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REVIEWS

Review

Investigating Cellular Recognition Using CRISPR/Cas9 Genetic Screening

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Neighbouring cells can recognise and communicate with each other by direct binding between cell surface receptor and ligand pairs. Examples of cellular recognition events include pathogen entry into a host cell, sperm–egg fusion, and self/nonself discrimination by the immune system. Despite growing appreciation of cell surface recognition molecules as potential therapeutic targets, identifying key factors contributing to cellular recognition remains technically challenging to perform on a genome-wide scale. Recently, genome-scale clustered regularly interspaced short palindromic repeats (CRISPR) knockout or activation (CRISPR-KO/CRISPRa) screens have been applied to identify the molecular determinants of cellular recognition. In this review, we discuss how CRISPR-KO/CRISPRa screening has contributed to our understanding of cellular recognition processes, and how it can be applied to investigate these important interactions in a range of biological contexts.

Highlights

Cells express adhesion and signalling molecules on their cell surface to communicate with neighbouring cells.

Cellular recognition is mediated by direct binding of specific cell surface proteins to trigger intracellular responses and is important for immune response and infectious disease.

CRISPR-KO and CRISPRa screens provide a genetic platform for identifying the molecular determinants of cell surface binding at a genome-scale to further our understanding of cellular recognition processes

Cellular Recognition Is the First Step in Cell Signalling

Cells express a diverse set of signalling molecules on their surface to sense and respond to their environment so that they respond appropriately as a function of their position within the organism. Cellular recognition occurs when specific sets of plasma membrane receptors from different cells bind to each other and trigger an intracellular signal that causes a change in cell state or behaviour [1]. Cell surface molecules involved in cellular recognition are increasingly being regarded as attractive therapeutic targets due to their role in initiating cellular responses and their accessibility to systemically administered biologics such as monoclonal antibodies. For instance, immune checkpoint molecules such as programmed cell death protein (PD)1, PD ligand (PDL)1, and cytotoxic T lymphocyte antigen (CTLA)4 have been the focus of recent cancer immunotherapy aimed at blocking immune evasion by cancer cells. Antibodies against PD1, PDL1, and CTLA4 disrupt immunoinhibitory interactions and enable T-cells to recognise and kill cancer cells more efficiently, resulting in tumour regression in some patients [2]. Cellular recognition is also particularly important in infectious disease, as demonstrated by the development of C-C chemokine receptor (CCR)5 inhibitors for antiretroviral therapy after CCR5 was identified as a coreceptor for human immunodeficiency virus [3], and ongoing work targeting molecules like merozoite surface protein 1, reticulocyte-binding protein homolog 5, and basigin (BSG), which are involved in erythrocyte recognition and invasion by *Plasmodium falciparum*, a parasite that causes severe malaria [4,5].

Despite growing interest in targeting cellular recognition processes for therapy, the study of molecular pathways contributing to cellular recognition remains challenging to perform at scale; not least due to the difficulties of solubilising amphipathic plasma membrane proteins while retaining low-affinity binding partners [6] (Box 1). This is in contrast to intracellular protein complexes that tend to form stable complexes that are soluble in aqueous solutions, making them more amenable to conventional high-throughput protein–protein interaction techniques such as yeast-two-hybrid and affinity purification with mass spectrometry (AP-MS). As a result, cell surface interactions are largely under-represented in most large-scale interaction datasets

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Box 1. Affinity of Cell Surface Interactions versus Intracellular Protein Complexes

The affinity of an interaction is often indicated by its equilibrium dissociation constant (K_D), with a higher K_D value indicating lower affinity since a higher concentration of ligand is needed to provide half-maximal occupancy at equilibrium. As an example, the K_D of nuclear-localised epigenetic factor tripartite motif-containing 28 (TRIM28) binding to Krüppel associated box (KRAB) domains in zinc-finger proteins is 8 ± 2 nM [44]. By contrast, the K_D of cell surface PD1–PDL1 and CD80-CTLA4 interactions are 8.2 ± 0.1and 0.26 ± 0.06 μM respectively, indicating lower affinities by 2–3 orders of magnitude [45,46].

compiled using these techniques [6], although this may improve with recent advancements that facilitate the isolation of plasma membrane-bound complexes using AP-MS [7].

Current Techniques for Cell Surface Interaction Screening

Several techniques have been developed or adapted specifically for the identification of cell surface interactions [8]. In many cases, these approaches rely on the ability to express the ectodomains of membrane-embedded receptors as soluble recombinant proteins that retain their extracellular binding activity (Figure 1A). These techniques include expression cloning [9], avidity-based extracellular interaction screening (AVEXIS) [10], protein microarrays [11], cDNA overexpression microarrays [12], CRISPR-KO [13], and CRISPRa screening [14]. For CRISPR-KO/CRISPRa screening, the soluble ectodomain of the ligand of interest is fused to a biotinylation sequence that is clustered around a fluorescently conjugated streptavidin molecule. This serves the dual purpose of enabling detection of the ligand of interest and increasing binding avidity to facilitate the detection of low-affinity interactions. These protein complexes are then used in cellular binding assays using fluorescence as a readout to indicate binding (Figure 1B). Where these approaches diverge is how candidate receptors are screened, but since the different techniques are beyond the scope of this review and have been extensively reviewed elsewhere [8,15], we only focus on the last two techniques: CRISPR-KO and CRISPRa screening.

CRISPR-KO Screening

Since the discovery of CRISPR in bacteria, these systems have been widely adopted for various applications such as genome-editing and large-scale screening for functional genetics and epigenetics (Box 2). The CRISPR/Cas9 system has been particularly successful as it only requires one nuclease (Cas9) and single guide RNA (sgRNA) to generate targeted gene knockouts (Figure 2A). Given the current ease of generating complex oligomer libraries containing thousands of sgRNAs, CRISPR/Cas9 technology has become the method of choice for genome-wide loss-of-function screens and has been used to identify functional gene interaction networks underlying biological

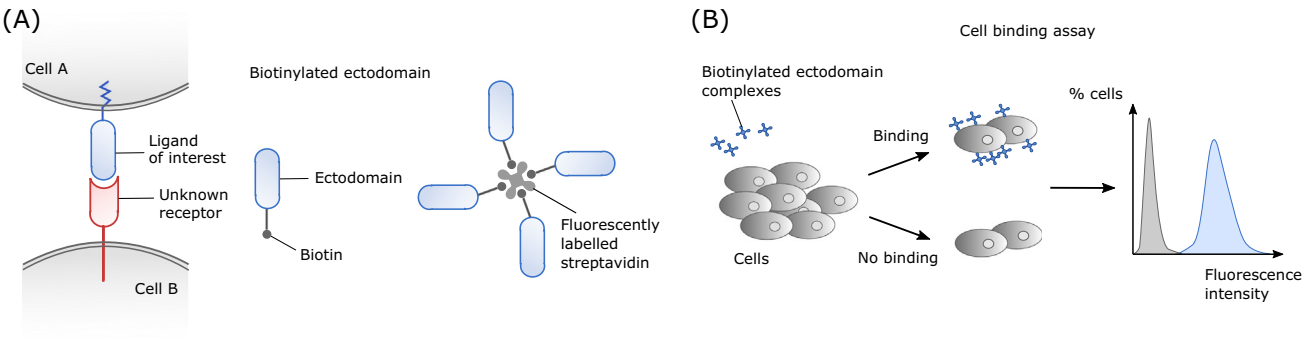

Figure 1. How Recombinant Bait Proteins Are Used for Cell Surface Receptor Identification in CRISPR/Cas9 Screens. (A) Cellular recognition is mediated by receptor–ligand interactions at the cell surface. To identify the receptor for a ligand of interest, the soluble ectodomain of the ligand can be produced recombinantly as a biotinylated bait protein and clustered around a tetrameric streptavidin molecule. (B) Cell binding assays use fluorescently labelled bait protein complexes to determine if a population of cells express a receptor capable of binding the ligand. Binding can be measured by flow cytometry using fluorescence intensity as a readout.

Box 2. How Do CRISPR/Cas9 Systems Work?

CRISPR genome editing systems are adaptive immunity strategies used by bacteria and archaea to provide protection against foreign genetic elements. The simplest among the CRISPR systems is type II, which utilises a single large multidomain effector protein (Cas9) guided by a coprocessed dual crRNA (CRISPR RNA): trans-activating crRNA (tracrRNA) molecule to perform foreign DNA recognition and cleavage. The crRNA and tracrRNA duplex can be substituted by an sgRN), a chimeric RNA performing the same function. The sgRNA can be engineered to target a DNA sequence of choice by altering the 20 bps at the 5′ end complementary to the target DNA sequence.

Once at the target site, the two endonuclease domains of Cas9, the HNH and the RuvC domain, cleave the two strands of DNA creating a double stranded break, which is repaired by the cellular DNA repair machinery either through nonhomologous end joining (NHEJ) pathway or the homology-dependent repair pathway. The NHEJ pathway, while being the preferred cellular pathway, is also error prone, which can lead to the generation of indel mutations leading to loss of gene function.

CRISPRa systems are derived from the principles of conventional type II CRISPR systems; however, the Cas9 protein is mutated such that it no longer cleaves DNA. This endonuclease dead Cas9 (dCas9) is fused to transcription activator domains, creating a programmable transcriptional activator. Upon binding of dCas9 to a promoter, transcriptional activators facilitate RNA polymerase recruitment to initiate transcription. A number of specific activation systems have been developed including the VP64-p65-Rta (VPR) system, the synergistic activation mediator (SAM) system, and the SunTag system [47]. A similar principle is also used in the CRISPR interference system (CRISPRi). dCas9 bound to a DNA sequence on its own creates steric hindrance to the transcriptional initiation or elongation machinery leading to transcriptional repression to an extent. Additional modifications to the dCas9 via fusing it to further transcriptional repressor protein domains such as KRAB or KRAB and MeCP2 increases the efficiency of this repression [48].

Genome-wide CRISPR-based screens utilise libraries of sgRNA that are designed to target every gene in the genome. In CRISPR-KO screens, Cas9 is targeted to the coding regions to create genome-wide knockout cell libraries, whereas in CRISPRa systems, sgRNAs are targeted to the promoter regions to create libraries of overexpressed genes. Such libraries provide a powerful platform to identify gene functions in an unbiased manner.

processes such as cell signalling and virus–host interaction [16–18]. CRISPR-KO screening now provides an alternative genetic platform to systematically investigate genes that contribute to cellular recognition on a genome-wide scale.

The procedure for performing cell surface receptor identification using genome-scale CRISPR-KO screening has been described [19]. A typical screen begins with the identification of a Cas9-expressing cell line that binds the ligand of interest (Figure 2B). Next, the cells are transduced with a lentivirus library containing sgRNAs targeting all protein-coding genes in the genome at a low multiplicity of infection (MOI) of 0.3 to ensure that, on average, each cell receives a single sgRNA. Due to the low MOI, not all cells are transduced, and untransduced cells must be removed by fluorescence-activated cell sorting (FACS) or antibiotic selection, depending on selection markers present in the lentiviral sgRNA expression vector. In this example, expression of blue fluorescent protein (BFP) indicates successful lentiviral transduction and therefore sgRNA expression (Figure 2B). Next, the transduced cell population is separated into control and treatment populations of equal numbers. The control population is used to determine the baseline frequency of each sgRNA within the population, which may vary between independent transductions, while the treatment population undergoes further selection using a binding assay as previously mentioned (Figure 1B). In this example, phycoerythrin (PE) fluorescence is used as a readout for binding (Figure 2B). Transfected cells (BFP^+) that have lost the ability to bind PE are segregated from the bulk population using FACS and sent for next-generation sequencing to determine the frequency of each sgRNA in the sorted population. The relative abundance of each sgRNA is then compared between sorted and control populations to identify genetic factors essential for binding of the protein ligand. The binding assay can be modified according to the specific aim of the screen; for instance, to study the determinants of receptor presentation on the plasma membrane, monoclonal antibodies targeting a known receptor could be used instead of a recombinant bait protein for sorting of the treatment population.

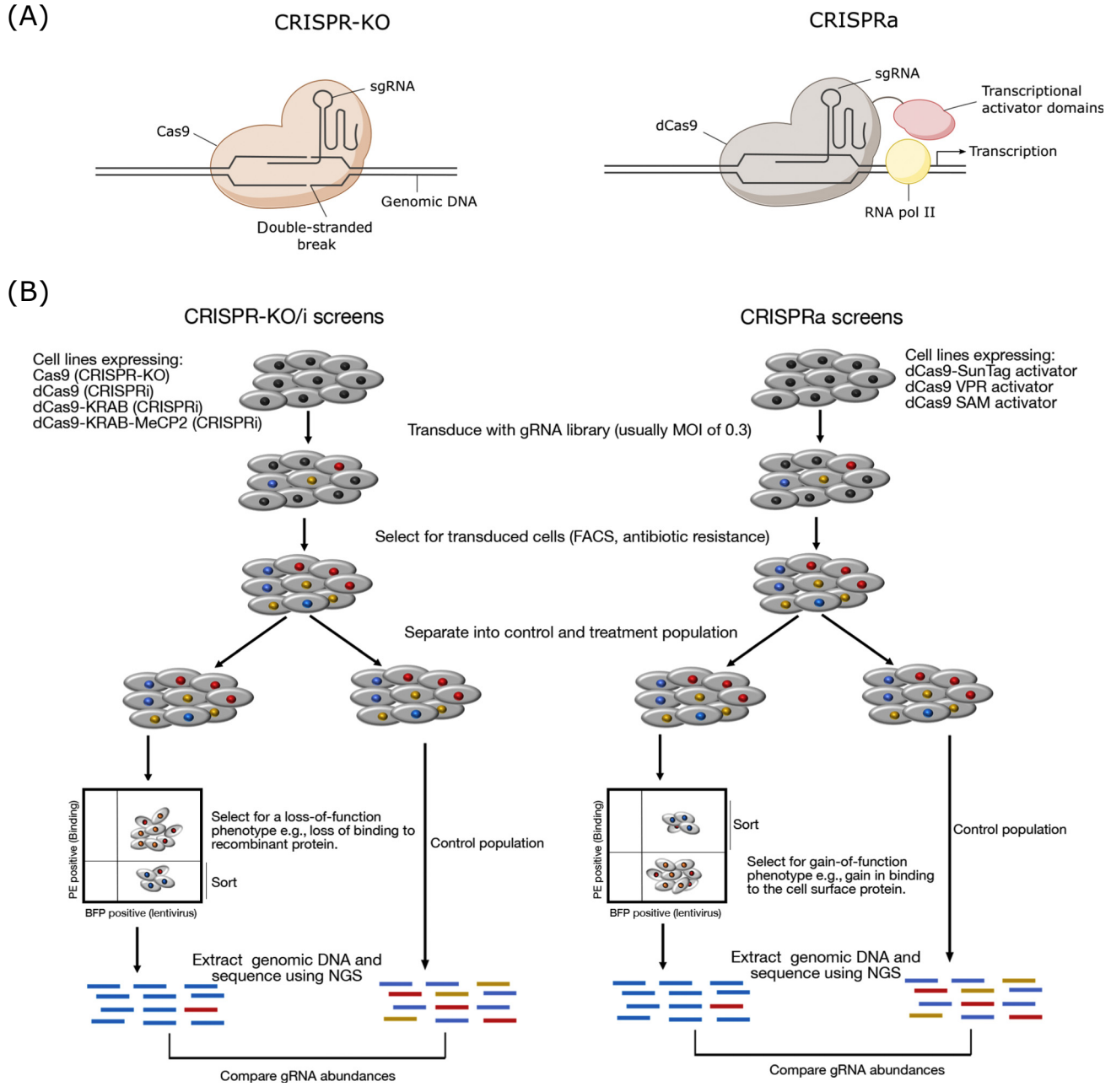

Figure 2. Investigating the Molecular Determinants of Cellular Recognition Using CRISPR-KO/ CRISPRi and CRISPRa Genetic Screens. (A) Schematic of how CRISPR-KO and CRISPRa systems work. Both systems are derived from Type II CRISPR/Cas systems from bacteria that are originally adaptive immunity strategies to protect against foreign genetic elements (Box 2). CRISPR-KO uses the RNA-guided nuclease Cas9 to create double-stranded breaks in genomic DNA at specific protein-coding loci as determined by a complementary guide sequence in the sgRNA. Errors in DNA repair of the double stranded break by nonhomologous end joining in the cell subsequently lead to frameshift mutations and then disruption of the target gene. By contrast, CRISPRa uses an RNA-guided but nuclease-inactive dCas9 fused to transcriptional activator domains to recruit transcription factors and RNA polymerase to the promoter regions of target genes to increase gene expression. (B) Diagram showing how CRISPR-KO/i and CRISPRa screens are conducted. CRISPRi utilises a catalytically inactive dCas9 protein to inhibit transcription and suppress gene expression (Box 2). A population of mutagenised cells are generated by transducing the relevant cell lines with a lentiviral sgRNA library and separating into treatment and control populations. The treatment population undergoes a cell binding assay and FACS to segregate cells that have either lost or gained

(Figure legend continued at the bottom of the next page.)

CRISPRa Screening

Rapid adoption of CRISPR/Cas9 technologies has driven the development of Cas9 variants designed to increase gene expression rather than disrupt or repress it (CRISPRa; Box 2). CRISPRa utilises a nuclease-inactive Cas9 fused to one or more transcriptional activator domains, which can be recruited to the promoter regions of target genes using an appropriate sgRNA (Figure 2A). The workflow for conducting CRISPRa screens is similar to that for CRISPR-KO screens, with a few key modifications such as the use of cell lines expressing 'dead' Cas9 (dCas9) activators (Figure 2B). Unlike CRISPR-KO screens, the cell line used for screening does not have to be able to bind to the protein of interest initially. Cells are also transduced with a CRISPRa lentiviral sgRNA library that contains sgRNAs targeting the promoter rather than coding regions of genes. For identifying cell surface interactions, focused CRISPRa libraries that only target genes encoding membrane proteins are available [14,20], although it is also possible to use genome-wide libraries [14,20]. Binding assays are conducted as previously described (Figure 1B) to detect transduced cells (BFP^+) that have acquired the ability to bind to a PE-labelled bait protein of interest (PE^+), and this population is isolated using FACS for sequencing and subsequent analysis. For more comprehensive details on conducting CRISPR-KO and CRISPRa screens in general, including tips for experimental design, normalisation, and statistical analysis, we refer the reader to the protocol paper by Joung *et al.* [21].

Contribution of CRISPR-KO/CRISPRa Screening to Our Understanding of Cellular Recognition

CRISPR-KO Screens Identify Factors That Directly and Indirectly Affect Binding

A cellular recognition event between two cells involves more than just the expression of the receptor and ligand in question. Binding also depends on receptor presentation at the cell surface, which may in turn rely on the presence of specific post-translational modifications (PTMs) and chaperone proteins. Unlike many other existing methods to study cellular recognition that only screen for directly interacting receptor–ligand pairs, genome-scale CRISPR-KO screens have the potential to reveal all genes that are required for correct display of the receptor in the plasma membrane. For instance, CRISPR-KO screening was used to identify CKLF-like MARVEL transmembrane domain containing 6 (CMTM6) as a master regulator of immune checkpoint inhibitor PDL1 expression on the plasma membrane [22,23], and solute carrier family 16 member 1 (SLC16A1) as a chaperone for expression of BSG, an erythrocyte receptor for *P. falciparum* [13]. Other CRISPR-KO studies also identified important PTMs such as glycosylphosphatidylinositol anchors and N-linked glycosylation required for cell surface localisation of innate immune receptors CD59 and galectin-3, respectively [13,24].

PTMs such as glycosylation, sulfation, and sialylation, can also act as coreceptors or modify binding properties directly. CRISPR-KO screens now present the possibility of detecting PTMs that are important for binding, and several such studies have identified heparan sulfate (HS), a polysaccharide modification commonly found on plasma membrane proteins, as a common coreceptor mostly in the context of host–pathogen interactions [13,25–27]. The same studies observed that HS binding was additive rather than codependent on other receptors, indicating that HS acts to enhance binding, while in some cases ligand binding was completely abolished in cells which are unable to form sulfated heparan moieties, suggesting that HS can also act as a receptor and mediate binding independently of a specific protein. CRISPR-KO screens have also

the ability to bind to the ligand of interest for CRISPR-KO/i or CRISPRa screens, respectively. Factors mediating binding are then determined by deep sequencing and comparison of sgRNA abundance in the sorted population to that in the control population. Abbreviations: BFP, blue fluorescent protein; CRISPR, clustered regularly interspaced short palindromic repeats; CRISPRa, CRISPR activation; CRISPRi, CRISPR inhibition; CRISPR-KO, CRISPR knockout; dCas9, dead Cas9; FACS, fluorescence-activated cell sorting; MOI, multiplicity of infection; NGS, next-generation sequencing PE, phycoerythrin; sgRNA, single guide RNA.

identified host factors involved in viral entry of murine norovirus, dengue virus, and hepatitis C virus, among others [16,28,29].

Limitations of CRISPR-KO Screening

While powerful, there are a few limitations of using CRISPR-KO screens to study cellular recognition. The main conceptual limitation of CRISPR-KO screening is that genes that are either essential for cell viability or functionally redundant are not identified. The longer the mutant cell library is kept in culture, the greater the chance that cells containing sgRNAs targeting genes essential for proliferation are depleted. However, essential genes contributing to cell surface recognition may be identified by performing phenotypic selection at an earlier time point after lentiviral transduction. Another limitation of the CRISPR-KO screen is the need to identify a cell line that binds to the protein of interest prior to screening. This often requires prescreening a large number of cell lines, which can be time consuming. Due to the high number of cell surface proteins that display HS-binding properties, we also recommend prescreening ligands for HS binding by comparing binding with an isogenic line harbouring a knockout in either SLC35B2 or EXTL3, which are key enzymes required for HS biosynthesis [13].

CRISPRa Screens Simultaneously Detect Multiple Binding Partners

Some of the shortcomings of CRISPR-KO screening can be addressed using the complementary CRISPRa approach. For instance, CRISPRa screens do not require extensive prescreening to identify a cell line that binds to the protein of interest, and CRISPRa libraries do not show depletion of sgRNA targeting genes detrimental to proliferation after extended periods in culture [14]. One of the advantages of CRISPRa screening over CRISPR-KO is the ability to detect multiple receptors in a single experiment, as was the case for the identification of interactions between neuronal receptor adhesion G-coupled receptor B1 (ADGRB1) and all three members of the reticulon-4 receptor (RTN4R) family [14]. In addition, CRISPRa screening has been applied to identify host cell factors such as N-acetylgalactosaminyltransferase 2 (B4GALNT2) and interferons that protect against viral infection by multiple subtypes of Influenza A virus and Zika viruses [30,31].

Limitations of CRISPRa Screening

By contrast to the CRISPR-KO approach which can reveal all nonessential, nonredundant genes required for the cell surface display of a receptor, CRISPRa screening is more suited to determine the gene encoding the directly interacting receptors themselves. Nonetheless, we have observed that CRISPRa screening of HS-binding proteins tends to identify syndecan (SDC)1 and 2 as binding partners, as they are HS proteoglycans [14]. The study that identified glycosyltransferase B4GALNT2 as a factor that prevented host-cell invasion by influenza A viruses through modifications of α2,3-linked sialic acid containing glycans [35] also indicated that CRISPRa screens can still be useful for detecting some forms of glycan involvement in cellular recognition.

CRISPR activation screens can also be limited by the receptors that can be expressed on the surface of the cells in a correctly folded form as transcriptional activation of a single gene might not be sufficient to achieve cell surface expression for proteins that rely on additional cellular accessory factors such as transporters and chaperones. For instance, while attempting to overexpress proteins that are restricted to erythroid cells such as rhesus factor antigen (Rh)D and SLC4A1 in immortalised HEK293 cells using the CRISPRa system, we observed that while we were able to achieve over 1000-fold overexpression at the mRNA level, this did not correspond to increases in protein expression at the cell surface [14]. One likely explanation is that HEK293 cells do not express accessory factors such as RhAG and glycophorin A, which are required for cell surface

localisation of RhD and SLC4A1, respectively [32,33]. Conducting screens in a more biologically relevant cell line such as immortalised erythroid lines might overcome this problem.

Functional Studies Complement Results from CRISPR/Cas9 Screens

Analysis of CRISPR/Cas9 screen data generates a ranked list of genes based on the likelihood that sgRNAs targeting that gene are enriched in the sorted treatment population as compared with the control population. In our experience, the results of CRISPR-KO and CRISPRa cell surface interaction screens are reproducible, with near complete overlap of top-ranked genes between replicates. As such, two replicates are generally sufficient for identifying receptors or key pathways involved in binding. However, the reader should be mindful that such screens are typically conducted on immortalised cell lines such as HEK293 cells that are easy to grow and manipulate, and that CRISPR-KO/CRISPRa screening may suffer from artefacts caused by off-target binding of Cas9 or ectopic overexpression of target proteins that have been previously demonstrated in cancer cell lines and yeast [34–36], although this has so far not been studied in cellular recognition screens. It is therefore crucial to validate the physiological relevance of any interactions found by CRISPR/Cas9 screens in more functionally relevant models. For instance, we recently identified HS as the binding partner of platelet inhibitory protein G6b using a CRISPR-KO screen, and subsequent *in vitro* and *in vivo* experiments in mice revealed that G6b-B when bound to multivalent HS side chains induces downstream signalling in platelets via tyrosine phosphatases Shp1 and Shp2 [37].

Concluding Remarks

On balance, CRISPR-KO and CRISPRa screens are robust and powerful tools for systematically investigating the molecular determinants of cellular recognition at a genome-wide scale (Figure 3, Key Figure). These approaches can go beyond identifying novel cell surface receptor–ligand pairs to detecting PTMs and chaperones that indirectly affect binding, as well as highlighting the role of glycans such as HS in cellular recognition. Nonetheless, a number of questions remain to be answered (see Outstanding Questions), a key one being: how well do the results from CRISPR/Cas9 screens in immortalised cell lines reflect what goes on in a physiological context *in vivo*? This is not as straightforward as it sounds, and while some studies have successfully demonstrated the relevance of interactions identified using CRISPR-KO/CRISPRa screening in mice models or explant cultures [31,37], others have struggled to find a suitable *in vivo* assay for validation.

Multiple CRISPR-KO screening studies have identified a number of HS-binding proteins, highlighting the importance of HS in cellular recognition. While HS has been known to play multiple roles in viral attachment, immune activation, and development [38], it is unclear how these interactions are independently regulated in the body and how specificity is achieved. To this end, CRISPR/Cas9 techniques could be applied to help delineate the differences between HS molecules displayed on the surface of different cell types and how that might affect cellular recognition. Using fluorescently labelled glycans [39], CRISPRa screening could also be used to generate a comprehensive list of HS-binding cell surface proteins, and compare it with that of other glycans such as chondroitin sulfate (Figure 3).

One aspect of cellular recognition that CRISPR/Cas9 screening does not currently address is how plasma membrane dynamics and receptor density affect ligand binding. For instance, active and inactive integrins have been shown to form nanoclusters with different spatial organisations [40]. CRISPR/Cas9 techniques are not suitable for such studies, but a generic cell surface ligand system has recently been developed to address this question [41].

Outstanding Questions

How can binding of a recombinant protein to the surface of immortalised cell lines be interpreted in a biological context.

What is the role of HS molecules displayed on the surface of multiple cell types in cell recognition events *in vivo*?

What role do receptor density and plasma membrane dynamics play in cellular recognition?

How can cellular recognition screens be performed in primary cells?

Can CRISPR/Cas9 screening be adapted to identify binding mediated by molecules that are poorly studied, for example glycolipids and exosomes?

Key Figure

Applications of CRISPR/Cas9 Screening for Cellular Recognition

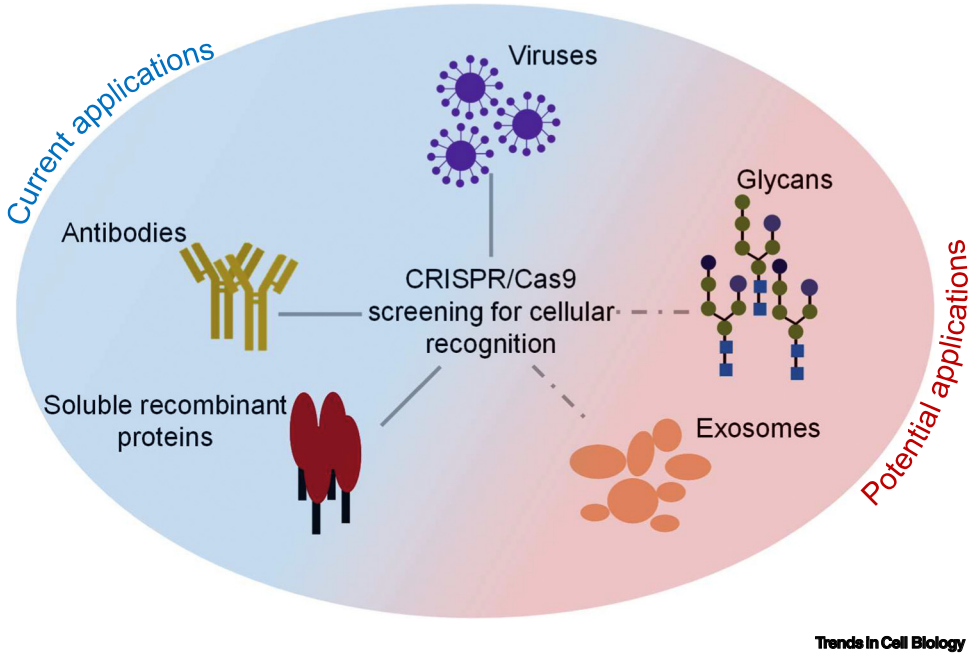

Figure 3. CRISPR/Cas9 screening has been applied to soluble recombinant proteins to identify cell surface receptors and to viruses to identify host factors that prevent viral infection [13,14,25–31]. Monoclonal antibodies have also been used in combination with CRISPR/Cas9 screening to identify essential factors for the correct presentation of key receptors or ligands at the cell surface [13,14,22–24]. Future applications for CRISPR/Cas9 genetic screening in cellular recognition include the identification of binding partners to common glycan modifications such as heparan and chondroitin sulfate, as well as dissection of pathways essential for exosome targeting and fusion. Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats.

Despite its limitations, we believe that CRISPR/Cas9 screening has potential for furthering our understanding of cellular recognition. CRISPR/Cas9 screens have already been performed in primary T cells [42], which could improve translatability of findings, while improvements in the synthesis and purification of glycolipids and exosomes could make them amenable for CRISPR/Cas9 screening to better understand their role in cell–cell communication in the near future (Figure 3). Exosomes represent an emerging class of therapeutics for a broad range of diseases, but the mechanisms by which they recognise and bind to target cells is still poorly characterised [43]. We therefore envision that CRISPR/Cas9 screening will continue to be a useful technique in the field of cellular recognition.

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