A Manual for Genome and Transcriptome Engineering

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***Abstract*—Genome and transcriptome engineering have emerged as powerful tools in modern biotechnology, driving advancements in precision medicine and novel therapeutics. In this review, we provide a comprehensive overview of the current methodologies, applications, and future directions in genome and transcriptome engineering. Through this, we aim to provide a guide for tool selection, critically analyzing the strengths, weaknesses, and best use cases of these tools to provide context on their suitability for various applications. We explore standard and recent developments in genome engineering, such as base editors and prime editing, and provide insight into tool selection for change of function (knockout, deletion, insertion, substitution) and change of expression (repression, activation) contexts. Advancements in transcriptome engineering are also explored, focusing on established technologies like antisense oligonucleotides (ASOs) and RNA interference (RNAi), as well as recent developments such as CRISPR-Cas13 and adenosine deaminases acting on RNA (ADAR). This review offers a comparison of different approaches to achieve similar biological goals, and consideration of high-throughput applications that enable the probing of a variety of targets. This review elucidates the transformative impact of genome and transcriptome engineering on biological research and clinical applications that will pave the way for future innovations in the field.**

***Index Terms*— Genome Editing, Transcriptome Editing, CRISPR-Cas Systems, RNA Therapeutics**

I. INTRODUCTION

Over the past decade, our ability to probe and manipulate nucleic acids has dramatically increased. Back in the 2010s, technologies such as CRISPR-Cas9 were just in their infancy, with basic mechanistic understanding of their function in human cells [1], [2], [3]. Jumping forward to 2024, we have already created FDA approved Cas9 based therapies (e.g. Casgevy [4]), and have branched out into a full suite of tools enabling precise DNA and RNA modifications. Engineering nucleic acids in situ is as much an art as it is a science, requiring careful consideration when electing an editing strategy and an intuition for the inherent tradeoffs and tolerances of each specific application. There are a number of excellent reviews covering the technology underlying the history and state-of-the-art tools in genome, epigenome, and transcriptome engineering including: [5], [6], [7] which we strongly encourage the reader to explore. Here, we aim to provide a guide for *tool selection* based on specific applications of interest, and suggest nuanced design factors to consider when choosing a strategy for genome and transcriptome engineering.

II. GENOME ENGINEERING

When engineering the genome, there are typically two edit classes of interest: changing gene function, and changing gene expression. Examples of gene function alterations include knocking out genes to study their function, inserting transgenes to introduce new genomic features, or in a therapeutic context removing premature stop codons to restore gene function. Examples of gene expression alterations are transient or inheritable repression/activation to a gene of interest. To facilitate efficient engineering of a desired phenotype, it is important to first identify the type of edit desired, the scale/size of the edit, required editing efficiency, and finally context dependent factors such as cell type specific editing and delivery considerations or off-target tolerance. This enables selecting the appropriate genome engineering tool, maximizing editing fidelity.

Two key motifs in genome engineering are that in general, (i) efficiency is a tradeoff with specificity and (ii) it is easier to delete than it is to add. Technologies for knocking out genes are relatively mature, the most common of which is CRISPR-Cas9 [8].]. Tools for gene knockouts have evolved to be highly reliable and multiplexable, enabling rapid querying of gene function in the transcriptional context of the cell. In a similar vein, kilo- to mega- base deletions are feasible with moderate efficiency [9], and gene repression via CRISPRi can be achieved robustly in most genomic contexts [10]. On the other hand, precise and efficient substitution/insertion of DNA fragments is still an active area of research, and also CRISPRa is more modest in potency [11]. Single to few nucleotide substitutions can be achieved with higher efficiency than insertions [12]. Furthermore, an overall rate-limiting step applicable to all forms of genome engineering is the packaging capacity and delivery efficiency of the editing tool into its target cell type. While we will not be deeply detailing those challenges in this review, we direct the readers to these excellent reviews on those challenges: ([13], [14], [15]).

Despite the remaining challenges towards complete genomic control, the genome engineering community has established a milieu of tools to achieve each of these desired edits, greatly enhancing our ability to probe and engineer biology. In the following sections, we will describe in greater detail the types of edits that can be made, the state-of-the-art tools for achieving these edits, and important context-specific considerations.

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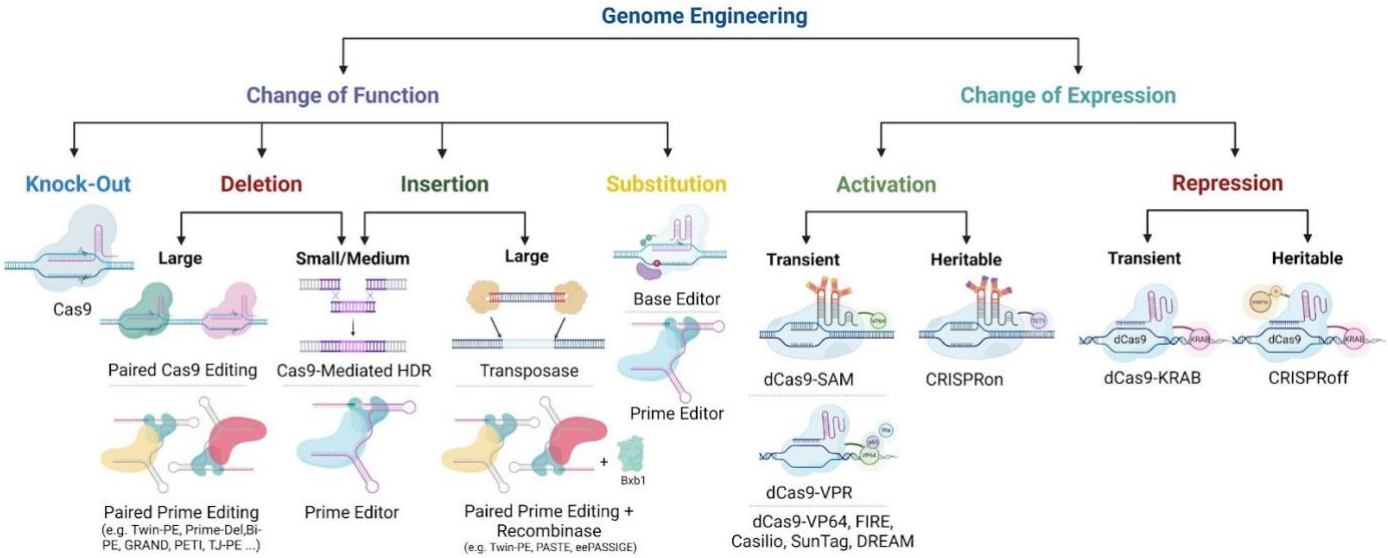

Figure 1. Genome Engineering Tool Selection Flowchart.

A. Change of Function

Generally, the three key genomic edits of interest are deletions, insertions, and substitutions. A fourth related class are knockouts, which leverage stochastic or directed insertion/deletion events (indels) to disrupt gene sequences. The most frequently used chassis for tools facilitating each of these edits is CRISPR-Cas9 . Cas9 is a ribonucleic protein with nuclease activity that uses a single guide RNA (sgRNA) to target and cleave DNA with single nucleotide resolution [2], [3]. Building upon this chassis by engineering Cas9 variants (such as Cas9 nickase [16][17]), fusing effector domains to Cas9 (such as reverse transcriptases [18] or deaminases [12]), and modifying the sgRNA architecture (such as prime editing gRNAs [19]) has enabled all four of these edit classes, from scales of a few nucleotides to thousands of base pairs.

1) Knockout

A classical example of altering DNA function is in performing gene knockouts. To study the specific biology of a gene, or to simulate a disease model of a mutated gene, it is often desirable to precisely inactivate (knock out) a gene of interest. The gold standard tool in this application is CRISPR-Cas9 for its (i) ease of use (ii) high modularity and (iii) precision.CRISPR-Cas9 operates by creating double-stranded breaks (DSBs) in DNA at specific locations guided by a single guide RNA (sgRNA).The sgRNA can be designed to create an edit at a desired genomic locus with single nucleotide resolution [2], [3]. In absence of a suitable repair template, the cell's DNA damage machinery in mammalian cells will typically repair DSBs though non-homologous end joining (NHEJ) [20]. NHEJ often introduces small nucleotide insertions or deletions (indels) that disrupt gene function, effectively knocking out the gene. This technique is referred to as CRISPRko. Notably, although the editing site of CRISPRko is precise, the edits induced by CRISPRko themselves are not. The sequences inserted by NHEJ are stochastic [21], often in a cell type dependent manner. Thus CRISPRko is only suitable for "chop and drop" applications where the exact resultant sequence is not of importance, only the inactivation of the target gene.

When performing a gene knockout via CRISPRko, there are several considerations a user must bear in mind. The first is that the mammalian genome is diploid, and thus to have a complete knockout both alleles of a gene must be edited. Precise editing requires robust pre-quantification of sgRNA potency, and may require using serial or simultaneous editing with multiple sgRNA targeting the same gene to ensure a complete knockout. Use of multiple sgRNA simultaneously has been shown to potentially create massive deletions between the target sites, whereas serial editing does not but takes commensurate longer time [22]. There are a number of computational predictive models that have been developed to assist users in selecting CRISPRko sgRNA successfully, substantially reducing the time to screen for potent sgRNA [23]. Some examples include CRISPick [24], CASPER [25], and CHOPCHOP [26]. Further, these models aim to maximize target specificity by minimizing target homology to off-target sequences. One factor to bear in mind is that there are restrictions on where Cas-based edits can be made as they require a protospacer adjacent motif (PAM) sequence downstream of the editing locus – in the case of SpCas9, the PAM is NGG (where N is any nucleotide and G is guanine). This is typically non-restrictive for most CRISPRko applications as (i) NGG is prevalent throughout the genome and it is typically immaterial where the exact indel is generated so long as it disrupts the coding sequence (CDS), and (ii) there have been Cas systems developed/discovered with relaxed/alternative PAM requirements and similar activities to wild-type Cas9 such as xCas9 (NG) [27], SpRY Cas9 (NYN/NRN) [28], and Cas12a (TTTV) [29].

A second key consideration is providing a selective advantage for the knockout of choice. CRISPRko efficiency is dependent on cell-type specific transfection permissibility and local chromatin structure of the gene of interest, and therefore is rarely 100% efficient [30]. In *in-vitro* settings, knock outs to surface receptors, secreted proteins, or in pre-engineered reporter cell lines can be selected via techniques such as flow-cytometry. However knockouts to genes that are intracellularly expressed often require selective pressure to isolate edited cells from a mosaic population. A common strategy for this is CRISPRko in conjunction with providing a donor template carrying an antibiotic resistance gene flanked by genome homology arms to promote Homology-Directed Repair (HDR) [31]. This technique leverages the cell's natural DNA damage repair mechanisms to simultaneously knock-out the gene of interest and knock-in a resistance gene to mammalian antibiotics such as puromycin, hygromycin, and geneticin (G418).One key limitation of HDR is that it is of relatively low efficiency, as NHEJ is the preferred repair mechanism in mammalian cells, and is limited to actively dividing cells [32]. However, (i) through passage cycles under antibiotic selection the edit containing population can be amplified, and (ii) HDR efficiency can be improved by suppressing NHEJ through overexpression of adenovirus 4 proteins E1B55K and E4orf6 [33] and stimulating HDR through overexpression of a RAD18 variant

[34]. Though time consuming, particularly to isolate isogenic knock-out populations, this method has been shown to reliably enrich CRISPRko induced knock-outs [35]. Further, there is active research in hastening this process, such as display of surface markers in cells with biallelic edits [35].

2) Deletion

For generating small deletions in a context tolerant to indels, a standard single sgRNA CRISPRko scheme can be used. In use cases where a scarless deletion is required, there are several choices. Classically, an HDR template with flanking homology arms and no insert can be used, although this is prone to the low efficiencies and HDR limitations as identified in the previous section.

Certain applications require large deletions on the kilo- to mega-base scale. Some examples of these applications include: studying the function of clusters of adjacent genes [36], modeling of large chromosomal deletions typical of cancer [37], investigation of non-coding DNA and long non-coding RNAs [36], and amelioration of diseases involving large insertions / duplications such as Huntington's [38]. For low-stakes applications tolerant to large indel formation and potentially chromosomal recombination [39] a standard dual flanking sgRNA + CRISPRko strategy is suitable. Recently a dual Cas9-Cas12a system has been used in a genome scale exon perturbation screen to delete whole exons, highlighting their fitness functions [40]. However, in applications requiring precision editing, such as in disease modeling or treatment, a new suite of tools have been developed to facilitate precise, large scale genomic deletions.

One solution is use of Type 1 CRISPR systems, which have been shown to facilitate up to ~100kb deletions. Type 1 CRISPR systems are reliant on the Cascade (CRISPR-associated complex for antiviral defense) complex to target DNA, a heteromeric protein complex consisting of multiple Cas proteins. For Cas3, the Cascade consists of Cas8, Cas11, Cas7, Cas5, and Cas6 [41]. Cas3-Cascade has been used for kilobase scale deletions by targeting the Cascade complex upstream of the target sequence, and leveraging Cas3 to translocate in the 3' to 5' direction, deleting DNA via its helicase-nuclease activity [42]. As this system only leverages a single targeting moiety, it is not susceptible to the same chromosomal recombination as Cas9, and is not associated with stochastic small indel formation. However, the location of deletion onset is not as precise as Cas9, and the length of deletion is not controllable. To control the length of deletion, Cascade has been fused with dimeric Fok-1 in a twin-guide scheme [43]. The key challenge with Cascade is the packaging and delivery of the complex, as it is composed of multiple protein subunits, though there is active research in compaction [44].

A more recent development is the use of paired Prime editing to facilitate deletions on the order of ~ <10kb. Generally, Paired Prime editing uses two pegRNAs to introduce two flanking nicks on the DNA, followed by reverse-transcription to form sequences with homology, which are then joined together, deleting the sequence in between. This method allows for more controlled and precise deletions compared to traditional CRISPR/Cas9 approaches, enabling ~95% indel-free editing, albeit with ~25% lower efficiencies [9]. Development of reliable paired prime editing strategies is a field of rapid development, with tools including Bi-PE [45], GRAND [46], PEDAR [47], PETI [48], PRIME-Del [49], Twin-PE [9], and TJ-PE [50]. The homology arms can be made complementary to each other (up to ~250 bp) to knock-in a desired sequence (e.g. attB recombinase site) as in TwinPE [9], or to the genome itself for scarless excision as in PRIME-Del [49]. Some key considerations are (i) use of a dual-PE system requires careful, non-trivial engineering of the pegRNAs, (ii) often requires a split-AAV strategy to deliver in vivo, (iii) is generally less efficient than dual-sgRNA CRISPRko, and (iv) has multiple features to optimize including prime editor binding site length, distance between knick sites, prime editing architecture (PE 2/3/4/5, SpCas9 nickase or nuclease), and reverse transcriptase template length. For detailed description and best practices when designing Prime editing experiments, we direct the reader to: [51].

3) Insertion

Insertion of novel genetic sequences into existing genomes is a fundamental goal of genome engineering and precision therapeutics, enabling us to correct mutated sequences and create new biology. Some examples of applications where genomic insertions are desirable include introducing selection markers to give a phenotypic advantage to cells of interest, rescuing the function of mutated genes by providing functional copies, and replacing erroneous DNA sequences with function restoring ones [52].

In applications where the exact genomic locus of integration is immaterial, such as integrating synthetic genetic circuits or generating reporter cell lines, there are several well established techniques. For integrations on the scale of ~ < 8kb, lentiviral integration is the preferred method for its simplicity and relative robustness. Though the locus of lentiviral integration is highly stochastic, the transduction efficiency is generally high across a spectrum of cell lines [53]. A well known drawback of lentiviral integration is the potential for insertional mutagenesis such as promoter shuffling and oncogene activation [54]. Payloads larger than 8kb up to ~300kb can typically be integrated via PiggyBac transposase [55], which integrates promiscuously into TTAA genomic loci.

For the most part, technologies to randomly integrate DNA sequences into the genome are mature and generally reliable in in-vitro settings, though there are still challenges remaining to optimize these techniques in-vivo. However, for most biologically and therapeutically relevant applications of the future, precise DNA insertions will be required. Engineering efficient and accurate edits is typically more challenging than deletions, as the target integration site must first be broken, deleted, or modified and then cellular machinery must be biased to favorably integrate the desired edit.

As previously outlined, the current go-to method for precise DNA integration is integration via homology directed repair (HDR). After selection of a genomic locus for insertion, a repair template is created where the desired sequence for insertion is flanked by homology arms complementary to the genomic locus where it will be inserted. Upon DNA damage to the locus via sgRNA-Cas9 mediated DSB, cellular machinery will, with low probability, use the repair template to correct the damaged DNA, integrating the desired novel sequence. HDR is compatible with a range of desired insertion sizes, from 10s to a few thousand base pairs [56]. For insertion sequences of ~10-100 bp, short single stranded oligodeoxynucleotides (ssODNs) can be provided as a template [57]. The key benefit is that these are relatively inexpensive to acquire at high concentrations, and do not require further cloning. For desired insertions on the ~100-4000 bp range, a double stranded DNA template is required [58].

When the only constraint on the desired integration locus is that it does not alter existing cell machinery, a common strategy is to flank the cargo with homology arms complimentary to a genomic safe harbor sites such as AAVS1, CCR5, and ROSA26.A key drawback of HDR mediated insertion is that it requires creating a DSB in the target site, and as HDR has low efficiency, there is a high probability that the target sequence will be stochastically mutated via NHEJ rather than rationally edited. This makes HDR techniques impermissible to applications intolerant to high indel rates, such as within actively transcribed genes. A recent study found that protection of the donor template by phosphorothioate groups can reduce TREX1 exonuclease mediated degradation of the donor, enabling a boost of HDR efficiency to ~25-30% across a wide range of genomic contexts [59].

For precision insertions, an emerging method of choice is Prime editing [18]. Prime editing leverages nicking (H840A) Cas9 fused to a reverse-transcriptase and a specialized prime editing guide RNA

(pegRNA) to facilitate precise deletions, insertions and substitutions. The 5' end of the pegRNA binds complementary to the target sequence, while the Cas9 nicks the DNA on the opposite strand. The 3' end of the pegRNA binds to the 3' exposed flap on the DNA, leaving a 3 bp exposed 5' flap. Upstream of the 3' end, the desired edit is engineered into the pegRNA. The reverse transcriptase (typically Moloney murine leukemia virus MMLV) then uses the 3' hybridized flap as a primer sequence to reverse-transcribe the pegRNA, including the desired edit of choice, which then through cellular repair machinery gets favorably integrated and replicated into the genome over the exposed, unedited 5' flap. To delete base pairs, the pegRNA is desired to bind downstream of the desired edit locus, and the reverse transcription template contains the sequence directly upstream of the desired edit locus, effectively "skipping" the region to be deleted during reverse transcription. With this method, deletions on the order of ~>80bp can be generated [18].

There have been several generations of improvements to Prime editors aimed at improving editing efficiency and decreasing the rate of undesirable edits. The first generation, PE1, suffered from poor editing efficiency which was improved by optimizing the reverse transcriptase design, yielding PE2. By including additional nicking sgRNA, PE3 and PE3b enhanced the editing efficiency further at the tradeoff of increased indel rate. PE4 and PE5 included a mechanism to bias cellular repair further towards the edit of choice by overexpressing a protein that inhibits DNA mismatch repair pathways. Further engineering to the Cas9-nickase yielded PEmax, and addition of 3' structural motifs such as TevopreQ1 stabilized the pegRNA yielding epegRNAs. In conjunction, PEmax with an epegRNA achieved ~45% editing efficiency with ~5% indel rate in HEK293T, and 35% editing efficiency with ~7% indel rate in human iPSCs, though efficiencies and indel rates are highly cell type and editing context dependent [19]. Prime editors can facilitate DSB-free integration of small fragments on the order of ~<44bp [18], though at limited and varying efficiencies. Recently, PE6a-g have been developed through phage-assisted continuous evolution (PACE) with enhanced properties over the PEmax generation. The PE6a and PE6b variants featured engineered reverse transcriptases that are 516-810 bp smaller than MMLV, achieving similar editing efficiencies to PEmax. PE6c and PE6d achieved editing efficiencies as high as 50% in HEK293T cells, leveraging a hairpin in the RTT. Further, these tools are compact enough to be packaged into a dual-AAV system, enabling potential therapeutic utility. Variants PE6e/f/g achieved 0.5-2 fold higher editing efficiency to PEmax across a variety of loci in N2a and HEK293T cells [60]. One additional consideration when performing insertions with prime editors is to ensure, if possible, that the reverse transcription template is PAM-less, removing the possibility of repeated editing of the same locus. Additionally, medium scale integration (100bp-1kb range [46]) can be facilitated via paired prime editing strategies such as TwinPE [9] albeit at modest to low efficiencies. Prime editing also facilitates delete-and-insert strategies where the reverse-transcriptase template encodes the insertion to "overwrite" the region to be deleted [18].

For precise integration of large (on the order of 10s to 100s of kb) fragments, there are a number of tools that have been developed, though rather expectedly suffer from poor efficiencies. The first generation of these tools involved fusing a dead-Cas9 (dCas9) incapable of nuclease activity to transposases such as Ginβ [61] , Sleeping Beauty [62], and Piggybac [63]. While scarless, the key limitation is that integration sites are transposase-specific, and often ubiquitous such as TTAA for piggybac, resulting in directed, but nevertheless nonspecific integration. Type V-K and 1-F CRISPR-associated transposases (CASTs) have also been used to mediate specific integration of fragments on the ~10-100kb range, although at efficiencies < 5% and leaving target site duplication scars [64] [65]. Recently, strategies using recombinase landing pads have increased in popularity, as recombinases such as Bxb1 can facilitate specific integration of large fragments with modest efficiencies. Use of recombinases however requires pre-integration of Bxb1 recombination sites (38bp AttB or 50bp AttP) into the target locus. Prime editing strategies such as Twin-PE knock-in have been leveraged to precisely integrate these recombination loci, and was built upon by PASTE where the Bxb1 recombinase was directly fused to the prime editor [66]. PASTE mediated context dependent efficiencies of ~5-60% and insertion of cargo of up to 36 kb without off target editing due to the high specificity of recombinases. As of date, the highest efficiency method is eePASSIGE, which features an enhanced Bxb1 recombinase with ~3.2 fold higher integration rate than WT Bxb1, and when directly compared to PASTE reported ~16 fold higher efficiency across 4 genomic loci in HEK293T [67]. Though there is still much work to be done to generate efficient, programmable, and multiplexable large scale genomic edits, the field is rapidly making progress towards these goals.

4) Substitution

Single to few nucleotide substitutions can be achieved with relatively high fidelity using base editors. In base editor systems, Cas9 nickase is fused to adenosine and cytidine deaminases, often accompanied by DNA repair biasing motifs. Current base editor systems can facilitate A-to-G, A-to-C, A-toT, C-to-T, and C-to-G conversions [12], [68], [69], [70], [71]. The current challenge with base editors is that although they can achieve on-target efficiencies with as high as 80% editing and off-target rates of under 1%, they contain an editing window [72]. That is, while base editors have an empirically determined preference for editing certain nucleotide positions, they may edit some, none, or all bases within a ~4-8 bp window, though active research is being done to prejudice editing outcomes for specific bases [73] [70]. Further, choosing a base editing strategy is a complex and context dependent decision process to which we direct the reader to: [5]. It is worth noting that conventional base editors cannot function to edit the mitochondrial genome, as mitochondria lack the machinery necessary to import sgRNAs. As a result, CRISPR-independent mitochondrial base editors have been developed such as DdCBEs [74], [75], which leverage a split deaminase targeted by transcription activator-like effectors (TALEs) to perform double stranded C-G to T-A conversions. More recently, mitoBEs [76] have been developed to enable single stranded A-to-G conversions.

Prime editors provide enhanced flexibility and specificity over base editors in the genomic substitution application as they enable all 12 possible substitutions and overcome potential limitations in base editor's editing windows with a reverse transcription template which enables precise specification of the substitution location. Further, prime editors can enable substitutions of larger than a few bases, to which base editors are restricted. Nevertheless, they are subject to the same caveats as discussed in the above section, including potential indel formation, off target editing, and modest efficiency. In applications that are intolerant to indel formation or require high editing efficiency, base editors should be the first modality attempted. Conversely, in applications requiring flexibility around the target locus and specificity of editing, prime editors would be preferred.

B. Change of Expression

Genome activation and repression techniques generally consist of two components: a targeting moiety and an effector domain. The targeting moiety is typically customized to a genome sequence of interest, directing the effector domain to modulate gene expression. The majority of modern targeting moieties consist of sgRNA-dCas9 due to the system's high modularity. A few notable alternatives include dCas12, TALENs and ZFs. While the use of dCas9 is standard as a targeting transcriptional, there exist a large variety of

effector domains to encompass a broad range of desired transcriptional modulations. Generally these can be split into gene activation and gene repression, further subdivided into transient and stable modes of transcriptional control. The potency of repressive, and to a greater extent activation tools are highly context dependent, and multiple tools may need to be assayed for the same locus in parallel to devise the optimal strategy.

1) Repression

The current gold-standard architecture for achieving robust transient transcriptional repression is a fusion of dCas9 to the zinc finger-imprinted 3 (ZIM3) KRAB domain [10]. In direct comparison with a number of other KRAB domains, the ZIM3 KRAB enabled robust transcriptional repression over a wide range of genomic contexts, and its small size is permissive to most delivery strategies. To achieve stable repression (inherited through > ~25 cell cycles), epigenome engineering strategies have been leveraged to engineer repressive epigenetic marks. Examples of attempts at this have included dCas9 fusions to LSD1 (demethylating H3K4me2 and deacetylating H3K27) [77], HDAC3 (H3K27 deacetylase) [78], and FOG1 (HE3K27 trimethylation) [79]. While these domains are capable of inducing repression, they are not particularly potent when compared to KRAB. CRISPRoff [80] [81] leverages KRAB and DNMT3A/3L to achieve potent and heritable epigenetic silencing of a locus, though DNMT3A was found to be cytotoxic. Using a similar architecture, durable silencing was achieved for nearly 1 year in mice [82], and toxicity has since been improved via updates to the effector domains e.g. CHARM [83].

2) Activation

There are a number of gene activation architectures that have been developed to attempt robust and persistent gene activation. It is generally considered in the field that robust activation is challenging to achieve across a broad range of genomic loci, particularly if the gene of interest has very low basal levels of transcription. Nevertheless, a large activation toolkit has been developed, with tools that have been shown to facilitate potent activation at certain genomic loci. Two architectures we highlight for first-pass attempts at potent transient activation are dCas9-SAM [84] and dCas9-VPR [85]. One key challenge with these architectures is their relatively large size (150-1400 bp for SAM ~1600 bp for VPR [7]), which may be impermissible for delivery. In these cases, a dCas9-VP64 [86] (~150bp) or a dCas9-dual VP64 [87] architecture (~300 bp) may be used, though these tools may not achieve the same activation effect size as SAM or VPR. Other notable activation architectures include dCas9-p300 [88], Casilio , SunTag [89], FIRE [90], and DREAM [91]. For heritable/durable activation of loci, similar epigenome engineering strategies as those used for repression have been leveraged. Fusion of dCas9 to the DNA dioxygenase TET1 has been shown to produce stable activation in mouse models [92]. CRISPRon leverages TET1 with the addition of p65-Rta to reactivate genes previously silenced via CRISPRoff [81].

3) Orthogonal Regulation

Some genome engineering applications require a cross between activation and repression, or change of function and change of expression. For example, it is enticing to study the co-regulation of two genes in a shared molecular pathway by inhibiting one gene and activating the other. A key limitation for this type of study is that with CRISPR-Cas based systems, the same CRISPR-Cas variant cannot be used for both activation and repression. This is because the scaffold for repressing and activating sgRNA may bind to either the repressing or activating Cas protein (if they are identical) and thus whether the desired target locus is activated or repressed cannot be controlled. A common strategy to overcome this is to use orthogonal editors – a different Cas variant for the activation and repression. For example, VPR-dSaCas9 may be used for activation and KRAB-dSpCas9 may be used for repression in tandem due to their different sgRNA scaffold specifications [93]. dCas9 and dCas12 may also be used in a similar way, and in RNA editing contexts Cas13a has been used with Csm6 [94]. For contexts where a change of function (e.g. base editing) is mixed with a change of expression (e.g. activation), a single-Cas9n dual-sgRNA strategy has been implemented wherein the first sgRNA facilitates the change of function, while the second binds to the post-edit region and recruits activating domains via aptamer loops [95].

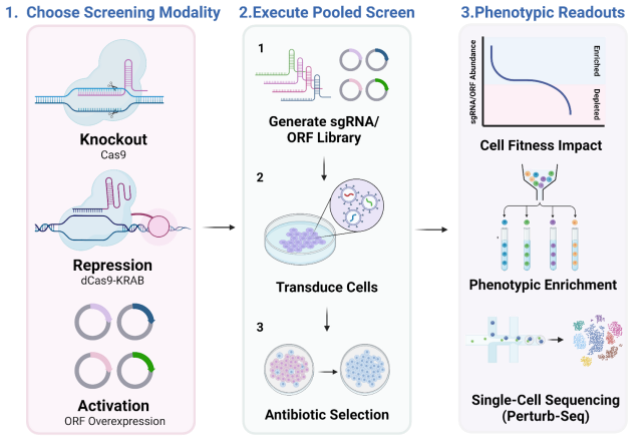

Figure 2. High Throughput Genome Sequencing Workflow.

4) High-Throughput Screening

High-throughput screens leverage the modularity of sgRNAs - as the variable targeting sequence and Cas-binding scaffold are independent - to perform tens to tens-of-thousands of perturbations in a parallel, pooled fashion. The high modularity of this approach has enabled massively parallel querying of fundamental genotype-phenotype relationships. By investigating the cellular fitness impact of perturbations, selecting for phenotypes of interest [96], or measuring observed phenotypes through single-cell RNA sequencing (a technique known as Perturb-Seq) [97], researchers can discover the functional role of genes (Figure 2). To do so requires generation of sgRNA libraries – a mixture of sgRNAs that in union cover all genes of interest – which is a labor-intensive process. Further, there is an interoperability challenge with sgRNA libraries. Generally, CRISPRko sgRNA target the CDS of a gene and are thus incompatible with other forms of CRISPR editing such as CRISPRa/i which tend to target near the transcription start site or promoter & enhancer regions. As a consequence, to study different aspects of gene regulation, separate sgRNA libraries will need to be created for each perturbing modality.

Further, for Perturb-Seq, there are nuances associated with the perturbing modality of choice. Choosing CRISPRko has benefits in that a large associated phenotypic change may be seen, however it may be challenging to isolate cells that received a perturbation. True knock-out events may be conflated with scRNA-seq associated dropout events, and as a result is is not possible to determine if a cell received a knockout by evaluating gene expression alone, though there have been a number of tools developed [98] to isolate these cells such as Mixscape [99]. CRISPRi's key advantage is that the extent of perturbation can be measured directly via comparing the expression of the perturbed gene in treated vs. control cells, though it may not yield as strong phenotypic results [100]. CRISPRa is challenging to robustly achieve across a range of genomic loci, and thus overexpression of these gene open reading frames is a preferred

approach [101] , though libraries of this form are challenging to create. Finally, regardless of perturbation strategy, there is still a lack of consensus on the correct way to carry out Perturb-seq experiments and analyze data [102].

5) Genome Engineering Future Directions

Since the characterization of Cas9 and its applications to genome engineering in 2013 [2], [3], [103], there has been substantial and rapid development over the past decade to develop tools enabling all basic forms of genome editing. Here, we have provided a roadmap for tool selection in the genome engineering context, and highlighted nuances to consider when electing a tool for use. The general tool selection decision tree is outlined in Figure 1. While there is still substantial work to be done towards enhancing efficiency, improving precision, and expanding capacity, the pace of developments will lead to a set of robust genome editing tools enabling near complete genomic control. It will be interesting to monitor which paradigm, one-tool-one-job (such as base editors) or one-tool-all-jobs (such as Prime editors), will eventually be the modality of choice. There have also been recent developments in RNA based DNA editing tools such as HYERs [104] and Bridge RNAs [105] which show high promise, though are still at the proof-of-concept stage.

III. TRANSCRIPTOME ENGINEERING

The transcriptome is the complete set of RNAs, both coding and non-coding, present inside a cell or organism [99]. Recently, the transcriptome has garnered more attention with the development of various tools to edit or silence certain transcripts. These tools fall under the umbrella of "transcriptome engineering" where scientists can precisely target the locus they are interested in to modulate the function of RNAs. Transcriptome engineering can help us understand a wide range of biological phenomena such as gene regulation or developmental biology. Furthermore, by analyzing the transcriptome we can decipher disease mechanisms and identify potential therapeutic targets.

RNA is transient, meaning that it is rapidly synthesized, processed, and degraded. Therefore, any changes made to the transcriptome are not permanent. This contrasts genome engineering with tools such as CRISPR-Cas9 which make permanent edits to knock down specific genes [107]. This is both a pro and a con of RNA editing technologies as repeat administration is oftentimes necessary to achieve sustained editing or knockdown. However, the reversible and adjustable nature of many transcriptome engineering tools make them much more translatable to clinical and therapeutic applications. As a result, a plethora of recent research has focused on the development and advancement of technologies in the field of transcriptome engineering.

A. RNA Targeting Cas Effectors

While Cas9 was extensively explored in the early 2000s its RNA-targeting counterpart Cas13 was not discovered until 2015. [108] Cas13 notably contains two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains which enable target RNA cleavage. Similar to Cas9 based systems a guide RNA, also known as a crRNA, is used to direct Cas13 to its target location to induce transcript cleavage (Fig 3). Cas13 is considered a class II CRISPR-Cas system as it only contains a single subunit effector complex [109]. Similarly, Cas9 also belongs to class II. These class II systems have been more thoroughly examined due to the more straightforward effector composition [110]. Abudayyeh et al. identified the class II type VI system which contains the sole effector C2c2 which is RNA targeting [111]. This later became known as Cas13a, but is important foundationally as these class II type VI systems are one of the only RNA targeting Cas effectors. There are various other Cas13 subtypes in addition to Cas13a such as Cas13b [112] and Cas13d [113]. While these are the most widely used there are many more which all function similarly to silence RNA transcripts via cleavage.

1) RNA Silencing via Cas13

RNA silencing via CRISPR-Cas13 is a powerful tool in the field of transcriptome engineering as opposed to Cas9 the Cas13 approach focuses on gene silencing at the post transcriptional level. This allows for discovery of potential therapeutics by knocking down aberrant transcripts, or studying how the knockdown of one gene may have transcriptome wide changes. Other RNA silencing technologies such as RNAi have been found to have high off target effects [114]. Targeting RNA with the CRISPR-Cas13 is a much more translatable alternative as it can perform at similar efficiencies, but with much higher specificity. Similar knockdown efficiencies (40-80%) for multiple endogenous genes were observed when comparing Cas13a to shRNA. It is key to note the researchers here identified hundreds of off-target effects using shRNA compared to none in the Cas13a condition [115]. In 2016, [111] the researchers observed both off-target effects and sequence-specific RNA degradation using Cas13a; however, these experiments were performed in vitro. Degradation of non-target RNAs was also observed [116], but again this was not in the context of mammalian cells. Similar collateral cleavage of off-target RNA was also observed in vitro using Cas13b, but only in the presence of the target sequence [112]. While in vitro applications are less therapeutically relevant than mammalian systems these off-target effects are still something that needs to be carefully monitored no matter the application system.

In addition to non-specific RNA cleavage another hurdle is delivery of Cas13 in vivo.The most therapeutically relevant delivery modality is Adeno Associated Viruses (AAVs), but there is a limit in packaging capacity [117]. One of the main challenges researchers have aimed to solve is discovering new Cas13 variants that are smaller allowing for more efficient AAV packaging [118]. One such example is Cas13d which is only 930 amino acids in size compared to the previously mentioned Cas13a which is 1,250 amino acids [119]. In addition to its smaller size Cas13d, also known as CasRx,

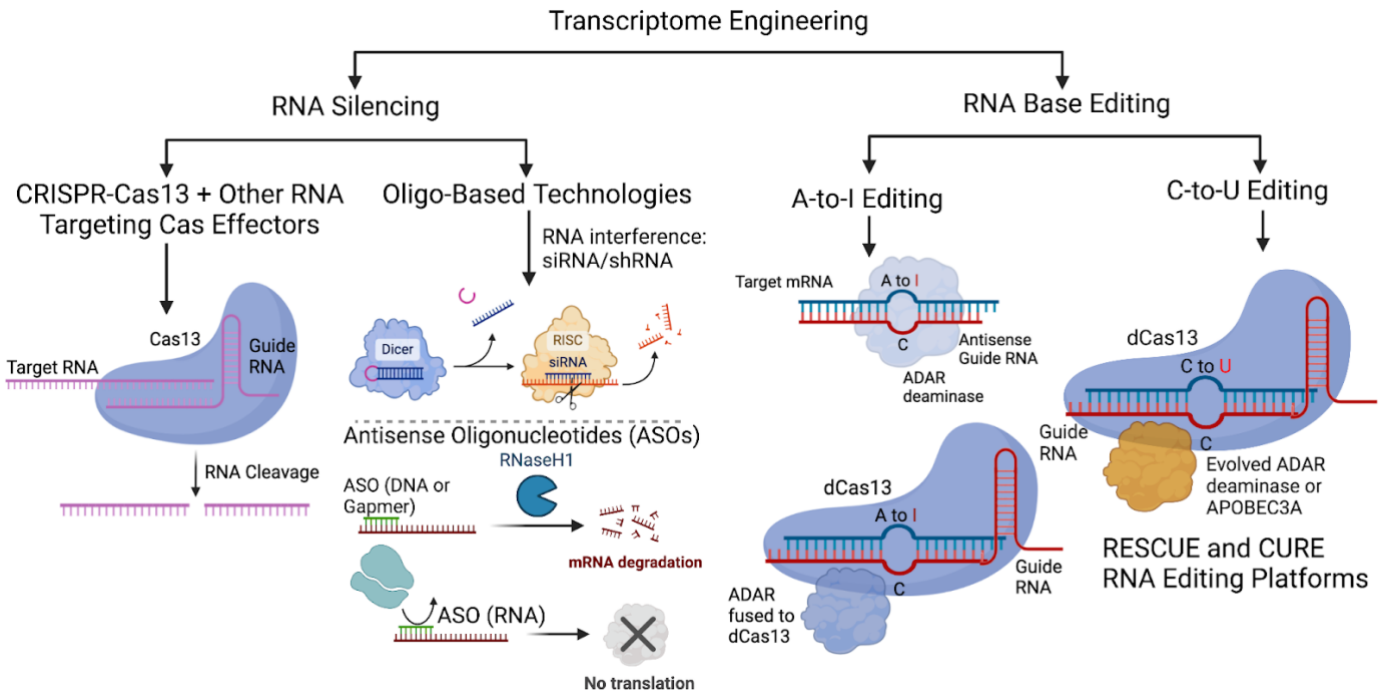

Figure 3. Transcriptome Engineering Tool Selection Flowchart.

exhibited increased knockdown efficiency compared to CRISPRi and shRNA. Additionally the in vivo results [115] indicated there were no off-target effects in mammalian cells using CasRx compared to ~900 significant off-target changes when using shRNA targeting the same transcript. Two other subtypes named Cas13X and Cas13Y were identified with sizes just under 800 amino acids [120]. Again, this novel family was able to show comparable knockdown efficiency to other Cas13 effectors when targeting various endogenous transcripts in mammalian cells with limited off target effects. Furthermore, a novel Cas effector termed Cas7-11 was recently used as an alternative to traditional Cas13 effectors. The name Cas7-11 is derived as this effector combines the Cas11 domain with various subunits from Cas7 [121]. Cas7-11 was found to have similar efficiencies for knockdown of endogenous targets in HEK293T cells compared to LwaCas13a, PspCas13b, and RfxCas13d. Notably, Cas7-11 was found to have significantly less off-target effects than shRNA or RfxCas13d. Again, this underscores the importance of monitoring off-target transcriptome effects when employing these Cas nuclease based systems.

2) High-Throughput Transcriptome Engineering

High throughput transcriptome engineering is a powerful tool to perturb multiple genes simultaneously. This can allow researchers to uncover genetic links, and understand how different families of genes work together in the transcriptome. Furthermore, this tool can be leveraged to determine optimal gRNA design and most effective target locations.

Specifically, a high throughput gRNA screen identified a “seed region” of Cas13d [122]. This “seed region” was defined as a short sequence in the crRNA that plays a key role in recognizing and binding to target RNA. Any mismatches in this region have been shown to cause much more drastic decreases in knockdown efficiency versus mismatches at other locations [123]. High throughput screens have also focused on tiling target RNA with an array of gRNAs. These screens have revealed high knockdown sensitivity depending on certain regions the gRNA is targeting; for example, strong negative correlation was shown between crRNA potency and cytosine nucleotides in the spacer sequence [124]. This underscores another main design consideration that needs to be taken into account when utilizing the CRISPR-Cas13 system. Combinatorial screening is a very powerful tool that has been exhaustively explored in the context of genome engineering. More recently these screening principles have been applied to the transcriptome field. A combinatorial screening platform named MEGA (Multiplexed Effector Guide Arrays) was used to analyze CAR T cells [125]. Through this system they were able to identify pairs of genes that work synergistically to regulate T cell function. Another technology named CaRPool-seq merged Cas13 RNA knockdown and Perturb-Seq [126]. This combinatorial screen allowed for elucidation of interactions between chromatin regulators involved in acute myeloid leukemia. Both of these Cas13 based screening methods have multiple advantages over traditional Cas9 knockdown screens. Mainly, altering DNA with Cas9 can have various genotoxic side effects such as large deletions and chromosomal truncation [39], [127]. By focusing on the transcriptome we can circumvent these issues by knocking down transiently made RNA. An example of a high throughput Cas13 knockdown screen examining cancer cell fitness can be seen in Fig 4.

3) Recent Developments and Future Directions

The recent work utilizing Cas13 has focused on alleviating the two potential pitfalls mentioned earlier. First, the possibility of off-target effects and second, size limitations for efficient packaging into AAVs. A 2024 paper utilized the protein structure prediction software AlphaFold to engineer mini variants of Cas13b and Cas13d [128]. These “mini” variants were able to recapitulate both RNA binding and cleavage activity as their full size counterparts. This highlights the

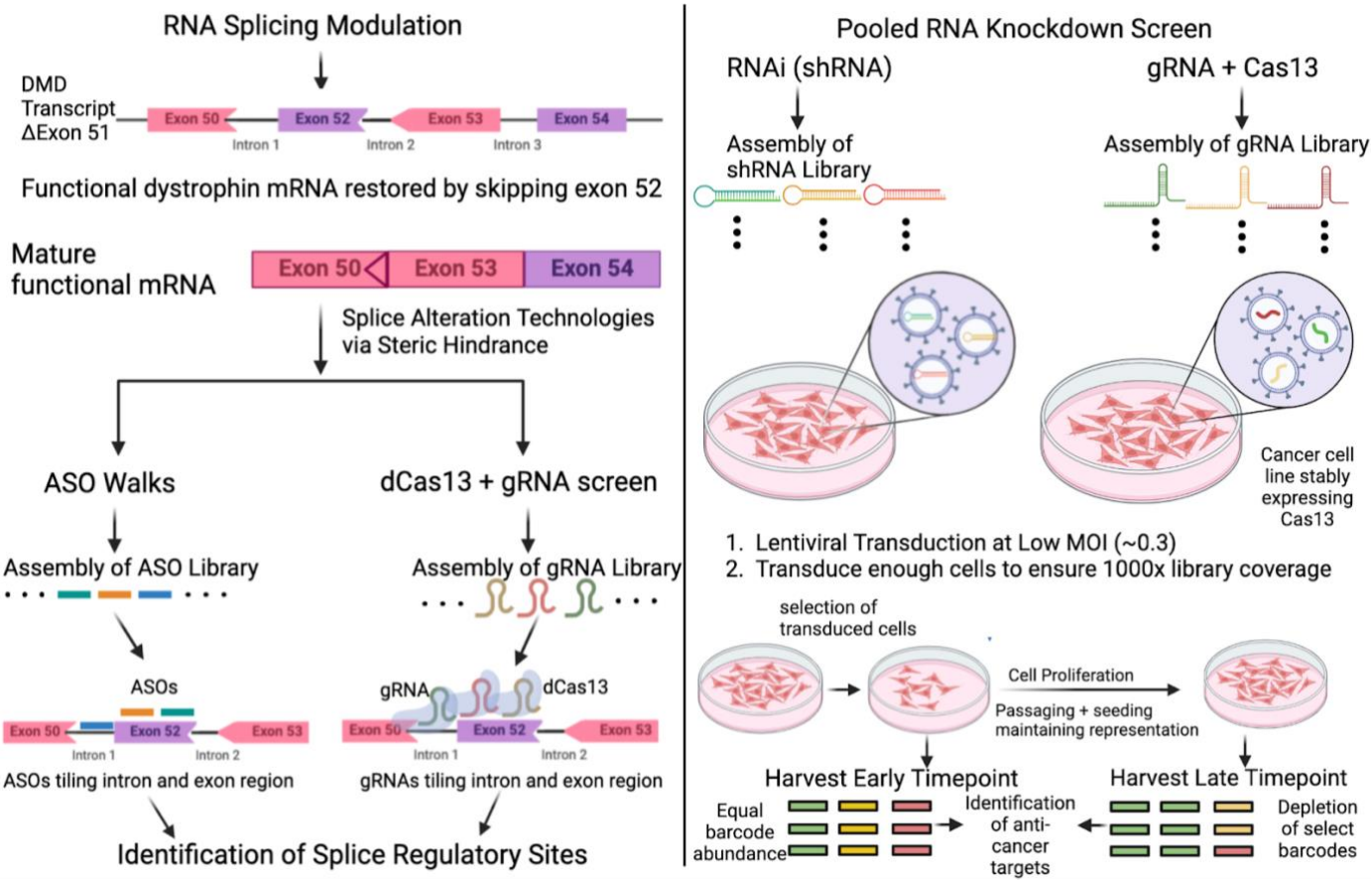

Figure 4. High Throughput Transcriptome Engineering Workflows.

power of computational approaches, which have been gaining increased attention, in discovering and novel Cas effectors. Furthermore, computational methods such as machine learning and deep learning have been used to predict both on-target and off-target activity of various guide sequences.

Another novel application of Cas13 effectors is targeting RNA viruses. Recent work has shown the ability of CRISPR-Cas13 to eliminate the EV-A71 RNA virus in vivo [117]. There is currently no treatment for EV-A71, also known as hand, foot, and mouth disease. Cas13 systems offer a very unique treatment modality as they have the ability to cleave both the viral RNA genome and viral mRNA.

Broadening the application of the CRISPR-Cas13 system from silencing endogenous transcripts to cleaving viral RNA opens the door for a whole new clinical application.

The field is still focused on developing Cas13 effectors with increased specificity for certain applications. For example, developing a specific CRISPR-Cas13 platform for a certain disease associated repeat sequence associated with ALS [129]. In addition, recent work has been focused on improving the translatability of CRISPR-Cas13 to patients. One of the main hurdles is the inflammatory immune response in response to the foreign Cas protein [130]. As a result, there is still need for improvement to Cas13 via certain chemical modifications to decrease this immune response, and allow for application to human patients.

Lastly, there is still a great deal of investigation into the off-target effects of the various Cas13 effector subtypes in different contexts.As mentioned earlier [115], [119] little off target-effects were reported using Cas13a and Cas13d when targeting endogenous transcripts in mammalian cells. Contrary to this recent work has identified off-target cleavage when using Cas13d in both drosophila and human cells [131]. One interesting finding was that off-targets effects seemed to be positively correlated with level of on target transcript. It is worth noting that the off-target cleavage results did vary based on cell type such as HeLa, HEK293, or drosophila. Thus, an important aspect in the biological system that must be considered when employing Cas13. Overall, the CRISPR-Cas13 system is a very powerful transcriptome engineering tool that has wide applications for RNA silencing.

B. RNA Silencing Technologies

1) Antisense Oligonucleotides (ASOs)

Long before the discovery of RNA targeting Cas effectors antisense oligonucleotides were the gold standard for RNA knockdown. As early as 1979 it was discovered that hybridization of a DNA oligo to a target RNA could induce RNase H mediated cleavage [132]. At their most basic level antisense oligonucleotides are chemically modified single stranded RNA or DNA around 16 to 30 base pairs in length [133]. They bind to target RNA using Watson-Crick base pairing, and can modulate RNA in a variety of ways [134]. The main method is via RNase H mediated cleavage which recognizes the DNA portion of the RNA-DNA hybrid that is formed when an ASO binds [135]. ASOs can also be ssRNA and function to inhibit translation by sterically blocking ribosomal machinery [136]. Both of these methodologies, although different mechanisms, work toward the same goal of mRNA silencing (Fig 3).

The main consideration when using ASOs is the chemical modifications that need to be incorporated to increase their effectiveness. As mentioned prior ASOs are single stranded oligonucleotide molecules, and as a result oftentimes have short plasma half-lives in vivo [137]. This is especially true in the case of ssRNA ASOs as this form of RNA is very prone to degradation. The

two most prominent modifications are phosphorothioate (PS) and 2'O-methoxyethyl (2'-MOE) [138], [139]. The PS modification replaces the non-bridging oxygen in the phosphate with a sulfur atom. This allows for broader distribution, and gives ASOs amphipathic properties enabling them to interact with various proteins facilitating cellular uptake [140]. The 2'-MOE ASO involves modification of the 2'-hydroxyl group on the sugar [141]. This modification has been shown to increase potency by improving binding affinity, and have better pharmacokinetic properties [142]. Specifically PS ASOs have been shown to have tissue half-lives of about 48 hours, while 2'-MOE PS ASOs have half-lives of 2 to 4 weeks [143]. This allows for much more infrequent dosing making applications more cost effective and safer for the patients. One thing that must be mentioned here is that 2'-MOE is an RNA modification, but complete RNA based ASOs cannot elicit RNase H cleavage. This is where the development of the "Gapmer" ASO comes from. Here the central region of the ASO is composed of DNA while the flanking nucleotides are RNA with the 2'-MOE modification [144].

ASOs offer a key advantage over the previously discussed CRISPR-Cas13 system. ASOs are much more translatable from lab to clinical use on patients due to reduced immunogenicity versus Cas13. Cas is a foreign protein, and thus its applications are limited in applying it to the patients. ASOs on the other hand are simply RNA or DNA so elicit much less of an immune response. The main downside here is that manufacturing ASOs is oftentimes very expensive. This is due to the extensive chemical modifications that need to be made before the ASO can be used which is a very energy-intensive, high waste process [145].

ASOs have been around for almost 50 years, but there are still a great deal of advancements being made in this field. Specifically, this research has focused on further modifications to both reduce potential toxicity and increase potency. For example, a group developed a new ASO termed BNAP-AEO that involved utilizing a modified sugar [146]. This modification both decreased CNS toxicity and enhanced silencing effects. Furthermore, recent work has focused on making high throughput experiments with ASOs more efficient. A biophysical screening strategy was developed that used various techniques such as differential scanning fluorimetry and surface plasmon resonance to determine ASO binding affinity in a high throughput manner [147]. Currently, there are over 20 FDA and EPA approved antisense drugs with many more being currently developed [148]. ASOs are such a powerful tool as in theory with the correct design they can target almost any endogenous RNA through simple Watson-Crick base pairing rules.

2) RNAi - Small Interfering RNA (siRNA)

siRNAs employ a similar mechanism to ASOs in that they bind to their target via Watson-Crick base pairing [149]. siRNAs are delivered as dsRNA where they are first cleaved by a dicer to form what is actually termed siRNA [150]. The siRNA forms a RISC complex which involves various other proteins. This siRNA and RISC complex binds to its target mRNA and induces cleavage thus silencing the transcript [151] (Fig 3).

SiRNAs suffer from the same pitfalls as ASOs. Specifically, they have relatively poor pharmacokinetic properties as once they are in their single-stranded RNA form they are very quickly degraded by nucleases [152]. As a result, incorporation of various chemical modifications can both increase potency and improve pharmacokinetics. The same 2'-MOE, 2'-O-Me and 2'-F sugar modification and PS backbone modifications that were discussed prior for ASOs have also been applied to siRNAs [153], [154].

Delivery systems for siRNA vary based on the desired application. Naked delivery involves using no delivery system at all, and in this case the siRNA is naturally filtered through the kidney [152], [155]. In addition to the kidney siRNAs rapidly accumulate in the liver which plays a main role in detoxification [156]. To reach other organs such as the eye local injections have been used to exhibit promising effects [157]. The main delivery system that has been explored involves using lipid based systems. Lipid nanoparticles (LNPs) are powerful in combination with siRNAs as they protect the siRNA from nucleases and combat renal clearance [158]. The first siRNA-based drug approved by the FDA by the name Patisiran (Onpattro) is formulated and delivered using LNPs [159].

Another modification which has garnered a lot of attention is N-acetylgalactosamine (GalNAc). This is a sugar that serves as a ligand for the asialoglycoprotein receptor (ASGPR) [160]. This receptor is only expressed on liver cells (hepatocytes) and in very high abundance. As a result this modification is highly effective for therapies where liver targeting is desired [161]. Companies have also developed antibody-oligonucleotide conjugates for improved delivery [162]. In a similar fashion to GalNAc these antibodies can be used for very specific targeting to certain tissues or cell types of interest by honing in a certain receptor.

3) RNAi - Short Hairpin RNA (shRNA)

shRNAs work using the same mechanism as siRNAs, but differ in how they are produced and processed. shRNA is processed within the cell into siRNA where it can then function to knock down genes [163]. The main benefit here over siRNA is that shRNA can be incorporated into plasmid vectors. Furthermore, they can be integrated with viral vectors allowing for host genome integration [164]. This allows for continuous production and a more long-term knockdown effect. shRNA is a single-stranded RNA molecule that folds on itself to form a double-stranded stem with a hairpin loop [165]. Similar to siRNA which is double stranded RNA the dicer functions to cleave this molecule into single strands known as mature siRNA.

Although shRNA is advantageous due to being more persistent, it is less translatable because it requires an expression vector. In the case of patient applications delivering plasmid or lentivirus is not as appealing as simply delivering synthetic RNA in the case of siRNA [54]. These safety concerns regarding delivery have hindered the translation of shRNA to clinic, but it still remains a powerful tool for gene therapy due to its long-lasting nature. Furthermore, both siRNA and shRNA have been shown to have significant off-target effects as guides can bind to other targets with partial guide similarity [166], [167]. Similar to the CRISPR-Cas13 system, high throughput RNAi screens can be used to analyze knockdown of a pool of targets. shRNA-expressing plasmids make these screens very straightforward as plasmid libraries can be quickly constructed for targets of interest [168], [169]. Then they can be utilized in a pooled manner to cells the same way gRNA libraries are. One of the most common screening types are barcode enrichment screens. In these types of screens gDNA is isolated, barcodes amplified, and sequenced using NGS [170]. An overview of one such screen applied to identification of anti-cancer gene targets is shown in Fig 4. These sort of fitness screens are a very common application of pooled RNA knockdown screens due to straightforward

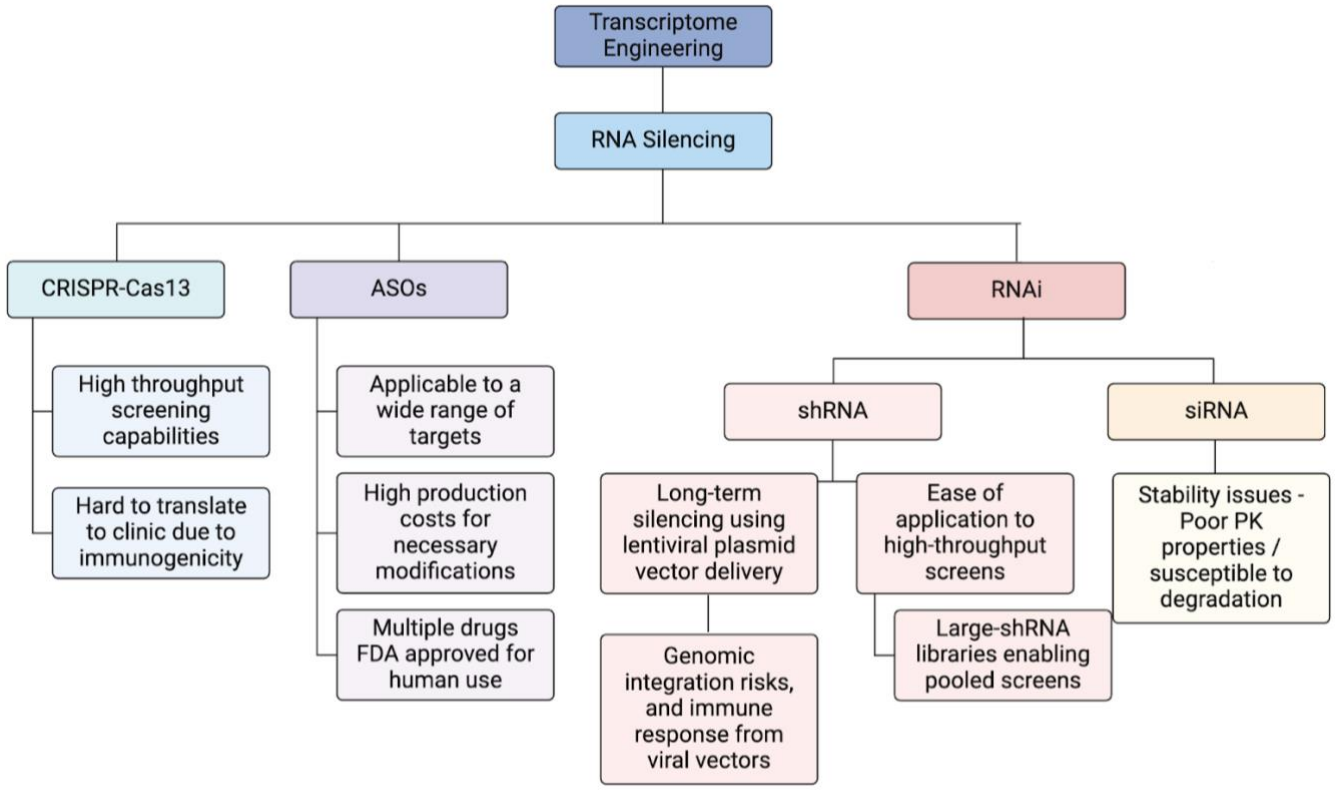

Figure 5. Transcriptome Engineering Tools in Depth.

readout in terms of barcode depletion. While viable, RNAi screens have been shown to have high off-target effects, and thus have fallen out of favor for the more modern CRISPR-Cas pooled screens (Fig 4).

A brief overview of the main RNA silencing technologies discussed above such as CRISPR-Cas13, ASOs, siRNA, and shRNA can be seen in Fig 5.

C. Splicing Modulation

1) Antisense Oligonucleotides

ASOs were discussed in depth prior as a well-studied method to silence transcript expression either through degradation or steric blockage of translation machinery. The ability of ASOs to modulate splicing was discovered later on in 1993 analyzing beta-thalassemia [171]. Specific mutations in beta-thalassemia cause aberrant splicing; however, targeting ASOs to the intron branch point led to restoration of correct splicing. ASOs can modulate splicing in a similar manner to how they inhibit translation through steric hindrance. Instead of blocking translation machinery they instead block splicing factors from binding to pre-mRNA [172].

A popular tool that has been employed in this sphere is “ASO walks” offering screens to determine which ASOs are most effective [173]. These walks tile intronic or exonic regions of pre-mRNA to potentially identify key splice sites. This tactic of ASO walks can be seen in Fig 4. One of the main diseases associated with aberrant splicing is spinal muscular atrophy (SMA). In SMA there is a loss of function of the SMN1 gene. The SMN2 gene has the same function, but there is a mutation that causes skipping of an exon 7 greatly reducing levels of function protein produced [174]. A two-step ASO walk was used first tiling exon 7 and its flanking introns using 15mers with 10 nt overlap [175]. Then going back and again tiling the hit regions with finer resolution of single nucleotide steps. This work led to the development of Spinraza (Nusinersen) which is an ASO targeting a splice silencing site in the intronic region after exon 7 [176]. A very similar two-step ASO walk screen was employed analyzing familial dysautonomia where a mutation in the IKBKAP gene causes exon 20 to be skipped [177]. This screen had the same goal of identifying ASOs that would increase exon 20 inclusion restoring normal gene function.

Similar screens have been done for other targets where the preferred therapeutic outcome was reduction of exon inclusion [178]. In the case of Duchenne Muscular Dystrophy the ASO would induce skipping of a certain exon leading to restoration of the correct translation frame [179]. Currently, four ASO therapeies have received conditional FDA approval for DMD and more are in clinical trials [180]. An example of how ASO walks can be applied to discovery of splice regulatory sites for treatment of DMD can be seen in Fig 4.

2) dCas13 + gRNA

dCas13 is a catalytically dead version of Cas13 which does not have the ability to perform transcript cleavage. Instead dCas13 has a role in blocking via steric hindrance similar to how RNA based ASOs function. Therefore, instead of using ASOs to modulate splicing, recent research has focused on utilizing dCas13 in combination with a gRNA for the same purpose. This is a much more efficient alternative to previously discussed ASO walks which can be expensive and time consuming [181]. High throughput screens of this nature can be used to identify splice regulatory sites [182] (Fig 4). Once these sites are identified ASOs, which are much more translatable to patients, can be developed that target the same location [181].

D. RNA Editing

1) Large Substitutions

Compared to genome engineering there are much fewer tools to perform edits at the RNA level. There are two main classes of edits that are explored at the transcriptome level which are large substitutions and then single base pair edits. One of the few mechanisms to make larger edits at the RNA level is trans-splicing. Trans-splicing involves correction of mRNA transcripts through introduction of another RNA molecule which can be spliced in as a replacement. Trans-splicing is similar to previously discussed splicing modulation in that both technologies involve tricking native splicing machinery (spliceosome) [183]. In the case of trans-splicing the key is avoiding cis splicing (normal) in favor of trans-splicing of the new replacement RNA. In normal cis splicing the exons are joined together, and the introns are spliced out. In trans-splicing splicing occurs in between two separate RNA transcripts one of which usually carries a repaired cDNA sequence. Trans-splicing involves the delivery of a PTM (pre-trans-splicing molecule) which has the replacement RNA, a binding domain to the target of interest, and then an intronic sequence that functions to facilitate splicing [183]. This intronic sequence is recognized by the spliceosome and other pieces of splice machinery to allow for the PTM to be spliced onto the target transcript of interest. An overview of the trans-splicing PTM design and workflow can be seen in Fig 6.

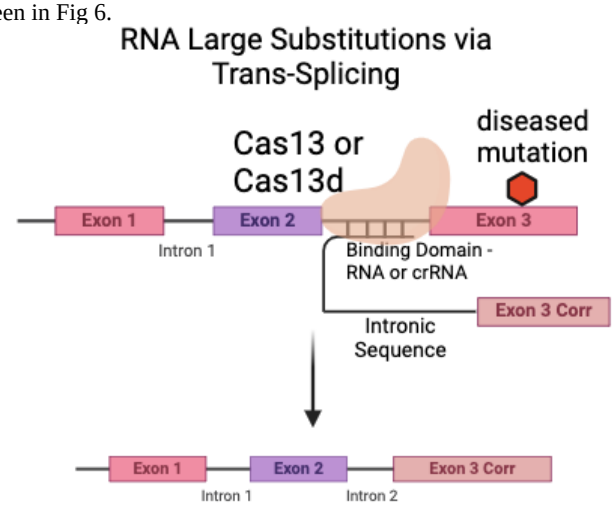

Figure 6. Trans-Splicing Workflow.

Trans-splicing is a field that has been around for 20+ years now, and has always been of great interest due to its versatility and ease of design. A corrective therapeutic that only involves one piece of RNA is very appealing; however, the field has been limited by poor efficiencies and off-target effects [184]. There have been a number of papers exploring the treatment of various diseases using trans-splicing to replace an exon or group of exons where there is a mutation. Some examples include cystic fibrosis, retinitis pigmentosa, and haemophilia A [185], [186], [187]. As mentioned prior these have all been limited by minimal efficiencies. In most of these cases in the PTM the binding domain ranged from 70-150bp complementary to the intron of the target mRNA transcript [188]. One major factor in trans-splicing efficiency revolved around not only the size of the binding domain, but also where on the intron the binding domain was targeted to. Here the binding domain serves a key role of bringing the PTM and the target transcript in close proximity so that trans-splicing can occur.

Trans-splicing for the last roughly 10 years has been a relatively quiet field; however, recently there have been a new wave of papers all focusing on a new method for highly efficient trans-splicing. These papers combined the power of the previously discussed RNA targeting Cas effectors with the versatility of trans-splicing. Either catalytically active or catalytically dead Cas effectors, or a combination of both were used in order to inhibit cis splicing and boost trans splicing. Fifils et al. utilized Rfx-dCas13d (catalytically dead) as a means to block native splicing machinery via steric hindrance [189]. This is the same mechanism discussed in the previous section of splicing modulation. Furthermore, since the cargo is being guided by a crRNA allowing for increased efficiency and specificity. Various efficiencies for endogenous targets ranging from ~20-50% were observed compared to sub 10% when using a solely RNA binding domain.

The PRECISE system explored by Schmitt Ulms et al. employed the Cas7-11 effector which was discussed prior as having multiple advantages over RfxCas-d13d in terms of decreased off-target effects [190]. There was a wide range in editing efficiency observed ranging from ~15% to 85%. Lastly, Chandrasekan et al. explored the RESPLICE system which combined dCasRx to inhibit cis splicing via steric hindrance with Cas7-11 to cleave the native transcript further decreasing cis splicing [191]. Similar efficiencies to the previously discussed papers were achieved for this dual Cas system. Although these papers did show increased efficiency over traditional RNA binding domain technologies they are less translatable to the clinic due to the immunogenicity of the Cas protein. Furthermore, efficiencies widely vary for different endogenous targets indicating there is a great deal of room for optimization. Nonetheless, trans-splicing remains a powerful technology to make large RNA edits as it is one of the only transcriptome engineering tools for this application.

E. RNA Base Editing

1) Adenosine deaminase acting on RNA – ADAR

RNA editing is a powerful tool as it enables transient editing rather than permanent genomic changes with technologies such as base editors. To enable RNA editing we can leverage the capabilities of RNA editing enzymes such as ADAR [192]. This enzyme is capable of catalyzing A-to-I editing on dsRNA where there is an A-C mismatch [193] (Fig 3). ADAR enzymes are naturally active in the body catalyzing posttranscriptional A-to-I editing of RNA transcripts. For example, ADARs have been shown to be especially active in the brain with high editing rates in neuronal cells. [194] As a result, this enzyme can be translated to a therapeutic context by directing its editing location. The most common strategy to perform this modification is utilizing an antisense guide RNA where there is a cytosine mismatch at the adenine location of interest [195], [196]. Furthermore, circular ADAR-recruiting guide RNAs (cadRNAs) have been leveraged for increased efficiency of A-to-I editing [197]. This can be attributed to prolonged guide persistence due to circularization which removes free ends that are degraded by exonucleases [198]. ADAR base editing has been validated both in vitro and in vivo, and has powerful applications in correcting point mutations. Specifically in Duchenne Muscular Dystrophy there is a premature stop codon in the dystrophin gene that can be corrected using ADAR technology [192]. The same paper also explored correction of ornithine transcarbamylase (OTC) deficiency where there is a G-to-A mutation leading to non-functional protein production. Overall, ADAR serves as a powerful therapeutic tool for a variety of diseases caused by specific point mutations. This year, a chemically modified RNA has entered clinical trials to address single-base editing to treat the genetic disorder alpha-1 antitrypsin deficiency (AATD) [199].

2) dCas13 + ADAR

ADAR has also been used in combination with dCas13 which is the catalytically inactive form of Cas13. The ADAR enzyme can be used to dCas13 for more targeted base editing (Fig 3). One such example is the REPAIR system. The main advantage of introducing

the Cas protein here is enhancement of target recognition and stringency [200]. Researchers here were able to achieve a roughly 30% correction rate which was similar to the editing efficiencies found in [192]. It was determined that there was a significant number of off target A-to-I edits [200]; therefore, a rational protein engineering approach was used to create REPAIRv2. Here the ADAR2 protein was mutated leading to a decrease in off-target effects and maintenance of on-target editing specificity. Recently it has been shown that mutagenizing positive amino acids of the ADAR2 protein can decrease off-target effects as this reduces general interactions with non-target RNAs [201].

3) C-to-U Editing

A system was developed termed RNA Specific C to U Exchange (RESCUE) which allowed for programmable C-to-U edits using an engineered ADAR2 enzyme [202]. The adenine deaminase domain of ADAR2 was evolved into a cytidine deaminase (Fig 3). RESCUE still retained A-to-I editing capabilities while now also being able to perform C-to-U editing. While this does enable multiplexing of edits there were still issues encountered with off-target editing.

In addition to ADAR other enzymes have been used for specific RNA editing. The APOBEC3A enzyme is a cytidine deaminase, and has been shown to catalyze C-to-U edits [203] (Fig 3). The APOBEC enzyme family targets ssRNA while ADAR targets dsRNA [204]. The CURE system was developed which combines APOBEC3A with dCas13 to perform high-specificity C-to-U edits [205]. It was reported that CURE had lower off-target effects than RESCUE, but there were limitations regarding editing of certain motifs such as AC and GC. Recent research has shown addition of a short histidine-rich domain to both RESCUE and CURE can increase on-target editing rates [206] with only slight increases in off-target effects. Despite challenges with efficiency and non-target transcript editing, RNA base editors remain versatile methodologies to correct a variety of cytosine point mutations.

IV. Conclusion

There have been rapid advancements in genome and transcriptome engineering over the past decade. The development of precise and versatile tools such as CRISPR-Cas systems, base editors, and prime editors has vastly improved our ability to edit the genome with high accuracy. In tandem, the advent of dCas9 activator and repressor fusions have enabled precise transcriptional control. These technologies can not only be used to probe gene function, but also develop novel therapeutics for a variety of diseases.

Transcriptome engineering has evolved a great deal starting with the classical RNA silencing tools such as ASOs and RNAi. Recent advances have shifted to using the CRISPR-Cas13 system and other RNA targeting Cas effectors for the same purpose with increased efficiency and specificity. Furthermore, precise systems for RNA base editing using ADARs or APOBEC have opened the door for a whole new class of transcriptome engineering.

The future of these technologies lies in increasing efficacy and reducing off-target effects which has shown to be an issue for both transcriptome and genome engineering tools. Furthermore, current research is focused on improving delivery modalities for these engineering tools to increase efficacy and safety profiles. Both reducing off-target effects and improving delivery modalities will allow for increased translation to clinical and therapeutic applications.

Overall, the continuous evolution of genome and transcriptome engineering tools is set to revolutionize our understanding of biology and the treatment of genetic diseases. As we overcome the current technical and delivery challenges, these technologies will likely become integral to both basic research and clinical practice, offering new avenues for precision medicine and innovative therapeutic strategies.

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