P, Brancati F, Klopocki E, Horn D, Kayserili H, Opitz JM, Laxova R, Santos-Simarro F, Gilbert-Dussardier B, Wittler L, Borschiwer M, Haas SA, Osterwalder M, Franke M, Timmermann B, Hecht J, Spielmann M, Visel A, Mundlos S. Disruptions of topological chromatin domains cause pathogenic rewiring of gene-enhancer interactions. *Cell.* 2015; 161:1012–1025. [PubMed: 25959774]
79. Blasco RB, Karaca E, Ambrogio C, Cheong TC, Karayol E, Minero VG, Voena C, Chiarle R. Simple and Rapid In Vivo Generation of Chromosomal Rearrangements using CRISPR/Cas9 Technology. *Cell Rep.* 2014; 9:1219–1227. [PubMed: 25456124]
80. Choi PS, Meyerson M. Targeted genomic rearrangements using CRISPR/Cas technology. *Nat Commun.* 2014; 5:3728. [PubMed: 24759083]
81. Maddalo D, Manchado E, Concepcion CP, Bonetti C, Vidigal Ja, Han Y-C, Ogrodowski P, Crippa A, Rekhman N, de Stanchina E, Lowe SW, Ventura A. In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. *Nature.* 2014; 516:423–7. [PubMed: 25337876]
82. Lekontsev S, Aligianni S, Lapao A, Bürckstummer T. Efficient generation and reversion of chromosomal translocations using CRISPR/Cas technology. *BMC Genomics.* 2016; 17:739. [PubMed: 27640184]
83. Drier Y, Cotton MJ, Williamson KE, Gillespie SM, Ryan RJH, Kluk MJ, Carey CD, Rodig SJ, Sholl LM, Afrogheh AH, Faquin WC, Queimado L, Qi J, Wick MJ, El-Naggar AK, Bradner JE, Moskaluk CA, Aster JC, Knoechel B, Bernstein BE. An oncogenic MYB feedback loop drives

alternate cell fates in adenoid cystic carcinoma. *Nat Genet.* 2016; 48:265–272. [PubMed: 26829750]

84. Bandopadhyay P, Ramkissoon LA, Jain P, Bergthold G, Wala J, Zeid R, Schumacher SE, Urbanski L, O'Rourke R, Gibson WJ, Pelton K, Ramkissoon SH, Han HJ, Zhu Y, Choudhari N, Silva A, Boucher K, Henn RE, Kang YJ, Knoff D, Paoletta BR, Gladden-Young A, Varlet P, Pages M, Horowitz PM, Federation A, Malkin H, Tracy AA, Seepo S, Ducar M, Van Hummelen P, Santi M, Buccoliero AM, Scagnet M, Bowers DC, Giannini C, Puget S, Hawkins C, Tabori U, Klekner A, Bognar L, Burger PC, Eberhart C, Rodriguez FJ, Hill DA, Mueller S, Haas-Kogan DA, Phillips JJ, Santagata S, Stiles CD, Bradner JE, Jabado N, Goren A, Grill J, Ligon AH, Goumnerova L, Waanders AJ, Storm PB, Kieran MW, Ligon KL, Beroukheim R, Resnick AC. MYB-QKI rearrangements in angiocentric glioma drive tumorigenicity through a tripartite mechanism. *Nat Genet.* 2016; 48:273–282. [PubMed: 26829751]
85. Vidigal, Ja, Ventura, A. Rapid and efficient one-step generation of paired gRNA CRISPR-Cas9 libraries. *Nat Commun.* 2015; 6:8083. [PubMed: 26278926]
86. Wong ASL, Choi GCG, Cui CH, Pregonig G, Milani P, Adam M, Perli SD, Kazer SW, Gaillard A, Hermann M, Shalek AK, Fraenkel E, Lu TK. Multiplexed barcoded CRISPR-Cas9 screening enabled by CombiGEM. *Proc Natl Acad Sci U S A.* 2016; 113:2544–9. [PubMed: 26864203]
87. Zhu S, Li W, Liu J, Chen C-H, Liao Q, Xu P, Xu H, Xiao T, Cao Z, Peng J, Yuan P, Brown M, Shirley Liu X, Wei W. Genome-scale deletion screening of human long non-coding RNAs using a paired-guide RNA CRISPR-Cas9 library. *Nat Biotechnol.* 2016; 34:1279–1286. [PubMed: 27798563]
88. Miles LA, Garippa RJ, Poirier JT. Design, execution, and analysis of pooled in vitro CRISPR/Cas9 screens. *FEBS J.* 2016; 283:3170–80. [PubMed: 27250066]
89. Peng J, Zhou Y, Zhu S, Wei W. High-throughput screens in mammalian cells using the CRISPR-Cas9 system. *FEBS J.* 2015; 282:2089–2096. [PubMed: 25731961]
90. Sanjana NE. Genome-scale CRISPR pooled screens. *Anal Biochem.* 2016; S0003–2697:30089–6.
91. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen TS, Heckl D, Ebert BL, Root DE, Doench JG, Zhang F. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science.* 2014; 343:84–7. [PubMed: 24336571]
92. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods.* 2014; 11:783–784. [PubMed: 25075903]
93. Zhou Y, Zhu S, Cai C, Yuan P, Li C, Huang Y, Wei W. High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. *Nature.* 2014; 509:487–91. [PubMed: 24717434]
94. Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, Smith I, Tothova Z, Wilen C, Orchard R, Virgin HW, Listgarten J, Root DE. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol.* 2016; 34:184–91. [PubMed: 26780180]
95. Chen S, Sanjana NE, Zheng K, Shalem O, Lee K, Shi X, Scott DA, Song J, Pan JQ, Weissleder R, Lee H, Zhang F, Sharp PA. Genome-wide CRISPR Screen in a Mouse Model of Tumor Growth and Metastasis. *Cell.* 2015; 160:1246–60. [PubMed: 25748654]
96. Parnas O, Jovanovic M, Eisenhaure TM, Herbst RH, Dixit A, Ye CJ, Przybylski D, Platt RJ, Tirosh I, Sanjana NE, Shalem O, Satija R, Raychowdhury R, Mertins P, Carr SA, Zhang F, Hacohen N, Regev A. A Genome-wide CRISPR Screen in Primary Immune Cells to Dissect Regulatory Networks. *Cell.* 2015; 162:675–686. [PubMed: 26189680]
97. Koike-Yusa H, Li Y, Tan EP, Velasco-Herrera MDC, Yusa K. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol.* 2013
98. Wang T, Wei JJ, Sabatini DM, Lander ES. Genetic screens in human cells using the CRISPR-Cas9 system. *Science.* 2014; 343:80–4. [PubMed: 24336569]
99. Shalem O, Sanjana NE, Zhang F. High-throughput functional genomics using CRISPR-Cas9. *Nat Rev Genet.* 2015
100. DeJesus R, Moretti F, McAllister G, Wang Z, Bergman P, Liu S, Frias E, Alford J, Reece-Hoyes JS, Lindeman A, Kelliher J, Russ C, Knehr J, Carbone W, Beibel M, Roma G, Ng A, Tallarico

- JA, Porter JA, Xavier RJ, Mickanin C, Murphy LO, Hoffman GR, Nyfeler B. Functional CRISPR screening identifies the ufm1ylation pathway as a regulator of SQSTM1/p62. *Elife*. 2016; 5:1–16.
101. Zhang R, Miner JJ, Gorman MJ, Rausch K, Ramage H, White JP, Zuiani A, Zhang P, Fernandez E, Zhang Q, Dowd KA, Pierson TC, Cherry S, Diamond MS. A CRISPR screen defines a signal peptide processing pathway required by flaviviruses. *Nature*. 2016; 535:164–168. [PubMed: 27383988]
 102. Marceau CD, Puschnik AS, Majzoub K, Ooi YS, Brewer SM, Fuchs G, Swaminathan K, Mata MA, Elias JE, Sarnow P, Carette JE. Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens. *Nature*. 2016; 535:159–163. [PubMed: 27383987]
 103. Wallace J, Hu R, Mosbrugger TL, Dahlem TJ, Stephens WZ, Rao DS, Round JL, O'Connell RM. Genome-Wide CRISPR-Cas9 Screen Identifies MicroRNAs That Regulate Myeloid Leukemia Cell Growth. *PLoS One*. 2016; 11:e0153689. [PubMed: 27081855]
 104. Ruiz S, Mayor-Ruiz C, Lafarga V, Murga M, Vega-Sendino M, Ortega S, Fernandez-Capetillo O. A Genome-wide CRISPR Screen Identifies CDC25A as a Determinant of Sensitivity to ATR Inhibitors. *Mol Cell*. 2016; 62:307–313. [PubMed: 27067599]
 105. Wang T, Birsoy K, Hughes NW, Krupczak KM, Post Y, Wei JJ, Lander ES, Sabatini DM. Identification and characterization of essential genes in the human genome. *Science*. 2015; 350:1096–101. [PubMed: 26472758]
 106. Shi J, Wang E, Milazzo JP, Wang Z, Kinney JB, Vakoc CR. Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. *Nat Biotechnol*. 2015; 33:661–7. [PubMed: 25961408]
 107. Schmid-Burgk JL, Chauhan D, Schmidt T, Ebert TS, Reinhardt J, Endl E, Hornung V. A genome-wide CRISPR (clustered regularly interspaced short palindromic repeats) screen identifies NEK7 as an essential component of NLRP3 inflammasome activation. *J Biol Chem*. 2016; 291:103–109. [PubMed: 26553871]
 108. Kiessling MK, Schuierer S, Stertz S, Beibel M, Bergling S, Knehr J, Carbone W, de Valliere C, Tchinda J, Bouwmeester T, Seuwen K, Rogler G, Roma G. Identification of oncogenic driver mutations by genome-wide CRISPR-Cas9 dropout screening. *BMC Genomics*. 2016; 17:723. [PubMed: 27613601]
 109. Hart T, Chandrashekhar M, Aregger M, Steinhart Z, Brown KR, MacLeod G, Mis M, Zimmermann M, Fradet-Turcotte A, Sun S, Mero P, Dirks P, Sidhu S, Roth FP, Rissland OS, Durocher D, Angers S, Moffat J. High-Resolution CRISPR Screens Reveal Fitness Genes and Genotype-Specific Cancer Liabilities. *Cell*. 2015; 163:1515–1526. [PubMed: 26627737]
 110. Arroyo JD, Jourdain AA, Calvo SE, Ballarano CA, Doench JG, Root DE, Mootha VK. A Genome-wide CRISPR Death Screen Identifies Genes Essential for Oxidative Phosphorylation. *Cell Metab*. 2016; S1550–4131:30433–8.
 111. Tzelepis K, Koike-Yusa H, De Braekeleer E, Li Y, Metzakopian E, Dovey OM, Mupo A, Grinkevich V, Li M, Mazan M, Gozdecka M, Ohnishi S, Cooper J, Patel M, McKerrill T, Chen B, Domingues AF, Gallipoli P, Teichmann S, Ponstingl H, McDermott U, Saez-Rodriguez J, Huntly BJP, Iorio F, Pina C, Vassiliou GS, Yusa K. A CRISPR Dropout Screen Identifies Genetic Vulnerabilities and Therapeutic Targets in Acute Myeloid Leukemia. *Cell Rep*. 2016; 17:1193–1205. [PubMed: 27760321]
 112. Steinhart Z, Pavlovic Z, Chandrashekhar M, Hart T, Wang X, Zhang X, Robitaille M, Brown KR, Jaksani S, Overmeer R, Boj SF, Adams J, Pan J, Clevers H, Sidhu S, Moffat J, Angers S. Genome-wide CRISPR screens reveal a Wnt-FZD5 signaling circuit as a druggable vulnerability of RNF43-mutant pancreatic tumors. *Nat Med*. 2016
 113. Park RJ, Wang T, Koundakjian D, Hultquist JF, Lamothe-Molina P, Monel B, Schumann K, Yu H, Krupczak KM, Garcia-Beltran W, Piechocka-Trocha A, Krogan NJ, Marson A, Sabatini DM, Lander ES, Hacohen N, Walker BD. A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors. *Nat Genet*. 2016; 49:193–203. [PubMed: 27992415]
 114. Sen DR, Kaminski J, Barnitz RA, Kurachi M, Gerdemann U, Yates KB, Tsao HW, Godec J, LaFleur MW, Brown FD, Tonnerre P, Chung RT, Tully DC, Allen TM, Frahm N, Lauer GM, Wherry EJ, Yosef N, Haining WN. The epigenetic landscape of T cell exhaustion. *Science*. 2016

115. Sanjana N, Wright J, Zheng K, Shalem O, Fontanillas P, Joung J, Cheng C, Regev A, Zhang F. High-resolution interrogation of functional elements in the noncoding genome. *Science*. 2016; 353:1545–1549. [PubMed: 27708104]
116. Diao Y, Li B, Meng Z, Jung I, Lee AY, Dixon J. A New Class of Temporarily Phenotypic Enhancers Identified by CRISPR/Cas9 Mediated Genetic Screening. *Genome Res*. 2016; 26:397–405. [PubMed: 26813977]
117. Wright JB, Sanjana NE. CRISPR Screens to Discover Functional Noncoding Elements. *Trends Genet*. 2016; 32:526–529. [PubMed: 27423542]
118. Korkmaz G, Lopes R, Ugalde AP, Nevedomskaya E, Han R, Myacheva K, Zwart W, Elkon R, Agami R. Functional genetic screens for enhancer elements in the human genome using CRISPR-Cas9. *Nat Biotechnol*. 2016; 34:192–198. [PubMed: 26751173]
119. Canver MC, Lessard S, Pinello L, Wu Y, Ilboudo Y, Stern EN, Needleman A, Galactéros F, Brugnara C, Kutlar A, Chen DD, Das PP, Cole MA, Zeng J, Kurita R, Nakamura Y, Yuan G, Lettre G, Bauer DE, Orkin SH. Variant-aware saturating mutagenesis using multiple Cas9 nucleases identifies regulatory elements at trait-associated loci. *Nat Genet*. 2017; doi: 10.1038/ng.3793
120. Kim HK, Song M, Lee J, Menon AV, Jung S, Kang YM, Choi JW, Woo E, Koh HC, Nam JW, Kim H. In vivo high-throughput profiling of CRISPR-Cpf1 activity. *Nat Methods*. 2016
121. Aguirre AJ, Meyers RM, Weir BA, Vazquez F, Zhang CZ, Ben-David U, Cook A, Ha G, Harrington WF, Doshi MB, Kost-Alimova M, Gill S, Xu H, Ali LD, Jiang G, Pantel S, Lee Y, Goodale A, Cherniack AD, Oh C, Kryukov G, Cowley GS, Garraway LA, Stegmaier K, Roberts CW, Golub TR, Meyerson M, Root DE, Tsherniak A, Hahn WC. Genomic copy number dictates a gene-independent cell response to CRISPR-Cas9 targeting. *Cancer Discov*. 2016; 2641:617–632.
122. Munoz DM, Cassiani PJ, Li L, Billy E, Korn JM, Jones MD, Golji J, Ruddy DA, Yu K, McAllister G, DeWeck A, Abramowski D, Wan J, Shirley MD, Neshat SY, Rakiec D, de Beaumont R, Weber O, Kauffmann A, McDonald ER, Keen N, Hofmann F, Sellers WR, Schmelzle T, Stegmeier F, Schlabach MR. CRISPR screens provide a comprehensive assessment of cancer vulnerabilities but generate false-positive hits for highly amplified genomic regions. *Cancer Discov*. 2016; 6:900–13. [PubMed: 27260157]
123. Cross BCS, Lawo S, Archer CR, Hunt JR, Yarker JL, Riccombeni A, Little AS, McCarthy NJ, Moore JD. Increasing the performance of pooled CRISPR-Cas9 dropout screening. *Sci Rep*. 2016; 6:31782. [PubMed: 27545104]
124. Thakore PI, D'Ippolito AM, Song L, Safi A, Shivakumar NK, Kabadi AM, Reddy TE, Crawford GE, Gersbach CA, D'Ippolito AM, Song L, Safi A, Shivakumar NK, Kabadi AM, Reddy TE, Crawford GE, Gersbach CA. Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. *Nat Methods*. 2015; 12:1143–1149. [PubMed: 26501517]
125. Fulco CP, Munschauer M, Anyoha R, Munson G, Sharon R, Perez EM, Kane M, Cleary B, Lander ES, Jesse M. Systematic mapping of functional enhancer-promoter connections with CRISPR interference. *Science*. 2016; 354:769–773. [PubMed: 27708057]
126. Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH, Guimaraes C, Panning B, Ploegh HL, Bassik MC, Qi LS, Kampmann M, Weissman JS. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell*. 2014; 159:647–661. [PubMed: 25307932]
127. Liu SJ, Liu SJ, Horlbeck MA, Cho SW, Birk HS, Malatesta M, Attenello FJ, Villalta JE, Cho MY, Chen Y, Mandegar A, Olvera MP, Gilbert LA, Conklin BR, Chang HY, Weissman JS, Lim DA. CRISPRi-based genome-scale identification of functional long noncoding RNA loci in human cells. *Science*. 2016
128. Amabile A, Migliara A, Capasso P, Biffi M, Cittaro D, Naldini L, Lombardo A. Inheritable Silencing of Endogenous Genes by Hit-and-Run Targeted Epigenetic Editing. *Cell*. 2016; 167:219–232.e14. [PubMed: 27662090]
129. Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK. CRISPR RNA-guided activation of endogenous human genes. *Nat Methods*. 2013; 10:977–979. [PubMed: 23892898]

130. Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol.* 2013; 31:833–8. [PubMed: 23907171]
131. Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature.* 1985; 317:230–4. [PubMed: 2995814]
132. Thomas KR, Folger KR, Capecchi MR. High frequency targeting of genes to specific sites in the mammalian genome. *Cell.* 1986; 44:419–428. [PubMed: 3002636]
133. Mansour SL, Thomas KR, Capecchi MR. Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature.* 1988; 336:348–52. [PubMed: 3194019]
134. Bibikova M, Carroll D, Segal DJ, Trautman JK, Smith J, Kim YG, Chandrasegaran S. Stimulation of homologous recombination through targeted cleavage by chimeric nucleases. *Mol Cell Biol.* 2001; 21:289–297. [PubMed: 11113203]
135. Smih F, Rouet P, Romanienko PJ, Jasin M. Double-strand breaks at the target locus stimulate gene targeting in embryonic stem cells. *Nucleic Acids Res.* 1995; 23:5012–5019. [PubMed: 8559659]
136. Gutschner T, Haemmerle M, Genovese G, Draetta GF, Chin L. Post-translational Regulation of Cas9 during G1 Enhances Homology-Directed Repair. *Cell Rep.* 2016; 14:1555–1566. [PubMed: 26854237]
137. Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR, Ploegh HL. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotechnol.* 2015; 33:538–42. [PubMed: 25798939]
138. Orthwein A, Noordermeer SM, Wilson MD, Landry S, Enchev RI, Sherker A, Munro M, Pinder J, Salsman J, Dellaire G, Xia B, Peter M, Durocher D. A mechanism for the suppression of homologous recombination in G1 cells. *Nature.* 2015; 528:422–426. [PubMed: 26649820]
139. Lin S, Staahl B, Alla RK, Doudna JA. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *Elife.* 2014; 3:1–13.
140. Chu VT, Weber T, Wefers B, Wurst W, Sander S, Rajewsky K, Kuhn R. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat Biotechnol.* 2015; 33:543–548. [PubMed: 25803306]
141. Yu C, Liu Y, Ma T, Liu K, Xu S, Zhang Y, Liu H, La Russa M, Xie M, Ma T, Ding S, Qi LS. Small Molecules Enhance CRISPR Genome Editing in Pluripotent Stem Cells. *Cell Stem Cell.* 2015; 16:142–147. [PubMed: 25658371]
142. Ge X, Xi H, Yang F, Zhi X, Fu Y, Chen D, Xu RH, Lin G, Qu J, Zhao J, Gu F. CRISPR/Cas9-AAV Mediated Knock-in at NRL Locus in Human Embryonic Stem Cells. *Mol Ther Acids.* 2016; 5:e393.
143. Wang J, Exline CM, DeClercq JJ, Llewellyn GN, Hayward SB, Li PWL, Shivak DA, Surosky RT, Gregory PD, Holmes MC, Cannon PM. Homology-driven genome editing in hematopoietic stem and progenitor cells using ZFN mRNA and AAV6 donors. *Nat Biotechnol.* 2015; 33:1256–1263. [PubMed: 26551060]
144. Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nat Biotechnol.* 2016
145. Pinder J, Salsman J, Dellaire G. Nuclear domain “knock-in” screen for the evaluation and identification of small molecule enhancers of CRISPR-based genome editing. *Nucleic Acids Res.* 2015; 43:9379–92. [PubMed: 26429972]
146. Dever DP, Bak RO, Reinisch A, Camarena J, Washington G, Nicolas CE, Pavel-Dinu M, Saxena N, Wilkens AB, Mantri S, Uchida N, Hendel A, Narla A, Majeti R, Weinberg KI, Porteus MH. CRISPR/Cas9 β -globin gene targeting in human haematopoietic stem cells. *Nature.* 2016
147. Paquet D, Kwart D, Chen A, Sproul A, Jacob S, Teo S, Olsen KM, Gregg A, Noggle S, Tessier-Lavigne M. Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature.* 2016; 533:125–9. [PubMed: 27120160]

148. Howden SE, Mccoll B, Glaser A, Vadolas J, Petrou S, Little MH, Elefanty AG, Stanley EG. A Cas9 Variant for Efficient Generation of Indel-Free Knockin or Gene-Corrected Human Pluripotent Stem Cells. *Stem Cell Reports*. 2016; 7:508–517. [PubMed: 27499201]
149. He X, Tan C, Wang F, Wang Y, Zhou R, Cui D, You W, Zhao H, Ren J, Feng B. Knock-in of large reporter genes in human cells via CRISPR/Cas9-induced homology-dependent and independent DNA repair. *Nucleic Acids Res*. 2016; 44
150. Renaud JB, Boix C, Charpentier M, De Cian A, Cochenne J, Duvernois-Berthet E, Perrouault L, Tesson L, Edouard J, Thinard R, Cherifi Y, Menoret S, Fontaniere S, de Crozé N, Fraichard A, Sohm F, Anegon I, Concordet JP, Giovannangeli C. Improved Genome Editing Efficiency and Flexibility Using Modified Oligonucleotides with TALEN and CRISPR-Cas9 Nucleases. *Cell Rep*. 2016; 14:2263–2272. [PubMed: 26923600]
151. Mikuni T, Nishiyama J, Sun Y, Kamasawa N, Yasuda R. High-Throughput, High-Resolution Mapping of Protein Localization in Mammalian Brain by in Vivo Genome Editing. *Cell*. 2016; 165:1803–1817. [PubMed: 27180908]
152. Nakajima K, Kazuno A, Kelsoe J, Nakanishi M, Takumi T, Kato T. Exome sequencing in the knockin mice generated using the CRISPR/Cas system. *Sci Rep*. 2016; 6:34703. [PubMed: 27698470]
153. Parikh BA, Beckman DL, Patel SJ, White JM, Yokoyama WM. Detailed phenotypic and molecular analyses of genetically modified mice generated by CRISPR-Cas9-mediated editing. *PLoS One*. 2015; 10:1–28.
154. Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, Dahlman JE, Parnas O, Eisenhaure TM, Jovanovic M, Graham DB, Jhunjhunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacohen N, Regev A, Feng G, Sharp PA, Zhang F. CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling. *Cell*. 2014; 159:440–455. [PubMed: 25263330]
155. Yoshimi K, Kunihiro Y, Kaneko T, Nagahora H, Voigt B, Mashimo T. ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. *Nat Commun*. 2016; 7:10431. [PubMed: 26786405]
156. Singh P, Schimenti JC. The genetics of human infertility by functional interrogation of SNPs in mice. *Proc Natl Acad Sci U S A*. 2015; 112:10431–6. [PubMed: 26240362]
157. Raghavan A, Wang X, Rogov P, Wang L, Zhang X, Mikkelsen TS, Musunuru K. High-throughput Screening and CRISPR-Cas9 Modeling of Causal Lipid-associated Expression Quantitative Trait Locus Variants. *bioRxiv*. 2016
158. Crane E, Bian Q, McCord RP, Lajoie BR, Wheeler BS, Ralston EJ, Uzawa S, Dekker J, Meyer BJ. Condensin-driven remodelling of X chromosome topology during dosage compensation. *Nature*. 2015; 523:240–244. [PubMed: 26030525]
159. Edwards SL, Beesley J, French JD, Dunning M. Beyond GWASs: Illuminating the dark road from association to function. *Am J Hum Genet*. 2013; 93:779–797. [PubMed: 24210251]
160. Spisak S, Lawrenson K, Fu Y, Csabai I, Cottman RT, Seo JH, Haiman C, Han Y, Lenci R, Li Q, Tisza V, Szallasi Z, Herbert ZT, Chabot M, Pomerantz M, Solymosi N, G.-O.E. Consortium. Gayther SA, Joung JK, Freedman ML. CAUSEL: an epigenome- and genome-editing pipeline for establishing function of noncoding GWAS variants. *Nat Med*. 2015; 21:1357–1363. [PubMed: 26398868]
161. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*. 2016; 533:420–424. [PubMed: 27096365]
162. Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, Tabata M, Mochizuki M, Miyabe A, Araki M, Hara KY, Shimatani Z, Kondo A. Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. *Science*. 2016; 102:553–563.
163. Ma Y, Zhang J, Yin W, Zhang Z, Song Y, Chang X. Targeted AID-mediated mutagenesis (TAM) enables efficient genomic diversification in mammalian cells. *Nat Methods*. 2016; 13:1029–1035. [PubMed: 27723754]
164. Hess GT, Fresard L, Han K, Lee CH, Li A, Karlene A, Montgomery SB, Bassik MC. Directed evolution using dCas9-targeted somatic hypermutation in mammalian cells. *Nat Methods*. 2016; 13:1036–1042. [PubMed: 27798611]

165. Findlay GM, Boyle EA, Hause RJ, Klein JC, Shendure J. Saturation editing of genomic regions by multiplex homology-directed repair. *Nature*. 2014; 513:120–3. [PubMed: 25141179]
166. Hashimoto T, Sherwood RI, Kang DD, Rajagopal N, Barkal AA, Zeng H, Emons BJM, Srinivasan S, Jaakkola T, Gifford DK. A synergistic DNA logic predicts genome-wide chromatin accessibility. *Genome Res*. 2016; 26:1430–1440. [PubMed: 27456004]
167. Nakade S, Tsubota T, Sakane Y, Kume S, Sakamoto N, Obara M, Daimon T, Sezutsu H, Yamamoto T, Sakuma T, Suzuki KT. Microhomology-mediated end-joining-dependent integration of donor DNA in cells and animals using TALENs and CRISPR/Cas9. *Nat Commun*. 2014; 5:5560. [PubMed: 25410609]
168. Maresca M, Lin VG, Guo N, Yang Y. Obligate Ligation-Gated Recombination (ObLiGaRe): Custom designed nucleases mediated targeted integration through non-homologous end joining. *Genome Res*. 2013; 23:539–546. [PubMed: 23152450]
169. Auer TO, Duroure K, De Cian A, Concordet J, Del Bene F. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Res*. 2014; 24:142–153. [PubMed: 24179142]
170. Hoshijima K, Jurynek MJ, Grunwald DJ. Precise Editing of the Zebrafish Genome Made Simple and Efficient. *Dev Cell*. 2016; 36:654–667. [PubMed: 27003937]
171. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, Wu J, Zhu J, Kim EJ, Hatanaka F, Yamamoto M, Araoka T, Li Z, Kurita M, Hishida T, Li M, Aizawa E, Guo S, Chen S, Goebel A, Soligalla RD, Qu J, Jiang T, Fu X, Jafari M, Esteban CR, Berggren WT, Lajara J, Nunez-Delicado E, Guillen P, Campistol JM, Matsuzaki F, Liu GH, Magistretti P, Zhang K, Callaway EM, Zhang K, Belmonte JCI. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature*. 2016; 540:144–149. [PubMed: 27851729]
172. Cheng AW, Wang H, Yang H, Shi L, Katz Y, Theunissen TW, Rangarajan S, Shivalila CS, Dadon DB, Jaenisch R. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res*. 2013; 23:1163–1171. [PubMed: 23979020]
173. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature*. 2015; 517:583–588. [PubMed: 25494202]
174. Chavez A, Tuttle M, Pruitt BW, Ben EC, Chari R, Dmitry TO, Haque SJ, Cecchi RJ, Kowal EJK, Buchthal J, Housden BE, Perrimon N, Collins JJ, Church G, Ewen-Campen B, Chari R, Ter-Ovanesyan D, Haque SJ, Cecchi RJ, Kowal EJK, Buchthal J, Housden BE, Perrimon N, Collins JJ, Church G. Comparison of Cas9 activators in multiple species. *Nat Methods*. 2016; 13:563–567. [PubMed: 27214048]
175. Simeonov DR, Gowen BG, Boontanrart M, Roth T, Lee Y, Chan A, Nguyen ML, Gate RE, Subramaniam M, Woo JM, Mitros T, Ray GJ, Bray NL, Curie GL, Naddaf N, Boyer E, Van Gool F, Schumann K, Daly MJ, Fahr KK, Ye C, Bluestone JA, Anderson MS, Corn JE, Marson A. Discovery of an autoimmunity-associated IL2RA enhancer by unbiased targeting of transcriptional activation. *bioRxiv*. 2016
176. Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, Gersbach CA. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol*. 2015; 33:510–7. [PubMed: 25849900]
177. Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, Sullender M, Ebert BL, Xavier RJ, Root DE. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat Biotechnol*. 2014; 32:1262–7. [PubMed: 25184501]
178. Haeussler M, Schönig K, Eckert H, Eschstruth A, Mianné J, Renaud J-B, Schneider-Maunoury S, Shkumatava A, Teboul L, Kent J, Joly J-S, Concordet J-P. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biol*. 2016; 17:148. [PubMed: 27380939]
179. Xu H, Xiao T, Chen C-H, Li W, Meyer Ca, Wu Q, Wu D, Cong L, Zhang F, Liu JS, Brown M, Liu XS. Sequence determinants of improved CRISPR sgRNA design. *Genome Res*. 2015; 25:1147–57. [PubMed: 26063738]
180. Fusi N, Smith I, Doench J, Listgarten J. In Silico Predictive Modeling of CRISPR/Cas9 guide efficiency. *bioRxiv*. 2015

181. Chari R, Mali P, Moosburner M, Church GM. Unraveling CRISPR-Cas9 genome engineering parameters via a library-on-library approach. *Nat Methods*. 2015; 12:823–826. [PubMed: 26167643]
182. Moreno-Mateos MA, Vejnar CE, Beaudoin J, Fernandez JP, Mis EK, Khokha MK, Giraldez AJ. CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. *Nat Methods*. 2015; 12:982–8. [PubMed: 26322839]
183. Housden BE, Valvezan AJ, Kelley C, Sopko R, Hu Y, Roesel C, Lin S, Buckner M, Tao R, Yilmazel B, Mohr SE, Manning BD, Perrimon N. Identification of potential drug targets for tuberous sclerosis complex by synthetic screens combining CRISPR-based knockouts with RNAi. *Sci Signal*. 2015; 8:rs9. [PubMed: 26350902]
184. Bae S, Kweon J, Kim HS, Kim JS. Microhomology-based choice of Cas9 nuclease target sites. *Nat Methods*. 2014; 11:705–706. [PubMed: 24972169]
185. Farboud B, Meyer BJ. Dramatic enhancement of genome editing by CRISPR/cas9 through improved guide RNA design. *Genetics*. 2015; 199:959–971. [PubMed: 25695951]
186. Ren X, Yang Z, Xu J, Sun J, Mao D, Hu Y, Yang SJ, Qiao HH, Wang X, Hu Q, Deng P, Liu LP, Ji JY, Li JB, Ni JQ. Enhanced specificity and efficiency of the CRISPR/Cas9 system with optimized sgRNA parameters in *Drosophila*. *Cell Rep*. 2014; 9:1151–1162. [PubMed: 25437567]
187. Chu VT, Graf R, Wirtz T, Weber T. Efficient CRISPR-mediated mutagenesis in primary immune cells using CrispRGold and a new Cas9 transgenic mouse line. 2016
188. Vejnar CE, Moreno-Mateos MA, Cifuentes D, Bazzini AA, Giraldez AJ. Optimization Strategies for the CRISPR-Cas9 Genome-Editing System. *Cold Spring Harb Protoc*. 2016; 2016:829–833.
189. Horlbeck MA, Gilbert LA, Villalta JE, Adamson B, Pak RA, Chen Y, Fields AP, Park CY, Corn JE, Kampmann M, Weissman JS. Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. *Elife*. 2016; 5:e19760. [PubMed: 27661255]
190. Stemmer M, Thumberger T, Del Sol Keyer M, Wittbrodt J, Mateo JL. CCTop: An intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. *PLoS One*. 2015; 10:1–11.
191. Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, Aryee MJ, Joung JK. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol*. 2015; 33:187–197. [PubMed: 25513782]
192. Frock RL, Hu J, Meyers RM, Ho Y, Kii E, Alt FW. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol*. 2014; 33:179–186. [PubMed: 25503383]
193. Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Mol Ther Nucleic Acids*. 2015; 4:e264. [PubMed: 26575098]
194. Tycko J, Myer VE, Hsu PD. Methods for Optimizing CRISPR-Cas9 Genome Editing Specificity. *Mol Cell*. 2016; 63:355–370. [PubMed: 27494557]
195. Guilinger JP, Thompson DB, Liu DR. Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. *Nat Biotechnol*. 2014
196. Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Keith Joung J. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*. 2016; 529:490–495. [PubMed: 26735016]
197. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F. Rationally engineered Cas9 nucleases with improved specificity. *Science*. 2016; 351:84–88. [PubMed: 26628643]
198. Kleinstiver BP, Tsai SQ, Prew MS, Nguyen NT, Welch MM, Lopez JM, McCaw ZR, Aryee MJ, Joung JK. Genome-wide specificities of CRISPR-Cas Cpf1 nucleases in human cells. *Nat Biotechnol*. 2016; 34:869–874. [PubMed: 27347757]
199. Kim D, Kim J, Hur JK, Been KW, Yoon SH, Kim JS. Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. *Nat Biotechnol*. 2016; 34:863–8. [PubMed: 27272384]
200. Gao Y, Xiong X, Wong S, Charles EJ, Lim WA, Qi LS. Complex transcriptional modulation with orthogonal and inducible dCas9 regulators. *Nat Methods*. 2016; 13:1043–1049. [PubMed: 27776111]
201. Liu KI, Bin Ramli MN, Woo CWA, Wang Y, Zhao T, Zhang X, Yim GRD, Chong BY, Gowher A, Chua MZH, Jung J, Lee JHJ, Tan MH. A chemical-inducible CRISPR-Cas9 system for rapid control of genome editing. *Nat Chem Biol*. 2016

202. Maji B, Moore CL, Zetsche B, Volz SE, Zhang F, Shoulders MD, Choudhary A. Multidimensional chemical control of CRISPR-Cas9. *Nat Chem Biol.* 2016;5–7.
203. Pawluk A, Amrani N, Zhang Y, Garcia B, Hidalgo-Reyes Y, Lee J, Edraki A, Shah M, Sontheimer EJ, Maxwell KL, Davidson AR. Naturally Occurring Off-Switches for CRISPR-Cas9. *Cell.* 2016; 167:1829–1838.e9. [PubMed: 27984730]
204. Jaitin DA, Weiner A, Yofe I, Lara-Astiaso D, Keren-Shaul H, David E, Salame TM, Tanay A, van Oudenaarden A, Amit I. Dissecting Immune Circuits by Linking CRISPR-Pooled Screens with Single-Cell RNA-Seq. *Cell.* 2016; 167:1883–1896.e15. [PubMed: 27984734]
205. Dixit A, Parnas O, Li B, Chen J, Fulco CP, Jerby-Arnon L, Marjanovic ND, Dionne D, Burks T, Raychowdhury R, Adamson B, Norman TM, Lander ES, Weissman JS, Friedman N, Regev A. Perturb-Seq: Dissecting Molecular Circuits with Scalable Single-Cell RNA Profiling of Pooled Genetic Screens. *Cell.* 2016; 167:1853–1866.e17. [PubMed: 27984732]
206. Adamson B, Norman TM, Jost M, Cho MY, Nuñez JK, Chen Y, Villalta JE, Gilbert LA, Horlbeck MA, Hein MY, Pak RA, Gray AN, Gross CA, Dixit A, Parnas O, Regev A, Weissman JS. A Multiplexed Single-Cell CRISPR Screening Platform Enables Systematic Dissection of the Unfolded Protein Response. *Cell.* 2016; 167:1867–1882.e21. [PubMed: 27984733]
207. Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS, Lim WA. Engineering Complex Synthetic Transcriptional Programs with CRISPR RNA Scaffolds. *Cell.* 2015; 160:339–50. [PubMed: 25533786]
208. Cox D, Platt RJ, Zhang F. Therapeutic genome editing : prospects and challenges. *Nat Med.* 2015; 21:121–131. [PubMed: 25654603]
209. Canver MC, Orkin SH. Customizing the genome as therapy for the β -hemoglobinopathies. *Blood.* 2016; 127:2536–2545. [PubMed: 27053533]
210. Maeder ML, Gersbach CA. Genome-editing Technologies for Gene and Cell Therapy. *Mol Ther.* 2016; 24:430–446. [PubMed: 26755333]

Highlights

- Multiple CRISPR-based methods exist to study non-coding DNA
- CRISPR-based methods can be used for gain- and loss-of-function studies
- CRISPR-based methods can be used for transcriptional activation or repression

Figure 1. CRISPR strategies to study non-coding DNA

a, Individual sgRNA mutagenesis targeting a chromatin mark (shown in red) and a transcription factor binding motif such as GATA. **b**, Targeted deletion using a dual sgRNA approach. Deleted sequence is indicated by red dotted lines. Inverted sequence is indicated by red arrows. **c**, Tiling sgRNA/saturating mutagenesis utilizing all sgRNA within genomic loci in a pooled screening format. **d**, CRISPR-mediated transcriptional repression. dCas9-KRAB and dCas9-LSD1 are shown. However, other repressive effectors are possible. Chromatin mark is shown in red and SpCas9 is shown in gray. **e**, Individual sgRNA with a homologous repair template for such applications as SNP insertion or reporter knock-in. Custom sequences from homologous repair templates are inserted by exploiting HDR or MMEJ. Red sequences indicate homologous sequence and SpCas9 is shown in gray. GFP reporter sequence is shown in green. **f**, Base editing. dCas9-AID is shown. However, other base editors are possible. SpCas9 is shown in gray. **g**, Homology-independent NHEJ-integration. Shared sequences on plasmid (black circle) and genomic DNA are shown in red. **h**, CRISPR-mediated transcriptional activation. dCas9-VP64 and dCas9-p300 are shown. However, other activation effectors are possible. Chromatin mark is shown in red and SpCas9 is shown in gray. **a–h**, All schema are shown with SpCas9. The blue line indicates the 20-mer sgRNA sequence.

Table 1

CRISPR approaches to study non-coding DNA.

<i>Approach</i>	<i># of sgRNA</i>	<i>Strategy</i>	<i>Throughput</i>	<i>Efficiency</i>	<i>Advantages</i>	<i>Disadvantages</i>
sgRNA mutagenesis via indel-prone NHEJ	1	LOF with genomic modification	Low-to-high	High	Efficient mutagenesis of individual loci; pooled screening	Narrow regions; often depend on some prior knowledge to design informative targets
sgRNA with homologous extrachromosomal template for HDR	1	GOF with genomic modification	Low	Low	Genome customization (e.g., variants, protein tagging)	Not suitable for non-dividing cells; low efficiency; low throughput of loci
sgRNA with homologous extrachromosomal template for MMEJ	1	GOF with genomic modification	Low	Low	Genome customization (e.g., variants, protein tagging)	Not suitable for non-dividing cells; low efficiency; low throughput of loci
sgRNA with nonhomologous plasmid (NHEJ-integration)	1	GOF with genomic modification	Low	High	Genome customization (e.g., variants, protein tagging); high efficiency	Low throughput of loci
Targeted deletion, inversion using dual sgRNA	2	LOF with genomic modification	Low-to-high	Low	Large regions; pooled screening	Low-resolution; multifocal indels as competing genomic outcome
Translocation induction using dual sgRNA	2	GOF with genomic modification	Low	Low	Creation of complex genomic rearrangements	Low efficiency; low throughput
Tiling sgRNA/Saturating mutagenesis	1	LOF/GOF with genomic modification	High	High	High-resolution, high-throughput; compatible with any nuclease	Variable resolution as function of underlying sequence, PAM availability and off-target potential
dCas9-KRAB	1	LOF without genomic modification	Low-to-high	High	Reduction without knockout (cell lethal study); pooled screening	Dosage; false negatives
dCas9-LSD1	1	LOF without genomic modification	Low-to-high	High	Reduction without knockout (cell lethal study); pooled screening	Dosage; false negatives
dCas9-VP64	1	GOF without genomic modification	Low-to-high	High	Pooled screening	Dosage; false negatives
dCas9-p300	1	GOF without genomic modification	Low-to-high	High	Pooled screening	Dosage; false negatives

LOF = loss-of-function, GOF = gain-of-function.