Trends in Parasitology

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Review

# The Past, Present, and Future of Genetic Manipulation in *Toxoplasma gondii*

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**_Toxoplasma gondii_ is a classic model for studying obligate intracellular microorganisms as various genetic manipulation tools have been developed in _T. gondii_ over the past 20 years. Here we summarize the major strategies for _T. gondii_ genetic manipulation including genetic crosses, insertional mutagenesis, chemical mutagenesis, homologous gene replacement, conditional knockdown techniques, and the recently developed clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 system. We evaluate the advantages and limitations of each of these tools in a historical perspective. We also discuss additional applications of modified CRISPR–Cas9 systems for use in _T. gondii_, such as regulation of gene expression, labeling of specific genomic loci, and epigenetic modifications. These approaches have the potential to revolutionize the analysis of _T. gondii_ biology and help us to better develop new drugs and vaccines.**

## Trends

The transfection technologies first introduced into *Toxoplasma gondii* two decades ago opened the way for molecular manipulation of the parasite.

Numerous genetic tools are now available for *T. gondii*, including a several strategies for conditional knockdown of essential genes.

The recently developed CRISPR–Cas9 system has been adapted to *T. gondii* to allow gene disruption and point mutations and to introduce epitope tags.

We review the advantages and disadvantages of the technologies currently available for genetic manipulation of *T. gondii* and discuss new CRISPR–Cas9-based systems that may be applied to *T. gondii* in the near future.

## Toxoplasmosis: A Zoonotic Disease in Need of Better Therapeutics

Toxoplasmosis is one of the most important zoonotic parasitic diseases, caused by the obligate intracellular protozoan *T. gondii*, which is capable of infecting all warm-blooded animals including humans [1,2]. It is estimated that one-third of the world's population is chronically infected with *T. gondii* [1,2]. Although *T. gondii* infection is usually asymptomatic in immunocompetent people, it can cause severe complications in immunocompromised individuals. Furthermore, infection during pregnancy can lead to miscarriage, stillbirth, or severe congenital defects including blindness, mental retardation, and hydrocephaly [1,2]. Unfortunately, the strategies used to prevent or cure *T. gondii* infection in humans or livestock are limited and not ideal [3]. An improved understanding of the biology of the parasite will facilitate the identification and characterization of new targets and strategies for intervention. The ability to genetically manipulate the genome of *T. gondii* is central to these advances. Therefore, understanding the molecular pathways of parasite pathogenesis and the ability to dissect gene function at the molecular level are crucial for developing effective vaccination strategies and better therapeutics.

Due to its medical importance and ease of growth in tissue culture, *T. gondii* has received considerable scientific and medical attention and is considered an important model organism for the study of obligate intracellular microorganisms [4]. A range of genetic tools has been developed to analyze gene functions in *T. gondii* [5,6]. Using *T. gondii* as a study model, we recapitulate and describe the currently available molecular genetic systems and potential applications of CRISPR–Cas9 systems in *T. gondii* to provide clues for genetic manipulation in other intracellular microorganisms.

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## Selectable Markers Available to Use with *T. gondii*

The ease of culturing the infectious stages of the parasite *in vitro*, along with the availability of numerous selectable markers and expression platforms that can be readily introduced into parasites by electroporation, makes highly tractable the study of the biology of *T. gondii* by genetic means (Figure 1). *T. gondii* can be easily propagated in cultured nucleated cells. The efficiency of introducing DNA into *T. gondii* by electroporation can be up to 15%, with less than 20% parasite viability loss during electroporation [7]. In general, most transfected parasites lose newly introduced DNA after a few generations if it is not integrated into the genome. Therefore, numerous selectable markers have been developed to select for stable transformants with heritable genetic changes [8–13]. Chloramphenicol acetyltransferase (CAT) [8], the pyrimethamine-resistant allele of dihydrofolate reductase–thymidylate synthase (*DHFR-TS*) [9], hypoxanthine–xanthine–guanine phosphoribosyltransferase (*HXGPRT*) [10], and bleomycin/phleomycin-binding protein (Ble) are the most commonly used systems for positive selection [11]. The uracil phosphoribosyltransferase (*UPRT*) and *SNR1* (TGME49_290860) loci, conferring resistance to 5-fluorodeoxyribose (FUDR) [12] and sinefungin [13], respectively, on inactivation, are frequently used for negative selections, such as for use in genetic complementation. *HXGPRT* can be used for both positive and negative selection but requires a *HXGPRT*-deficient strain for positive selection [10]. Fluorescent reporter genes can also be used for enriching transformed parasites with fluorescence-activated cell sorting (FACS) [14]. Moreover, selectable markers can be removed from the *T. gondii* genome by Cre recombinase if they are flanked by *loxP* sites [15].

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## Forward Genetic Tools Available for *T. gondii*

Forward genetics – approaches used to identify the genetic basis of a specific phenotype – have been successfully employed in *T. gondii* in several ways, including genetic crosses, chemical mutagenesis with whole-genome sequencing (WGS), and random insertional mutagenesis. *T. gondii* isolates from Europe and North America have an unusual population structure comprising three dominant clonal lineages (types 1, 2, and 3) [16]. However, with more strains isolated from other parts of the world, it was recently shown by Su *et al.* [17] that the *T. gondii* population structure is more complex than simply three dominant clonal lineages and can be divided into 16 haplogroups within six major clades. Despite being similar genetically, their biological traits can be broadly different [18]. In most laboratory mice, type 1 strains are highly virulent, with $LD_{100} = 1$, whereas type 2 and type 3 strains are less virulent, with $LD_{50} = 10^2$ and $10^5$, respectively [16–18]. Additionally, the host genetic background also affects the virulence of *T. gondii*, such as polymorphism of the mouse immunity-related GTPases [19]. Forward genetic approaches based on sexual crosses in cats were first demonstrated by the Pfefferkorn laboratory in 1980 using two drug-resistant lines [20]. Subsequent crosses were created by several laboratories for use in quantitative trait locus (QTL) analysis, which led to the discovery of genes responsible for the phenotypic differences between the types 1, 2, and 3 clonal strains. For example, ROP18 and ROP5 were identified as virulence factors by genetic mapping using progenies derived from $1 \times 3$ and $2 \times 3$ and $1 \times 2$ and $2 \times 3$ crosses, respectively [21–24]. Several other genes have also been identified using genetic crosses (e.g., *ROP16* [25], *GRA15* [26], *GRA25* [27]), demonstrating the potential value of this approach. Despite these strengths, this forward genetic approach does not allow the identification of non-polymorphic genes that are conserved in all strains. Another major hurdle for genetic crosses is the use of cats to enable sexual reproduction of the parasites. Performing genetic crosses in cats is rather intractable and requires special handing and biological containment due to the highly infectious oocysts. To overcome this limitation, the development of *in vitro* systems is needed to allow *T. gondii* sexual recombination in cultured cells such as intestinal epithelia cell lines derived from the cats. If successfully developed, such methods would dramatically accelerate genetic-cross studies. Given these limitations, it is unsurprising that only a few crosses have been conducted to date [21–27]. However, with the affordability of WGS in *T. gondii* genetic analysis of progenies derived from crosses became much easier [28].

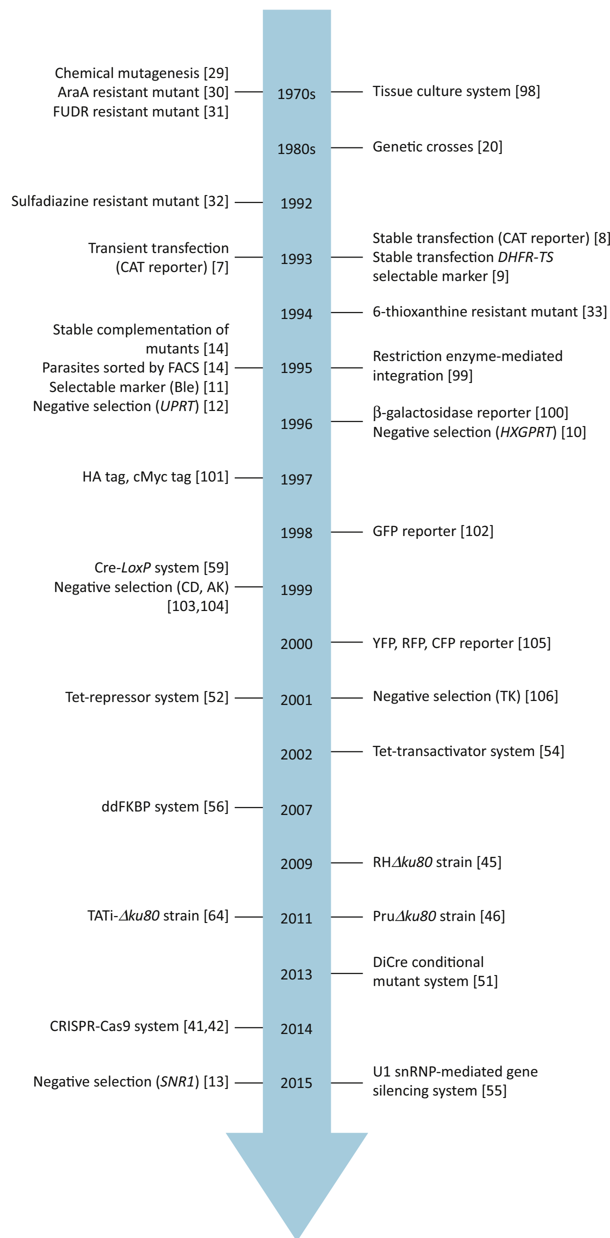


Figure 1. Timeline of Key Molecular Tools Available for the Study of *Toxoplasma gondii*. Abbreviations: AK, adenosine kinase; AraA, adenine arabinoside; Ble, bleomycin/phleomycin-binding protein; CAT, chloramphenicol acetyltransferase; CD, cytosine deaminase; *DHFR-TS*, dihydrofolate reductase–thymidylate synthase; FACS, fluorescence-activated cell sorting; FUDR, 5-fluorodeoxyribose; *HXGPRT*, hypoxanthine–xanthine–guanine phosphoribosyltransferase; *SNR1*, sinefungin-resistant protein; TK, thymidine kinase; *UPRT*, uracil phosphoribosyltransferase [98–106].

Another forward genetic approach broadly used in *T. gondii* is chemical mutagenesis, which was first adapted for *T. gondii* by the Pfefferkorn laboratory in the 1970s [29]. Since then, a series of drug-resistant mutants and temperature-sensitive mutants have been generated, such as adenine arabinoside-resistant mutants [30], FUDR-resistant mutants [31], sulfonamide-resistant mutants [32], and 6-thioxanthine-resistant mutants [33]. These studies were critical for the identification of selectable markers. This method leads to mutations in the genome that may

confer special properties on the mutants, such as temperature sensitivity, or lead to the inactivation of affected genes, which may be useful to study essential genes responsible for a particular phenotype such as parasite growth, egress, or cell cycle regulation [34–37]. Chemical mutagenesis is often used to construct libraries for genetic screens to sample a large number of mutants and has the ability to sample a larger mutational space than genetic disruptions, including functional alleles with partial or different activities. However, this approach is highly nonspecific and chemical treatment usually results in multiple mutations; therefore, it is challenging to precisely identify the affected genes.

In addition to chemical mutagenesis, random insertional mutagenesis is also frequently used for library construction and genetic screens. The highly active nonhomologous end joining (NHEJ) activity of wild-type *T. gondii* parasites allows random integration of selectable markers into the genome to produce insertional mutant libraries. This strategy has been used to identify genes involved in various aspects of *T. gondii* biology [38–40]. For example, a random insertion-based gene-trapping method was used to identify the *BSR4* gene involved in bradyzoite development [38]. GFP tagging with random insertional mutagenesis proved a powerful approach to identify novel proteins localized to a specific subcellular organelle, such as ROP4 targeted to the rhoptries [40]. Although this random insertional mutagenesis approach is attractive in constructing genome-wide mutant libraries for genetic screens, it is not suitable for analyzing a specific gene of interest (GOI). In this case site-specific modification is required and NHEJ-mediated insertion is often random and lacks site specificity. However, it was recently shown that CRISPR–Cas9 enhances site-specific nonhomologous integration of foreign DNA in wild-type *T. gondii* [41,42], which allows highly efficient and specific gene inactivation by insertional mutagenesis.

## Traditional Reverse Genetic Strategies Used in *T. gondii*

In the post-genomic era, understanding of *T. gondii* biology has been greatly accelerated by the use of reverse genetic strategies, which is an approach to discover the function of a gene by analyzing the phenotypic consequences after disruption or modification of its sequence. To replace, mutate, or knock out endogenous genes or to introduce exogenous sequences to a specific location in the genome, homologous targeted sequences may be included in the transformation construct to drive site-specific alteration. Unfortunately, homologous recombination is not favored over nonhomologous integration in wild-type *T. gondii*, even with long homologous sequences [43]. In *T. gondii*, as in many other eukaryotes, DNA double-strand breaks (DSBs) are repaired by NHEJ or the homology directed repair (HDR) pathways [44]. The error-prone NHEJ simply stitches together broken DNA ends while introducing short insertions or deletions at the repair site, which may lead to gene inactivation. Alternatively, the 'error-free' HDR can precisely introduce desired alterations to a given sequence through a homologous template to guide the repair. Several studies have indicated that the NHEJ pathway is preferentially used by *T. gondii* [38,40,43,45,46]. It was later shown that disrupting the Ku80 protein significantly reduced NHEJ activity and markedly increased the success of homologous gene replacements (300–400-fold) [45,46]. It was also shown that relatively short homology flanks (500 bp) were sufficient for homologous gene replacement in this system [45,46]. Although only two Ku80-deficient *T. gondii* strains (RH*ΔKu80* and Pru*ΔKu80*) have been generated so far, these two strains have contributed greatly to functional analysis of *T. gondii* genes [47,48]. However, the Ku80 protein is critical to many cellular activities, including chromosome stability. Inactivation of Ku80 results in increased sensitivity to DSBs, which limits the use of selection methods that cause DSBs, such as phleomycin (a glycopeptide antibiotic that binds and intercalates DNA to inhibit the growth of many prokaryotic and eukaryotic organisms) [11], as well as other applications that may require NHEJ, such as meiotic recombination in oocyst development or telomere stability [44,45,49,50].

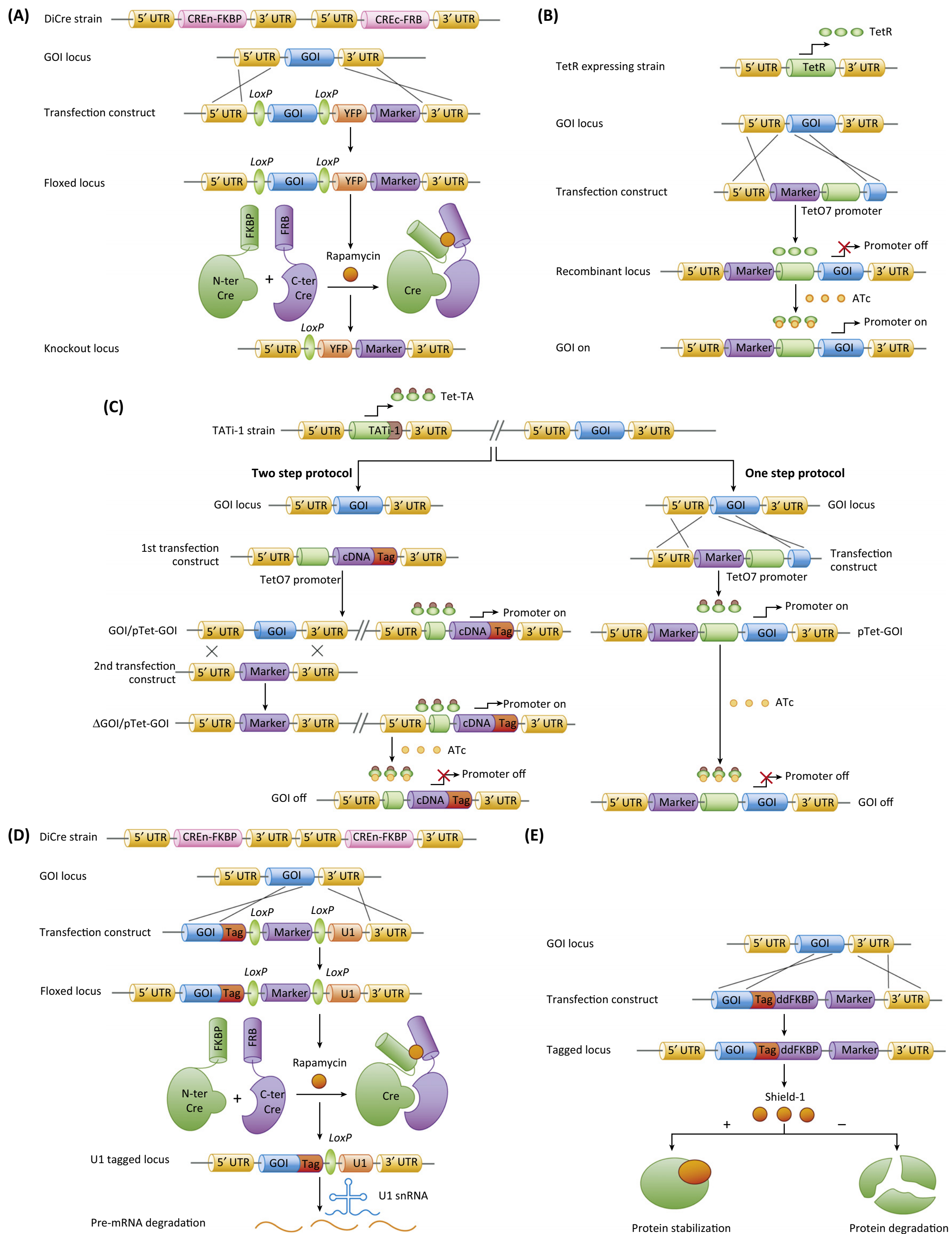


Figure 2. Conditional Manipulation of Gene Expression in *Toxoplasma gondii*. (A) Conditional deletion of a gene of interest (GOI) by the DiCre system. The endogenous GOI is modified by the insertion of two *loxP* sites. The DiCre strain expresses Cre, which is split into two inactive moieties (N-terminal Cre is fused to FKBP and C-terminal Cre is fused to FRB). Addition of rapamycin leads to dimerization of the subunits and reconstitutes the active Cre recombinase, which catalyzes recombination between the two *loxP* sites leading to irreversible deletion of the GOI. (B) Conditional regulation of gene expression by a tetracycline-repressor system. The GOI is modified by the insertion of a Tet operator sequence (TetO) adjacent to the transcription start. In the absence of anhydrotetracycline (ATc), the ectopically

*(Figure legend continued on the bottom of the next page.)*

## Strategies for the Analysis of Essential Genes

Essential genes, the most promising drug candidates due to their required functions, cannot be disrupted by classical gene-knockout approaches. To study such genes, we may temporally regulate their expression or conditionally remove their coding sequences. Several strategies have been successfully developed in *T. gondii* to control gene expression at different levels, including a site-specific recombination system [51], a tetracycline-inducible system [52–54], a U1 small nuclear ribonucleic particle (U1 snRNP)-mediated gene-silencing system at the transcriptional level [55], and a degradation domain fusion system at the protein level [56]. Chemical genetics can also be used to study gene functions without altering the coding sequences. For an extensive review of their advantages and disadvantages, see [57].

### Site-Specific Recombination System (DiCre)

Cre–*loxP* technology is extensively used in various model systems for spatial and temporal excision of DNA sequences flanked by *loxP* sites [58]. The most challenging aspect of the use of Cre–*loxP* technology in *T. gondii* is obtaining tight control of Cre enzymatic activity [59]. To achieve this, the DiCre system was developed (Figure 2A) [51]. In this system, the GOI endogenous locus is replaced by *loxP* sites flanking the GOI coding sequence, followed by YFP and a selectable marker. This allows easy screening of gene-knockout parasites through YFP expression, as excision of the GOI flanked by *loxP* sites shifts YFP proximal to a promoter inducing its expression. Additionally, Cre can be split into two inactive fragments fused to the rapamycin-binding proteins FRB and FKBP. These separate domains can then be efficiently heterodimerized by addition of rapamycin, leading to the reconstitution of Cre enzymatic activity and deletion of genes flanked by *loxP* sites with very high success rates (20–90%) [51]. Using this approach, conditional knockout mutants of MyoA, MIC2, and ACT1 have been generated and analyzed [51]. The DiCre system can achieve high levels of gene depletion (up to 100%) without the major concern of leaky expression commonly seen in other systems such as the Tet-based systems described below, but DiCre constructs may be difficult to build and deletion is irreversible.

### Tetracycline-Inducible System

The tetracycline-repressor system is a widely used strategy to modulate gene expression in eukaryotes [60]. This system comprises two regulatory elements: the Tet operator (TetO), placed close to the promoter of a GOI, and the ectopically expressed Tet repressor (TetR). Addition of anhydrotetracycline (ATc) abolishes the binding of TetR to TetO and consequently allows the initiation of transcription (Figure 2B) [52,61]. When the Tet system was first adapted to *T. gondii*, it allowed gene expression in the presence of ATc; therefore, it was not ideal for the regulation of essential genes because it required the constant growth of parasites in ATc-containing media, which can lead to revertants that are refractory to regulation [52,53]. This system has other applications such as the generation of dominant-negative mutants [52,53]. To overcome this problem, the tetracycline-inducible transactivator system was developed and has been widely used to study essential genes in *T. gondii* (Figure 2C) [54]. In this system, an artificial

expressed Tet repressor (TetR) binds to TetO and represses transcription, while addition of ATc abolishes TetR–TetO binding and consequently allows transcription. (C) Conditional regulation of gene expression by a tetracycline-inducible transactivator system in the TATi-1 strain. In this system, a minimal silent promoter is placed upstream of the GOI and a TetO sequence is placed upstream of the minimal silent promoter. The TATi-1 strain expresses Tet-transactivator protein (Tet-TA), a fusion of the Tet repressor and a transactivating domain that binds to TetO and induces transcription of the GOI. Addition of ATc prevents Tet-TA binding to TetO and transcription is silenced. Left: Two-step approach for strain construction to study the GOI in the TATi-1 line. The first step is to introduce an extra copy of the GOI into the genome under Tet-regulatable promoter control; the second step is to delete the endogenous GOI by homologous recombination. Right: One-step strain construction to regulate GOI expression in TATi-1 that replaces the endogenous promoter with the Tet-regulatable promoter. (D) Conditional regulation of transcriptional expression by a U1 small nuclear ribonucleic particle (U1 snRNP)-mediated gene-silencing system in the DiCre strain. The endogenous GOI locus is modified at the 3′ end by the introduction of a selection cassette flanked by *loxP* sites and U1 snRNA recognition sequences. Addition of rapamycin induces Cre recombinase activity and removal of the selection cassette, shifting the U1 snRNA recognition sequences adjacent to the termination codon of the GOI. This leads to recruitment of the U1 snRNP and degradation of the pre-mRNA of the GOI. (E) Conditional regulation of protein stability by a destabilization domain fusion system. The endogenous GOI is fused to the destabilization domain (ddFKPB) at the N or C terminus. Addition of Shield-1 leads to stabilization of the fusion protein and the function of the GOI is maintained. Removal of this ligand results in rapid degradation of the fusion protein and loss of GOI function.

transcriptional activating domain was fused to TetR to form a tetracycline-dependent transactivator (Tet-TA), which activates the expression of genes from TetO-containing promoters in the absence of ATc. Addition of ATc to this system abolishes Tet-TA–TetO binding, leading to repression of gene expression [54]. A parasite line (TATi) expressing Tet-TA was used to study the functions of many genes, including MyoA, AMA1, and CDPK1 [54,62,63]. Two approaches were used to generate conditional mutants with this system. The first approach requires two steps: introduction of an extra copy of the GOI under the control of ATc and subsequent deletion of the endogenous gene. The second approach directly replaces the endogenous promoter of the GOI by a Tet-regulatable promoter. The availability of the TATi-*Δku80* strain made the second approach even easier [64]. One concern regarding the Tet-TA system is that the gene depletion is often not 100% in the presence of ATc and residual expression may mask the phenotypes associated with the GOI.

### U1 snRNP-Mediated Gene-Silencing System

The use of a U1 snRNP-mediated gene silencing system was recently reported in *T. gondii* (Figure 2D) [55]. This system relies on the positioning of a U1 snRNA recognition sequence into the 3′-terminal exon or adjacent to the stop codon of the GOI, which leads to the degradation of pre-mRNA and efficient depletion of the GOI. To optimize this system for the analysis of essential genes and to achieve temporal regulation, it is used in combination with DiCre technology to conditionally place the U1-recognition sequences next to the stop codon of the GOI. Addition of rapamycin leads to the deletion of the *loxP*-flanked sequences that had separated the U1-recognition sequence from the GOI stop codon, resulting in their juxtaposition and subsequent degradation of the GOI transcripts [55]. Using this technology, *vps26*, *chc1*, and *drpC* were successfully silenced in *T. gondii* [55]. One drawback that is often seen with DiCre–U1 technology is that the introduction of *loxP* sites into the 3′ untranslated region (3′-UTR) can itself affect the expression of the GOI and, probably because of this, tagging genes with U1 has largely not been fruitful, with a success rate of about 20% [55].

### Regulation of Protein Stability

The conditional approaches discussed above are relatively slow in silencing genes, prompting the need to develop a system that could rapidly regulate protein stability in *T. gondii* (Figure 2E) [56]. This alternative system is based on fusing a protein of interest to the N or C terminus of a rapamycin-regulated destabilization domain such as ddFKBP. The fusion protein is stabilized in the presence of the rapamycin analog Shield-1 (Shld1) but is rapidly degraded in its absence [65]. Although this system has fast degradation kinetics and is suitable for the generation of dominant-negative mutants, it may not be suitable for the regulation of proteins residing in organelles because the degradation of fusion proteins requires the proteasome, which resides in the cytosol.

### Chemical Genetics

All of the abovementioned strategies require modification of the GOI locus in one way or another to dissect its function. Chemical genetics, by contrast, uses small molecules to inhibit the function of a gene product to assess its biological consequence. As in classic genetics, chemical genetics can also be performed in a forward or reverse direction. In recent years, both forward and reverse chemical genetics have been utilized to study the biology of *T. gondii*. For example, Compound 1 was identified in a cell-based screen (forward chemical genetics) to inhibit the growth of *T. gondii* and other coccidian parasites *in vitro* and *in vivo* [66–68]. Subsequently, the biological relevant target of Compound 1 was confirmed to be protein kinase G (PKG) in experiments testing the sensitivity of wild-type and gatekeeper-mutant (T761Q) parasites to Compound 1 [68]. Well-known examples of reverse chemical genetics include the use of bumped kinase inhibitors to study the functions of calcium-dependent protein kinases (CDPKs) [69]. Protein kinases typically contain relatively large gatekeeper residues (a residue in the ATP-binding pocket) but these can be mutated to small residues such as glycine without

compromising the kinase activity. Kinases with small gatekeeper residues allow the docking of bulky (bumped) inhibitors to the ATP-binding pocket and are sensitive to such inhibitors. CDPK1 comes with a glycine residue at the gatekeeper position and therefore is naturally sensitive to bumped inhibitors. Utilizing this unique property, 3-MB-PP1, a bumped kinase inhibitor, was used to assess the biological functions of CDPK1. The results from chemical genetic studies were in good agreement with those from classic molecular genetics [63,69]. One obvious challenge for chemical genetics is the identification of small compounds with adequate specificity, which can be difficult. For example, more recent studies suggest that PKG is not the only target of Compound 1 [70].

## CRISPR–Cas9-Mediated Genome Editing in *T. gondii*

Although traditional genetics strategies have been effective in yielding important insights into *T. gondii* biology, these strategies require complex plasmid constructions and are difficult to use in non-laboratory strains. Recently, programmable nucleases were engineered for genome editing purposes, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and RNA-guided engineered nucleases (RGENs) such as Cas9. Although ZFNs and TALENs have been used for several years in other models, they were never developed in *T. gondii*, which may be due to the high cost and laborious design. The CRISPR–Cas9 system is widely used for genome editing in various organisms, allowing high-throughput functional genomics analysis [71,72]. Like ZFNs and TALENs, CRISPR–Cas9 has the ability to induce site-specific DSBs at the targeted site, which enhances recombination by several orders of magnitude [73].

CRISPR systems are used by bacteria and archaea as a defense tool against invading viruses and plasmids [74]. Among the various CRISPR systems, the type II CRISPR system from *Streptococcus pyogenes* is the most extensively studied [75,76]. In this system, CRISPR RNA (crRNA) forms an RNA duplex with a *trans*-activating crRNA (tracrRNA) to guide the endonuclease Cas9 to a targeted sequence to introduce DSBs. For use as a genome editing tool, the RNA duplex can be replaced by a single guide RNA (sgRNA) [76]. Due to the targeted specificity of DSBs and ease of design, CRISPR–Cas9-based technologies have revolutionized genome editing in various organisms, including *T. gondii* [13,15,41,42,77–80]. Shen *et al.* [41] showed that CRISPR–Cas9 not only can knock out genes in the laboratory-adapted RH strain but also can disrupt the *rop18* gene in the low-passage natural-isolate GT1 strain, which had been refractory to traditional reverse genetics approaches. When CRISPR–Cas9 generates DSBs in a targeted sequence, a selectable marker can be integrated into the DSB site by NHEJ or HDR [41]. Sidik *et al.* [42] demonstrated that the DSBs generated by CRISPR–Cas9 can be used to introduce point mutations or epitope tags. They also showed that HDR induced by CRISPR–Cas9 is very efficient in the RH*ΔKu80* strain, which allows genetic manipulation even without drug selection [42]. Both studies used a single plasmid to express the Cas9 nuclease and the sgRNA and are available from Addgene (Plasmid #54467 and Plasmid #52694). Further studies modified the original plasmid to express two sgRNAs for the efficient deletion of large fragments (such as the *rop5* locus, a large tandemly repeated region with multiple copies of *rop5* [79]). It was also used to rapidly produce double or triple CDPK knockouts [15]. Furthermore, several web-based tools for selecting sgRNA sequences and predicting specificity have been recently made available (for *T. gondii*, http://www.e-crisp.org and http://grna.ctegd.uga.edu/) [81]. These advances will significantly enhance our ability to manipulate the *T. gondii* genome.

## CRISPR–Cas9: A Powerful Tool to Understand Parasite Biology

As a model organism for apicomplexan parasite biology, the study of *T. gondii* is highly influenced by the availability of genetic tools used to manipulate its genome. Over the past 20 years, tremendous progress and significant improvements have been made in this regard. Currently there are various tools available to inactivate nonessential genes and to manipulate essential genes for functional dissection. However, from a practical point of view, techniques that

allow genome editing with higher efficiency at a larger scale and that are less time consuming are desirable. For disruption of nonessential genes, the CRISPR–Cas9 system is in general the best among the currently available approaches. First, it is efficient and specific and can be used in almost any strain. Second, using a dual guide RNA strategy can precisely generate large deletions and multiple-gene knockouts with high efficiency. Third, CRISPR–Cas9 can be used to generate genome-wide mutant libraries, which is extremely useful and should be pursued [82].

Due to the haploid nature of the *T. gondii* genome, analysis of essential genes can be performed by regulating the expression of a single allele. An ideal system for such applications should offer reversible, rapid, and tight (fully on or fully off) regulation of protein levels. Unfortunately, none of the approaches currently available fulfills all of these requirements. Nonetheless, it should be noted that the CRISPR–Cas9 system can be modified and used for regulating targeted gene expression [83,84]. This can be achieved by mutating Cas9 into a catalytically inactivated enzyme (dCas9), which can be repurposed as a RNA-guided DNA-binding protein (Figure 3). Furthermore, other RNA molecules with special properties can be fused to sgRNA without affecting its binding to dCas9 [83]. Consequently, a transcriptional activator and repressor can be directed to promoter regions to regulate gene expression by fusing them to dCas9 itself or recruitment via RNA–sgRNA fusion. Spatially and temporally controlled expression of dCas9 or sgRNA expression is also promising for the of study essential genes [85,86]. Additionally, CRISPR–Cas9 can be modified to effectively manipulate the RNA transcripts only without

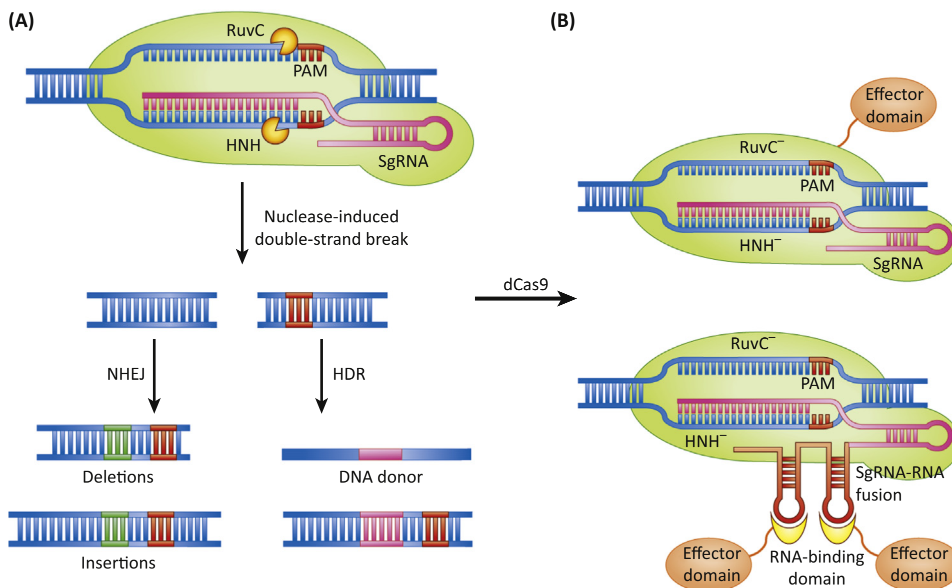


Figure 3. The Power of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–Cas9-Based Systems. (A) Gene editing with the CRISPR–Cas9 system. Cas9 is guided to the targeted DNA sequence by a single guide RNA (sgRNA) and its nuclease domains (RuvC and HNH) generate blunt-ended double-strand breaks (DSBs) in targeted DNA. The blunt-ended DSBs can be repaired by either nonhomologous end joining (NHEJ) or homology directed repair (HDR). The error-prone NHEJ mechanism can result in small insertion or deletion (indel) mutations. If homologous sequences are available, HDR can be used to repair the DSBs and introduce any desired modifications, from nucleotide substitutions to complete gene replacement at the targeting site. (B,C) The CRISPR–Cas9 system has been exploited in numerous, diverse applications by using a catalytically inactive Cas9 (termed dCas9). Either the dCas9 (B) or the sgRNA (C) can be fused with other molecules to recruit various effector proteins to a specific locus. These include the introduction of a transcriptional repressor (KRAB) or activator (VP64) to regulate gene expression, fluorescent proteins to visualize chromosomal structure and dynamics, histone acetylases to alter epigenetic modifications, and affinity tags to isolate specific genomic regions and associated proteins.

disrupting the corresponding DNA template [87]. If applied to *T. gondii*, such approaches may provide alternative ways to study essential genes in a high-throughput fashion.

In addition to transcriptional regulation, CRISPR–Cas9 systems have other applications. dCas9 fused with an enhanced GFP has been applied to visualize specific genomic loci in living cells [88], which could be used to study genomic architecture and dynamics during *T. gondii* replication and differentiation. dCas9 fused with affinity tags may be used for the purification of specific chromatin-containing regions [89]. Furthermore, dCas9 has been used for targeted epigenome editing in human cell lines [90], and similar applications may be useful in *T. gondii*.

One major problem with the CRISPR–Cas9 system is the generation of off-target mutations [91,92]. However, this problem is not a huge concern in *T. gondii* due to the relatively small size of its genome [93]. Furthermore, many strategies have been exploited to overcome this problem, such as double-nicking CRISPR–Cas9, dimeric *FokI*–dCas9 nucleases, high-fidelity Cas9 nucleases, and truncated sgRNAs [94–97].

## Concluding Remarks

Understanding the functions of specific genes in *T. gondii* and the roles they play in parasite development and pathogenesis is the most direct way to identify drug targets and develop new vaccines. Various genetic tools have been developed to study gene functions in *T. gondii*, yielding important insights into the parasite's biology. However, most of the traditional technologies have limitations and are not suitable for generating mutants on a large scale. The malleable design, high efficiency, and simplicity of the CRISPR–Cas9 system have made it an unprecedented genome engineering tool in a variety of cells and organisms. The use of this system in *T. gondii* is still in its infancy, yet it has already proved to be a powerful tool for targeted gene disruption. As an easy and affordable tool, it makes feasible high-throughput functional analysis of *T. gondii* genes by establishing individual CRISPR-based gene mutant libraries. In conclusion, we believe that ongoing efforts and future advances in the CRISPR–Cas9 technology have the potential to revolutionize the field of parasitology (see Outstanding Questions).

### Outstanding Questions

What are the endogenous mechanisms that make some *Toxoplasma gondii* strains refractory to gene manipulation by traditional reverse genetics approaches?

How can we fully combine traditional genetics strategies with the CRISPR–Cas9 system to study *T. gondii* biology?

How can we modify the CRISPR–Cas9 system to study essential genes in *T. gondii*?

Will CRISPR–Cas9-based strategies be used to explore the functions of RNA and long noncoding RNAs and manipulate the epigenome in *T. gondii*?

How can the CRISPR–Cas9 system be further improved to allow genetic manipulation of *T. gondii* with higher efficiency and throughput?


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