# Identifying Genetic Drivers of Innate Immunity Using Custom Targeted CRISPR Screens


## ABSTRACT

Phagocytosis is a vital cellular process where a cell engulfs (eats) a large particle with its outer cell membrane. The process is controlled by a network of interacting biochemical pathways and biomechanical cellular processes. Gaining a better understanding of this process will enable a better understanding of disease clearance from the body, and homeostasis. CRISPR knock-out screens provide a remarkably powerful tool with which to investigate highly coordinated cellular processes like phagocytosis. Using genome-wide CRISPR knockout screens, followed by focused CRISPR screens, researchers at Boehringer Ingelheim probed the genetic drivers of human monocyte phagocytosis when the cells were challenged with Staphylococcus aureus. The focused CRISPR screen, which used a custom high-quality guide library synthesized on Twist Bioscience's DNA synthesis platform, confirmed known phagocytosis genes involved in actin cytoskeleton regulation. Moreover, this customized screening library revealed additional genes that control phagocytosis, including several involved in UDP glycosylation.


## INTRODUCTION

Phagocytosis is an essential cellular process used by multicellular organisms to engulf and ingest large particles, that are then digested in an organelle called a phagosome. This process is used to clear pathogens, remove damaged or dead cells, and eliminate cell debris[1]. During phagocytosis, the cell recognizes the target particle using interactions between its cell receptors and ligands on the particle surface[2]. Recognition results in coordinated changes in the cell's cytoskeleton and cell membrane, leading to membrane rearrangements that engulf the target[3].

Dysregulation of phagocytosis can cause a loss of homeostasis and result in disease. Some immune cells, including monocytes, macrophages, neutrophils, eosinophils, and dendritic cells, are able to perform phagocytosis and eliminate pathogens, but some pathogens evade phagocytosis to persist in the host organism[2]. The function of some of the genes that control phagocytosis, such as those that regulate actin restructuring, are understood, but many of the biochemical mechanisms underlying phagocytosis remain unknown[4,5].

CRISPR knockout screens provide a powerful tool for interrogating complex cellular processes. The targeted creation of loss-of-function mutations allows for the efficient characterization of genotype-phenotype relationships. While single guide RNA (sgRNA) libraries can be purchased, sgRNA libraries for further confirmation of hits must be custom designed and engineered. In order to create a focused sgRNA library, specific oligonucleotide sequences (oligos) encoding sgRNAs are synthesized.

This application note dives into recent research from Boehringer Ingelheim that utilized Twist Bioscience's silicon-based DNA synthesis platform to produce high-quality, customized oligos for CRISPR screens. The researchers performed a series of CRISPR knockout experiments to identify genes that control phagocytosis of *Staphylococcus aureus* by human monocytes (THP-1)[5]. A genome-wide knock-out screen was used to initially identify potential genes involved in phagocytosis. A comparison of the sgRNAs in cells with high versus low rates of phagocytosis revealed a subset of genes that were potentially involved in phagocytosis. These genes were the subject of a focused CRISPR screen, which identified the genes most likely associated with phagocytosis. Finally, high-confidence hits were validated with single-gene knockouts. This workflow exemplifies the value of custom CRISPR screens to further home in on potential hits to reduce the burden of validating large lists of genes that often result from genome-wide experiments.

## WORKFLOW

### Engineering THP-1 iCas9 Cells

In order to control Cas9 activity, the cells were engineered to contain the TET-inducible Cas9-GFP expression system, which only becomes active in the presence of doxycycline (Figure 1). The THP-1 cell line was chosen because it spontaneously phagocytoses without the need for activation techniques. In addition, the lentiviral particle used to transduce cells with sgRNAs were designed to co-express murine surface protein Thy1.1, which allows for the purification of sgRNA containing cells with magnetic-activated cell sorting (MACS).

### Loss of Function Experiments With CRISPR Knockout

Three CRISPR knock-out experiments were used to identify and then validate genes involved in phagocytosis.

- A genome-wide CRISPR screen was used to interrogate 18,187 genes, based on a custom sgRNA design. For this initial screen, a coverage of six sgRNAs per gene was used, plus an additional 500 non-targeting controls. The THP-1 iCas9 cells were transduced at a rate of 33.9% with a cell guide coverage of 3,000X.
- Once a subset of 831 genes had been identified in the genome-wide screen, a second focused screen was conducted targeting these sequences. Twist Bioscience synthesized the custom oligo pool that encoded the sgRNA library. This second CRISPR screen also used approximately six guides per gene and 500 non-target controls, resulting in 5,407 sgRNAs, and a cell guide coverage of >36,000X.
- The high confidence hits from the second screen were subjected to single-gene knockout using the best performing sgRNAs.

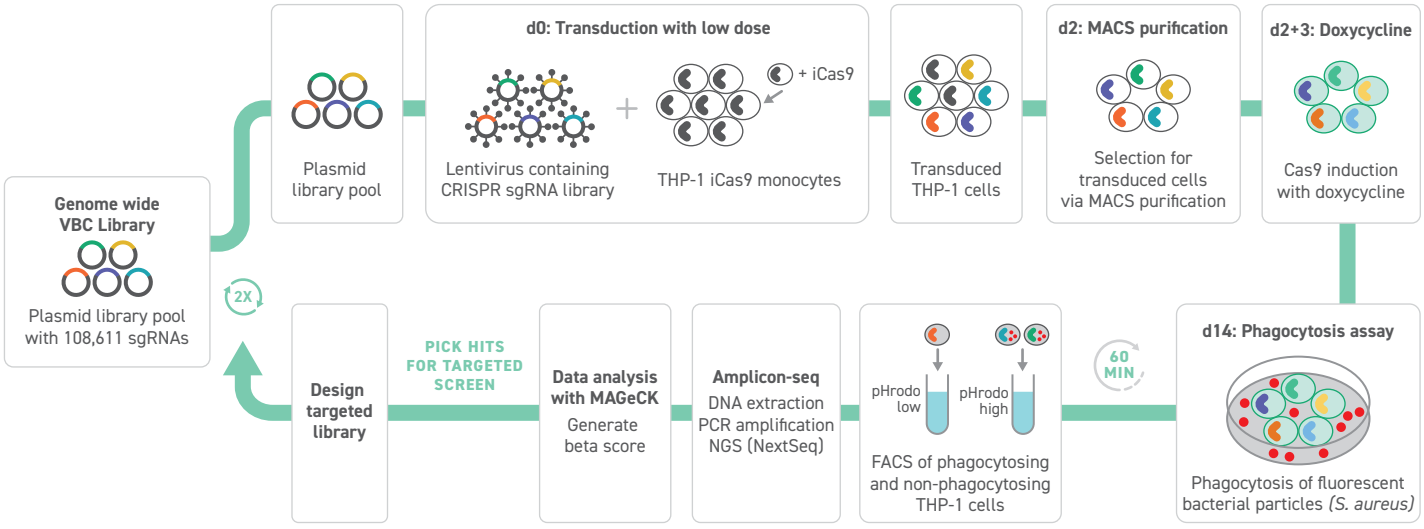


**Figure 1. Experimental workflow for both the genome-wide and focused CRISPR screens.** This workflow included the production of THP-1 iCas9 cells, sgRNA library design, CRISPR knockout screens, the phagocytosis assay, amplicon sequencing, and data analysis. The oligo pools for the focused screen were designed and engineered with Twist Bioscience's technological platforms.

### THP-1 iCas9 Cell Transduction and Phagocytosis Analysis

To conduct the genome-wide and focused CRISPR screens, the sgRNA libraries were transduced into THP-1 iCas9 cells via a lentiviral vector (Figure 1). The transduced cells were enriched with MACS and then Cas9 expression was switched on with doxycycline. The effect of the knockout screen on phagocytosis was measured 12 days after doxycycline activation. The transduced, enriched cells were cultured with pHrodo red-labeled *S. aureus* and allowed to phagocytose for 1 hour. Fluorescence-activated cell sorting (FACS) was employed to sort cells based on the signal intensity of pHrodo, creating cell populations with high and low rates of phagocytosis. After genomic DNA extraction, the sgRNAs in the resulting populations were amplified with PCR and sequenced with a Next-Seq 550 instrument (Illumina). Data analysis using the MAGeCK software identified guides associated with high and low levels of phagocytosis.

## RESULTS

### Pinpointing the Genes Involved in Phagocytosis

The initial genome-wide CRISPR screen interrogated over 18,000 genes, and, of these genes, the sgRNAs of 211 genes were depleted in the transduced cells (Figure 2). These genes were identified as essential genes whose loss of function was lethal to that cell population. Of the remaining 17,976 genes, 831 genes were identified as potentially important for phagocytosis of *S. aureus*. A second, focused sgRNA pool, created with Twist's custom oligos, further interrogated the 831 genes identified in the genome-wide screen. Leveraging highly accurate customized sgRNA pools, the secondary screen was used to filter out false-positive hits from the genome-wide screen. From the secondary CRISPR screen, 75 high confidence hits were identified, of which 28 activated and 47 inhibited phagocytosis.

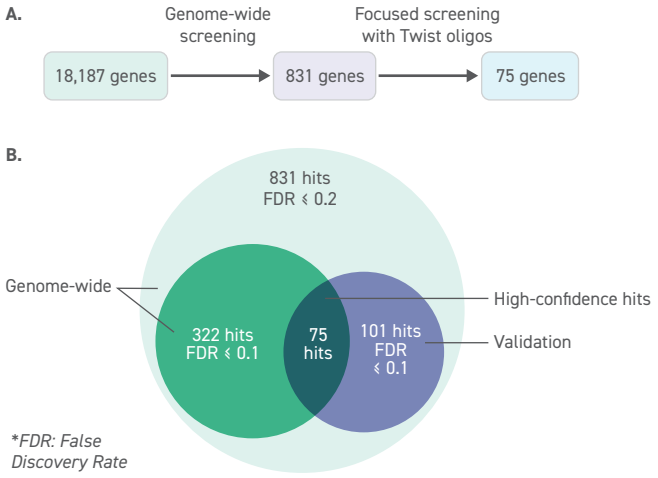


**Figure 2. Successive elimination of genes not involved in phagocytosis through a genome-wide screen and focused screen. A.** The genome-wide screen interrogated more than 18,000 genes and identified 831 hits. Of these hits, the secondary screen identified 75 high-confidence hits. **B.** In order to identify genes involved in phagocytosis, data from the genome-wide and secondary screens were analyzed with MAGeCK software. A total of 831 genes were returned from the genome-wide screen (FDR* ≤ 0.2). When these 831 hits were interrogated with the secondary screen, a total of 101 hits were identified (FDR ≤ 0.1). A final analysis of the genome-wide screen using an FDR ≤ 0.1 identified 322 hits. The genome-wide and secondary screens were cross-referenced, yielding 75 high confidence hits.

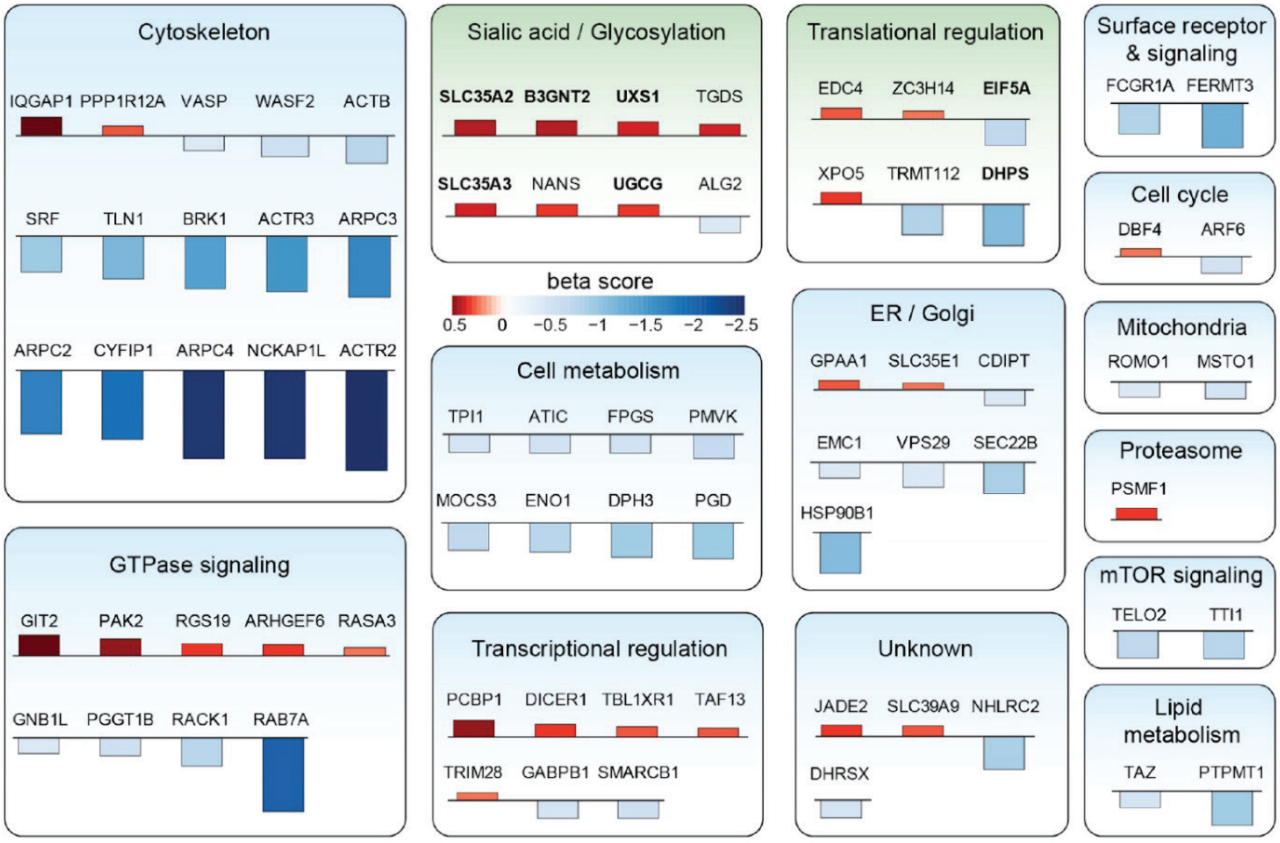


**Figure 3.** The 75 high-confidence hits were classified by biological function. Genes shown in bold were further analyzed with single-gene knockouts. The bars represent the beta score for each gene, which is a measure of the degree of selection imposed by the phagocytosis assay. Negative and positive beta values indicate genes whose knockout impairs or activates phagocytosis, respectively. Genes in bold are selected for single-gene knockout validation.

Analysis of the two knock-out screens showed depletion of several sgRNAs in cells with high rates of phagocytosis. The depleted sgRNAs target genes involved in the processing and regulation of the actin cytoskeleton during phagosome membrane invagination. The affected genes included members of the actin-related protein complex (Arp2/3), including ACTR2, ACTR3, and ARPC2, 3, and 4, and the WAVE complex, including NCKAP1L, CYFIP1, BRK1. As the Arp2/3 and WAVE complexes are essential to the control and formation of the actin cytoskeleton, their loss of function led to decreased rates of phagocytosis. In addition, sgRNAs targeting lysosome movement regulator RAB7A were also depleted in cells with high rates of phagocytosis.

Biological pathway analysis identified genes related to the actin cytoskeleton, epithelial adherens junctions, integrin signaling, Fcγ receptor-mediated phagocytosis, and several other signaling pathways (Figure 3). Of these genes, 10 produced proteins localized in the plasma membrane, 13 in the nucleus with most of the remaining genes producing proteins localized in the cytoplasm. When the genes were sorted into functional groups, the most commonly disrupted cellular functions involved cytoskeleton control, endoplasmic reticulum and Golgi body control, GTPase signaling, glycosylation, cell metabolism, and transcriptional and translational regulation. It is not surprising that genes involved in cytoskeleton restructuring and regulation affect phagocytosis since the dynamic activity of the actin cytoskeleton is essential for phagosome formation. Interestingly, the loss of function of five genes related to UDP glycosylation resulted in an increase in phagocytosis, a previously unidentified association.

**Validation of High Confidence Hits**

In order to further investigate some of the novel high confidence hits, five genes involved in glycosylation (SLC35A2, SLC35A3, B3GNT2, UXS1, and UGCG) and two involved in translational regulation (EIF5A and DHPS) were subjected to single-gene CRISPR knockout. The resulting cells were assayed for phagocytosis with *S. aureus*, *Escherichia coli*, and zymosan A. The single-gene knockouts showed that the loss of function of EIF5A and DHPS reduced phagocytosis of the three substrates. Thus, EIF5A and DHPS may indirectly contribute to the control of actin cytoskeleton restructuring[6]. Additionally, knockout of the UDP glycosylation genes SLC35A2 and 3, UXS1, and UGCG resulted in increased phagocytosis of the substrates. The relationship between glycosylation and phagocytosis is not currently understood.

## CONCLUSIONS

The accurate and uniform pools of oligos available from Twist Bioscience are ideal for the production of custom, focused CRISPR screens. Twist Bioscience's oligo pools have no size limitations and allow for the design and production of truly custom screens. Secondary, custom screens often follow genome-wide screens and are extremely useful for ruling out the involvement of vast numbers of genes in complex, cellular processes. In this application note, researchers used Twist Bioscience's Oligo Pools to build a secondary CRISPR screen targeting specific genes hypothesized to regulate the activity of phagocytosis with great accuracy. In doing so, they were able to avoid the cost and time impact associated with validating the complete list of hits generated by their genome-wide screen. An initial pool of 831 genes was refined by over 90% to a much more manageable 75 genes.

The highlighted study identified a number of genes that affect phagocytosis in human monocytes. Not surprisingly, genes that alter the function of proteins critical to cytoskeletal restructuring (WAVE, ARP2/3) affected the rate of phagocytosis (Figure 4). Additionally, RAB7A impacts phagocytosis by facilitating the maturation of phagosomes.

A number of other genes previously not known to be associated with phagocytosis were also identified. One of these genes, DHPS, is involved in post-translational modification of the amino acid lysine in another of the genes, EIF5A—a translational elongation factor. Furthermore, this study is one of the first to recognize the importance of UDP glycosylation in phagocytosis. The discovery of the relationship between these genes and phagocytotic phenotypes provides new avenues for research of this complex and vital cellular process.

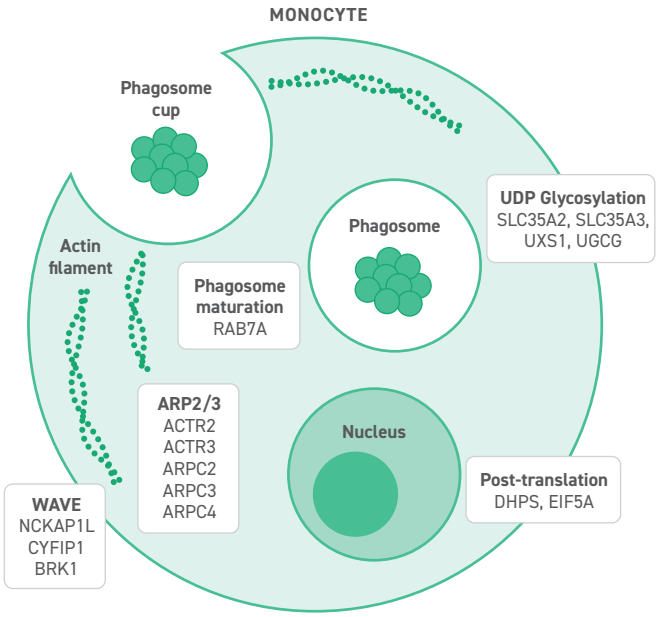


**Figure 4.** Genes that are involved in phagocytosis identified through CRISPR loss-of-function screens. The genes are grouped by their biological function. Collectively, the WAVE and ARP2/3 genes affect the actin cytoskeleton structure. The effect of UDP glycosylation on phagocytosis is not understood yet.

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