and full-length cDNA allowed transformation of the gene into a cultivated spring wheat variety, where it conferred resistance to stem rust.

Witek *et al.*[5] pursued a similar approach to clone resistance genes specific for potato late blight, a major potato disease that is currently managed by multiple pesticide applications during the growing season. They also used RenSeq to capture a library of gene sequences with weak homology to known R genes[6] but combined the method with long sequence reads using Pacific Biosciences technologies. Long reads and ~20× coverage with high-accuracy short reads enabled *de novo* assembly of the NLR repertoire from a previously unsequenced, highly divergent wild relative of cultivated potato. Rather than using mutagenesis, Witek *et al.*[5] relied on initial bulk-segregant genetic mapping of the resistance locus followed by fine mapping to distinguish between three linked R gene clusters. Identification of the cognate R gene was completed by expressing full-length clones of candidate R genes in *Nicotiana benthamiana* leaves and identifying the gene that conferred protection to *Phytophthora infestans* challenge. A transgenic potato line harboring the resultant R gene was resistant to *P. infestans* infection.

The third paper, by Kawashima *et al.*[3], accesses R genes from a donor species that cannot be crossed with the target crop. All commercial soybean cultivars are susceptible to Asian soybean rust, caused by *Phakopsora pachyrhizi*, which is prevalent in South America. Fungicide use in this region has subtantially increased owing to the incidence of Asian soybean rust. The authors take advantage of the observation that some accessions of pigeonpea are fully resistant to Asian soybean rust. One pigeonpea accession was found to be resistant to all of the 80 diverse soybean rust isolates that were tested. The relevant R gene from this accession was cloned by genetic mapping of closely linked markers, isolation of genomic BAC clones carrying the candidate gene region and shotgun sequencing to identify the four NLR-encoding genes on the BACs. One of these candidate genes, *CcRpp1*, produced rust-resistant genetically modified soybeans.

The methods described in these papers expedite R gene isolation, but caveats remain. For example, some R gene products 'guard' a second host protein[1,2], and for R genes from less-related plant species, both the R gene and the gene encoding the guarded protein may need to be engineered into a susceptible plant. Also, in a mutagenesis-based screen such as that adopted by Steurnagel *et al.*[4], some susceptible plants may arise from mutations in genes other than R genes, and susceptible R gene mutants may be difficult to detect if more than one R gene in the mutagenized parent recognizes the pathogen used for mutant screening. Screens for R genes may be most successful when resistance elicitation can be attributed to a single pathogen effector; such efforts require more initial research but are already well underway for many plant pathogens[7]. Use of isolated effectors may be especially preferable if it leads to identification of R genes that target those effectors that are most common in, and least readily jettisoned by, the pathogen population[7].

Most researchers agree that to increase the durability of R gene efficacy it is important to release only plants that carry at least two stacked R genes that are effective against the same pathogen strains[1,2,8]. Individual pathogen isolates must harbor two rare mutations in virulence proteins to successfully reproduce on a plant that harbors two stacked R genes. Indeed, Kawashima *et al.*[3] stress that soybeans expressing *CcRpp1* should be released to growers only as part of a multicomponent resistance package, as rusts that overcome *CcRpp1* resistance might otherwise arise within a few growing seasons. R genes pyramided as a single multigene cluster offer the further advantage of expediting plant breeding by generating co-inheritance at a single locus.

Taken together, these papers[3–5] present methods to substantially expand the pool of available R genes. The next step will require wider acceptance of technologies that move R genes from one plant to another to combat plant disease. In addition, current rules often require expensive 'event-by-event' retesting and recertification each time a previously approved transgene is transformed into a different plant[9,10]. A more rigorous science-based system for risk assessment would be likely to streamline the reuse of R genes in different plants. Unfortunately, engineering of improved R gene repertoires, with concomitant benefits in reduced pesticide application, may currently be feasible only in high-profit crops.

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Comparing CRISPR and RNAi-based screening technologies

Benjamin E Housden[1] & Norbert Perrimon[1,2]

Two studies provide an experimental side-by-side comparison of genetic screening methods.

High-throughput loss-of-function screens are well-established approaches for characterizing genotype to phenotype relationships. Currently, both RNA interference (RNAi) and CRISPR-based screening approaches are used, but so far no systematic side-by-side comparison of their relative merits has been performed. In this issue, studies by Evers *et al.*[1] and Morgens *et al.*[2] perform viability screens in cell lines to assess how reliably and effectively the two methods identify essential genes. Both studies attest to the high sensitivity of CRISPR-based knockout screens, but differ in their assessment of the relative performance of RNAi screens.

RNAi screens in either arrayed or pooled format have been employed to study many different biological questions, ranging from basic biological processes such as signaling or cell morphology to identification of drug targets for human diseases for many years now[3]. However, the technology has several limitations. For instance, many RNAi reagents result in inefficient knockdown of the target gene, leading to false-negative results. In addition, the prevalence of off-target effects, where additional genes are unintentionally perturbed, leads to false-positive results. Such issues have led to poor reproducibility between screens[4] and considerable effort has gone into developing modified

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reagents and approaches to overcome these limitations. For example, short hairpin RNA (shRNA) libraries today contain many independent constructs targeting each gene. By pooling results from each of these reagents, it is possible to overcome limitations of individual reagents to identify high-confidence hits. Nevertheless, it is still necessary to perform extensive follow-up analyses to isolate the most robust hits.

More recently, the toolbox for loss-of-function screens has been expanded by the development of CRISPR-based genome editing methods. In 2013, the bacterial immune system, CRISPR, was repurposed for disruption of genome sequence and genome engineering in mammalian cells[5,6]. CRISPR generates small, targeted insertions or deletions in genomic DNA, which in the coding sequence of a gene has the potential to cause loss of gene function. The technology was quickly adapted for high-throughput use, following guidelines developed over the years for RNAi screens[7]. One of the key differences between CRISPR and RNAi is that CRISPR results in most cases in true loss-of-function effects, which is in contrast to RNAi that generally causes hypomorphic effects. Thus, CRISPR is expected to generate stronger and more consistent phenotypes and, therefore, more robust screen output, though this may be complicated by the creation of heterozygous cells or hypomorphic mutations caused by non-frameshift mutations. Early reports of CRISPR-based screens demonstrated both increased sensitivity and reduced off-target effects compared to RNAi[7]. Understandably, this technology was seen by many as a replacement for RNAi.

The utility of CRISPR screening has now been demonstrated experimentally by results from several studies. For example, a recent screen for essential genes in human cells identified many more hits than previously obtained from RNAi-based screens[8]. In addition, correlation of hits between this screen and other mutagenesis-based methods in human or yeast cells was high, indicating that the results are likely robust. In contrast, a study that used both CRISPR and RNAi-based screens to investigate mechanisms of cytotoxicity of the GSK983 antiviral drug demonstrated that the combination of these two approaches was more powerful than either alone because each method identified only a subset of the relevant genes[9], illustrating that we should not yet discard RNAi-based methods in favor of CRISPR.

Given the various options now available to perform loss-of-function screens, it has become important to assess the relative strengths and weaknesses of the approaches and carefully consider whether one is more appropriate for a given biological question. The studies by Evers *et al.*[1] and Morgens *et al.*[2] now provide a much-needed experimental side-by-side comparison of the two methods.

Both groups used a similar approach to compare the two technologies. First, positive and negative control genes were selected based on the same previously defined set of gold standard essential and non-essential genes[10]. Next, dropout screens were performed using libraries of CRISPR single guide RNA (sgRNA) and shRNA reagents in pooled format in combination with deep sequencing to identify essential genes. Reagents targeting the 'gold standard' control genes were used to assess the relative sensitivity and stringency of each approach. However, although the approaches were broadly similar between the two studies, conclusions differed regarding the relative strengths of CRISPR and RNAi. Evers *et al.*[1] found that the CRISPR screen outperformed the shRNA screen, whereas Morgens *et al.*[2] found that the two technologies performed similarly, though they do note that the CRISPR screen identified more essential genes not present in the gold standard controls.

Although the approaches used by these two studies initially appear similar, there are differences in experimental design, which likely contribute to the contrasting conclusions (**Fig. 1**). For example, the two studies used different shRNA and sgRNA libraries with varying numbers of reagents per gene and different design characteristics. For RNAi, Morgens *et al.*[2] used an ultra-complex shRNA library with 25 reagents per gene that they previously showed outperforms less complex libraries, such as the one used by Evers *et al.*[1] (4.8 reagents per gene). In contrast, the CRISPR library used by Morgens *et al.*[2] was less complex with only four reagents per gene, whereas Evers *et al.*[1] generated 10 sgRNAs per gene. It is well established that shRNA library complexity contributes to the quality of screen output and so these differences in the number of reagents used likely contribute to the stronger relative performance of RNAi in the Morgens *et al.*[2] study. Both studies attempted to address this using computationally downsampled libraries to equalize the number of reagents per gene considered for analysis. In both cases, the conclusions did not change significantly. It therefore remains unclear whether differences in library complexity contributes to the variable performance of each technology.

Although these two studies disagree on the general conclusion, CRISPR performed robustly in both cases, indicating that issues, such as library complexity, are less important with this technology. Together, these studies suggest that the selection of control genes and screening libraries used have a greater effect on screen quality than the loss-of-function approach chosen. However, Morgens *et al.*[2] found that although shRNA and CRISPR screens performed equally well for the detection of essential genes, overlap between results was more limited than expected. In addition, hits from each screening method were clearly enriched for different gene ontology (GO) categories, indicating that the limited overlap is not simply due to false-positive results. The targets therefore fall into three categories. The first category includes genes that were identified by both technologies, which likely reflect true essential genes. The second category includes genes that were identified only by CRISPR. Morgens *et al.*[2] show that these genes are enriched for GO categories associated with transcription (e.g., RNA polymerase and mediator complex) and therefore may reflect the dependence of shRNA production on continued transcription. Additionally, some of these hits may represent cases where the hypomorphic effects of RNAi are insufficient to cause a detectable phenotype. The third category, genes identified only by RNAi, is the most puzzling. The authors suggest several possible explanations for these hits. For example, some genes may have their most potent viability phenotype in a hypomorphic state, perhaps due to disruption of protein complex stoichiometry. Another possible reason for these differences may be non-specific effects caused by shRNA overexpression or Cas9 nuclease activity. In particular, Cas9 activity could induce a potent DNA damage response and overexpression of shRNAs may affect the microRNA pathway. In addition, some null mutations may not produce phenotypes due to compensation by upregulation of genes with redundant functions. Whatever the explanation for these intriguing results, the limited overlap between hits from CRISPR and

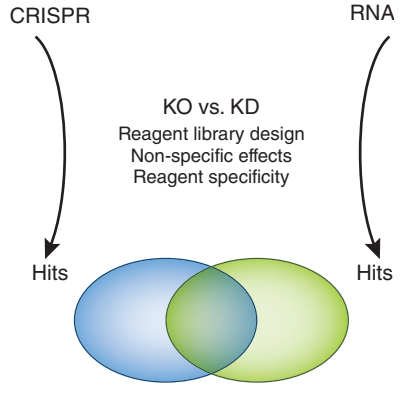

Figure 1 Relative performance of loss of function screens is dependent on many factors. For example, the technology used may lead to knockout (KO) or knockdown (KD) effects. Additionally, factors such as reagent library design, non-specific effects of the technology or reagents will affect the subset of true hits identified.

RNAi screens highlights the need to compare multiple approaches to fully understand the underlying biology.

Moving forward, it is likely that the approach of combining results from CRISPR and shRNA screens will be invaluable to improve our understanding of many biological processes. Related to this, Morgens *et al.*[2] report a computational framework that can be used to combine results from these two screening methods and demonstrate that this considerably improves discrimination between positive and negative control genes.

Finally, it will be interesting to see how CRISPR and shRNA screens compare when tested on more challenging assays, such as developmental phenotypes or the identification of therapeutic targets. When considering such complex biological systems, it is likely that the limitations of each approach will be more pronounced and combining results will prove even more valuable.

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The minimal genome comes of age

Claudia E. Vickers

A genome smaller than any known natural or reduced genome has been designed, synthesized and shown capable of sustaining self-replication in a free-living environment.

In 1973, geneticist Wacław Szybalski was asked during a panel discussion what he would like to be doing in the "somewhat non-foreseeable future." Szybalski's reply: "Up to now we are working on the *descriptive* phase of molecular biology.… But the real challenge will start when we enter the *synthetic* phase…. We will then devise new control elements and add these new modules to the existing genomes or build up wholly new genomes … [and] finally other [synthetic] organisms"[1]. Szybalski foresaw with remarkable clarity the initiation and progression of synthetic biology, a field that focuses on the design and construction of new biological parts and devices. Synthetic biology encompasses a broad variety of tools, approaches and applications, but its ultimate promise, as Szybalski noted, is in synthetic genomics. One aim of synthetic genomics is to devise a minimal genome—that is, a genome that contains only the genes required to sustain free-living self-replication. A functional minimal genome would help broaden understanding of the requirements for life and demonstrate the ability to build synthetic organisms, and it might lead to biotechnological applications. The latest step in that long road was taken recently with a publication in *Science* by Hutchison *et al.*[2], which describes a functional, minimal synthetic bacterial genome that is the smallest yet reported.

For more than two decades, researchers at the J. Craig Venter Institute (JCVI) have been at the forefront of synthetic genomics and efforts toward a minimal genome[3–5]. To realize the minimal genome project, it was necessary to first define a minimal set of genes, generate a complete synthetic minimal genome and demonstrate effective transplantation of the genome into a recipient cell. *Mycoplasma* bacteria (class Mollicutes), which lack a cell wall and have very small genomes, were chosen as the starting model. At 583 kilobase pairs (kbp) and only 525 genes, the genome of *Mycoplasma genitalium* was considered to have close to the minimal gene set required to sustain a free-living existence. Transposon mutagenesis was used to identify a minimal set of 375 essential genes in *M. genitalium* grown under laboratory conditions[3,5]. In 2007, the *Mycoplasma mycoides mycoides* genome was successfully transplanted into a *Mycoplasma capricolum* cell, replacing the native genome and supporting self-replication[6]. The following year, the capability for chemical synthesis of whole genomes was established using the *M. genitalium* genome[7]. However, *M. genitalium* is very slow growing, which hindered progress. In 2010, researchers turned to the faster-growing *M. mycoides mycoides* (1,079 kbp and 901 genes). They synthesized the genome and transplanted it into an *M. capricolum* cell[8]. This produced *M. mycoides* JCVI-syn1.0, the first self-replicating synthetic cell. The JCVI researchers have now refined the minimal gene set and minimized the JCVI-syn1.0 genome to produce JCVI-syn3.0, a functional cell with a minimal genome[2].

The genome of JCVI-syn3.0 is 531,560 bp long and contains 473 genes (438 encode proteins; 35 encode RNA)—smaller than that of any known natural or reduced free-living cell. Strikingly, the biological functions of 149 genes of the minimal genome remain unknown. To create JCVI-syn3.0, Hutchinson *et al.*[2] developed a 'design–build–test' (DBT) cycle that relies on iterations of *in silico* design, genome construction and wet lab testing (**Fig. 1a**). The authors cycled through just four DBT iterations to produce JCVI-syn3.0. The genome of JCVI-syn3.0 is half the size of the JCVI-syn1.0 genome and 50 kbp smaller than that of *M. genitalium*, but almost twice the size of minimal genome estimates based on comparative genomics and shared gene sets. The authors explain that the inclusion of "quasi-essential" genes needed for "robust growth" under laboratory conditions accounts for the larger-than-expected genome size, concluding that JCVI-syn3.0 therefore represents "a working approximation of a minimal cell"[2].

Hutchinson *et al.*[2] also explored the potential for genome reorganization. They completely rearranged a one-eighth segment of the JCVI-syn3.0 genome into a designed, logic-driven, ordered structure in which genes responsible for different functional processes (DNA repair, transcription, translation, membrane functions, nucleotides, glycolysis and 'other') were grouped together. This is the first broad-scale attempt to re-engineer evolved biology at a genomic level according to human design principles, and thus realizes Szybalski's prediction. Clearly, probing the function of every gene required for a functional minimal genome may shed light on the fundamental aspects of the requirements for life, but there are potential biotechnological applications of these findings as well.

One potential application for synthetic genomics is the development of chassis cells: self-replicating minimal machines that can be tailored to synthesize specific products by adding 'bolt-on' genetic modules[9]. Minimal genomes are one starting point, albeit not a prerequisite, for chassis cells. Reducing genome complexity should theoretically enable precise control over regulatory and metabolic programs, improve genome stability, and simplify metabolic modeling, all of which would aid

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