Investigating expression of a human optimized *cas9* transgene in *Neurospora crassa*

Natalie Burrell*, Nicholas A. Rhoades*, Amy Boyd, Jim Mierendorf, Aykhan Yusifov, Austin Harvey, Kevin Edwards, Laura Vogel, and Thomas M. Hammond.

School of Biological Sciences, Illinois State University, Normal, Illinois 61790

*These authors contributed equally to this work.

¹Corresponding author:

Thomas M. Hammond

School of Biological Sciences

Illinois State University

Normal, IL 61790

Phone: 309-438-2602

Email: tmhammo@ilstu.edu

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Cas9 in *Neurospora crassa*

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ABSTRACT

The CRISPR-associated Cas9 enzyme is used in molecular biology to engineer the genomes of a wide range of organisms. While Cas9 can be injected or transfected into a target cell to achieve the desired goal, there are situations where stable expression of Cas9 within a target organism is preferable. Here, we show that the model filamentous fungus *Neurospora crassa* is recalcitrant to heterologous expression of a human-optimized version of *Streptococcus pyogenes cas9*. Furthermore, partial optimization of *cas9* by synonymous codon exchange failed to improve its expression in the fungus. Finally, we show that transgene expression can be detected when *cas9^{HS}* sequences are placed in the 3' UTR regions of transgene-derived mRNAs, but not when the same sequences are in the translated part of the transgene-derived mRNA. This finding suggests that the primary obstacle to high *cas9^{HS}* expression levels in *N. crassa* is translational in nature.

INTRODUCTION

The availability of CRISPR-associated (Cas) systems for use in genome engineering has accelerated research on organisms that were historically difficult to engineer with traditional techniques (Knott and Doudna 2018; Wang *et al.* 2020; Zhu *et al.* 2020). Cas technology has also accelerated the field of gene driver research, where it offers the possibility of engineering synthetic gene drivers for the control of disease spreading organisms (Esvelt *et al.* 2014). For these reasons, we sought to establish a robust *Streptococcus pyogenes* Cas9-based system for use in the model filamentous fungus *Neurospora crassa*.

MATERIALS AND METHODS

Strains and media

Strains are listed in Table 1. Vogel's minimal medium (VMM) (Vogel 1956) with and without 2% agar was used for vegetative propagation of all strains. L-histidine was added to VMM at 500 mg / L when needed to support growth of *his-3* strains. Hygromycin B (GoldBio, H-270) was included in media at concentrations of 200 µg / ml to select for hygromycin-resistant transformants. Crosses were performed on synthetic crossing medium (pH 6.5) with 1.5 % sucrose (Westergaard and Mitchell 1947).

N. crassa transformation

Transformation of *N. crassa* was performed as described by Rhoades et al. (2019b), except that the recovery step was omitted when transformants were selected for histidine prototrophy. *N. crassa* strain P8-42 was transformed with pAH41.4, a plasmid containing *tef-1(p)-cas9^{HS}*. Transformants were selected by their ability to grow on medium lacking histidine.

Transformation vectors v76, v77, v95, v96, v97, v98, v100, v101, v102, v103, v104, v124, v125, v126, v161, v163, and v193, which convert a *tef-1(P)-cas^{9Hs}* transgene to a *ccg-1(P)-gfp-cas^{9Hs}* transgene, were transformed into strain ISU-4145. Transformants were selected for resistance to hygromycin B. Transformation vectors v76-stop, v77-stop, v95-stop, v96-stop, v97-stop, v98-stop, v100-stop, v101-stop, v102-stop, v103-stop, v104-stop, v124-stop, v126-stop, v161-stop, v163-stop, v193-stop, v296-stop, v307-stop, v308-stop, and v309-stop, which convert a *tef-1(P)-cas^{9Hs}* transgene to a *ccg-1(P)-gfp-stop-cas^{9Hs}* transgene, were transformed into strain ISU-4145. Transformants were selected for resistance to hygromycin B. Plasmids pNR206.2 and pNR207.1 contain *gfp-cas^{9NcC213}* and *gfp-cas^{9NcC60}* transgenes, respectively, and these plasmids were used to transform strain P8-43. Transformants were selected for histidine prototrophy. Homokaryotic strains were isolated from transformants by single spore purification (from conidium or ascospores) and confirmed to be homokaryotic with polymerase chain reaction (PCR)-based genotyping assays.

Transgene construction and insertion

tef-1(P)-cas^{9Hs}: A *tef-1(P)-cas^{9Hs}*-containing plasmid (pAH41.4) was constructed by amplifying the *N. crassa tef-1* promoter region (*tef-1[P]*) from *N. crassa* genomic DNA with primers 598 and 599 by PCR and inserting the PCR product into the *SpeI* site of Plasmid 43802 (Addgene, DiCarlo *et al.* 2013). The resulting plasmid was used as the template for PCR-based amplification of *tef-1(P)-cas^{9Hs}* with primers 610 and 589. The PCR product was inserted into the *NotI* site of the *his-3*-targeting plasmid pTH1150.11 (GenBank MN872812.1) to produce plasmid pAH41.4. Primer sequences are listed in Table S1. Sequences of *N. crassa* genes, such as *tef-1* and the *tef-1* promoter region, were obtained from FungiDB (Stajich *et al.* 2012).

gfp-cas9^{Hs}: The *gfp-cas9^{Hs}* and *gfp-stop-cas9^{Hs}* transgenes, including all truncated versions (e.g., *gfp-cas9^{HsC1209}*, *gfp-stop-cas9^{HsC1078}*, etc.) were constructed by double-joint PCR (DJ-PCR) (Hammond *et al.* 2011) with primers and templates described in Tables S1 and S2.

gfp-cas9^{NcC213}: Primers 2021 and 2022 were used to amplify a single PCR product containing *hph*, *ccg-1(P)* (McNally and Free 1988), *gfp*, and a *(GA)₅*-linker from plasmid pTH1117.12 (GenBank JF749202.1). Primers 1980 and 624 were used to amplify a single PCR product containing *cas9^{NcC213}* from pNR177.2 (described below). The two products were fused by PCR, and the fusion product was used as a template for amplification with primers 2024 and 2025. The amplified product was digested with *NotI* and cloned into the *NotI* site of the *his-3*-targeting plasmid pTH1150.11 to create plasmid pNR206.2.

gfp-cas9^{NcC60}: The *gfp-cas9^{NcC60}* transgene was assembled similarly to the *gfp-cas9^{NcC213}* transgene except that primer 1980 was exchanged for primer 1983. The final amplification product was cloned into the *NotI* site of pTH1150.11 to create plasmid pNR207.1.

Codon optimization

We used a publicly available RNAseq dataset (SRR1055991, Wu *et al.* 2014) to identify 100 of the most highly expressed protein-coding genes in the *N. crassa* nuclear genome (Table S3) by aligning RNA sequences to all predicted *N. crassa* protein coding genes as described in Samarajeewa *et al.* (2017). Genes with the highest “reads per kilobase exon model” (RPKM) values (Mortazavi *et al.* 2008) were considered to be the most highly expressed. Relative

adaptiveness (RA) values (Sharp and Li 1987) were then calculated for each codon (Table S4). RA values were calculated by dividing the observed frequency of each codon in the set of 100 highly expressed genes by the frequency of the most common synonymous codon in the same set of genes. Codon optimization of *cas9* for expression in *N. crassa* was then performed by replacing *cas9^{His}* codons in *cas9^{His}* as follows: 1) the synonymous codon with the highest RA value was used for all occurrences of amino acids C, E, F, H, I, K, L, M, N, Q, W, and Y, as well as the stop codon; 2) the synonymous codon with the second highest RA value was used for all occurrences of A, D, G, P, and R; and, 3) a combination of synonymous codons with either the first or second highest RA values was used for amino acids S, T, and V. Use of the second most optimal codon for some amino acids helped reduce the overall GC content of the optimized sequence. Reducing GC content allowed the optimized sequence to be synthesized as a gBlock® (Integrated DNA technologies). A gBlock® DNA fragment containing the optimized *cas9^{Nc}* sequence (Figure S1) was cloned to pJET1.2 to create pNR177.2.

Visualization of GFP in conidia by fluorescence microscopy

Fluorescence microscopy and imaging was performed with a Leica DMBRE microscope or a Leica SP8 confocal microscope. For imaging with a Leica DMBRE system, *N. crassa* cultures were incubated on VMM for 1-2 days at 32°C followed by 2-4 days at room temperature. Conidial suspensions were placed on a standard microscope slide for imaging. A 40× objective was used. GFP-signal was collected with a 20 second exposure for all strains. Cropped raw images were used without additional modifications. For imaging with the Leica SP8 confocal microscope, the same growth conditions were followed as stated for imaging with the Leica DMBRE system. Conidial suspensions were prepared, and then incubated in a 37°C shaker for 2

hours before imaging. Conidial suspensions were placed on a standard microscope slide for imaging. All the images were acquired with the same settings so that they could be directly compared. A 63×/1.40 oil objective was used, with a white light laser set to a wavelength of 488 nm. Fluorescence was detected between the wavelengths of 500–560nm, with 8× line averaging. Images were assembled in Photoshop; GFP is shown with original contrast.

Quantification of GFP in conidia by flow cytometry

Fresh *N. crassa* cultures were prepared by qualitative transfer of conidia to 2.5 ml of solid VMM slants in 16×100 mm glass culture tubes. The inoculated culture tubes were incubated for two days in a 32°C incubator and 5–6 days at room temperature on a laboratory bench top. Sterile wood applicators were used to transfer conidia to 1× PBS (137 mM NaCl, 2,7 mM KCl, 10mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) (Figure S2). Conidial suspensions were then analyzed with a BD FACSMelody system (equipped with 488 nm, 561 nm, and 640 nm lasers) and FACSCorus software. Conidia were gated on FSC and SSC and MFI of GFP histograms were analyzed using a 1.5 neutral density filter. Raw MFI values are provided in Table S5.

RESULTS

A *cas9^{His}* transgene is poorly expressed in *N. crassa*

We began our studies by constructing a *cas9^{His}* transgene (Fig. 1A) and inserting it downstream of *his-3* on chromosome I (Fig. 1B). We then constructed a second transgene, *gfp-cas9^{His}*, to express a GFP-Cas9 fusion protein (Fig. 1, C–E). This second transgene was constructed to allow us to determine if Cas9, which includes an SV40 nuclear localization signal on its C-terminus, localizes to the *N. crassa* nucleus. However, when we examined the *gfp-cas9^{His}* strain

by fluorescence microscopy, we failed to detect a GFP signal (Fig 2, A–B). Because we focused our assays on *N. crassa* conidia (asexual spores), we considered the possibility that the promoter used to drive expression of *gfp-cas9^{Hs}* (*ccg-1[P]*) was insufficient for expression of the fusion protein in this cell type. Thus, as a control, we examined conidia from a *gfp-sad-6* transgene-carrying strain. SAD-6 is a meiotic silencing by unpaired DNA (MSUD) protein (Samarajeewa *et al.* 2014) and it was chosen as a control for Cas9 expression because it is larger than Cas9 (210 kD vs 159 kD) and because, like the *gfp-cas9^{Hs}* transgene, the *gfp-sad-6* transgene is driven by the *ccg-1* promoter. In contrast to GFP-Cas9, GFP-SAD-6 was easily detected by fluorescence microscopy (Figure S3, compare A and B). These observations suggest that the *ccg-1* promoter should be sufficient for expression of GFP-Cas9 in *N. crassa* conidia.

Partial codon optimization of *cas9* for expression in *N. crassa*

The above results suggest that *cas9^{Hs}* sequences are poorly expressed in *N. crassa*. To identify a segment of *cas9^{Hs}* that could be expressed more robustly in the organism, and concomitantly identify regions of *cas9^{Hs}* that prevent its robust expression, we dissected the *cas9^{Hs}* coding region with a series of *gfp-cas9^{HsC#}* transgenes (Fig. 1D). “C#” specifies the number of Cas9 amino acids in the GFP-tagged and N-terminally truncated Cas9 protein (for example, *cas9^{HsC60}* encodes only the C-terminal 60 amino acids of Cas9. The number “60” does not include the seven amino acids of the C-terminal NLS). We constructed 16 of these *gfp-cas9^{HsC#}* transgenes and examined GFP levels in conidia from the *gfp-cas9^{HsC#}* transgene-carrying strains by flow cytometry. Interestingly, we found that all transgenes containing more than 212 *cas9^{Hs}* codons failed to produce a GFP signal at detectable levels and that GFP levels increased rapidly as the number of *cas9^{Hs}* codons approached zero (Fig 2, C and D; Fig. 3A).

We considered the possibility that robust expression of *cas9^{Hs}* in *N. crassa* is restricted by the organism's synonymous codon biases. To test this hypothesis, we altered the *cas9* coding sequence in the *gfp-cas9^{HsC213}* and *gfp-cas9^{HsC60}* transgenes to produce equivalent *gfp-cas9^{NcC213}* and *gfp-cas9^{NcC60}* transgenes (Figure S3). Specifically, we replaced codons that are found at low frequency in highly expressed *N. crassa* mRNAs with synonymous codons that are found at high frequency in *N. crassa* mRNAs (see methods). Interestingly, despite replacing 120 of 220 codons to make *cas9^{NcC213}* (213 *cas9^{Hs}* codons, 7 *nls* codons, and 1 stop codon) and replacing 40 of 68 codons to make *cas9^{NcC60}* (60 *cas9^{Hs}* codons, 7 *nls* codons, and 1 stop codon), neither transgene produced more GFP fusion protein than their human-optimized counterparts (Fig. 4).

Translation of *cas9^{Hs}* sequences negatively influences expression of *gfp-cas9^{Hs}* transgenes

The tendency of GFP levels to increase as the length of *cas9^{Hs}*-coding sequence decreases suggests that low GFP levels are due to problems encountered during translation of *cas9^{Hs}* sequences by *N. crassa* ribosomes. To test this hypothesis, we constructed a set of *gfp-stop-cas9^{HsC#}* transgenes. These transgenes are identical to the *gfp-cas9^{HsC#}* transgenes except that they contain a stop codon between the *gfp* and *cas9^{Hs}* coding sequences. Interestingly, we found that the placement of a stop codon between the *gfp* and *cas9^{Hs}* coding sequences increased the level of GFP produced by all transgenes, but not in a completely uniform manner (Figure 3B and Table 3). For example, GFP levels appeared to increase around two maxima, one represented by the *gfp-stop-cas9^{HsC656}* transgene and the other by the *gfp-stop-cas9^{HsC4}* transgene (Figure 3B).

DISCUSSION

In this study, we have presented evidence demonstrating that a human-optimized *cas9^{Hs}* transgene is poorly expressed in *N. crassa*. Our major findings are as follows: 1) a *gfp-cas9^{Hs}* transgene is poorly expressed relative to a control transgene *gfp-sad-6*, 2) removing the majority of *cas9^{Hs}* coding sequences from the *gfp-cas9^{Hs}* transgene (e.g., with *gfp-cas9^{HsC#}* transgenes) improves expression levels, 3) increasing the number of *N. crassa*-“preferred” codons does not improve expression of at least two segments of the *cas9* coding region, and 4) *cas9^{Hs}*-coding sequences negatively influence transgene expression in a translation dependent manner.

Our data suggest that the relatively large size of a GFP-Cas9 fusion protein should not prevent its detection by our chosen cytological and flow-cytometry-based methods because both methods allowed us to detect a GFP-SAD-6 fusion protein. Importantly, the *gfp-cas9^{Hs}* and *gfp-sad-6* transgenes use the same promoter (*ccg-1[P]*) and the same *gfp* coding sequences. This suggests that transcription rates of both transgenes should be relatively similar, as should the efficiency of ribosome loading onto the mRNAs derived from each transgene (e.g., because the mRNAs should have identical 5' UTRs). However, the dissimilarities between the *gfp-cas9^{Hs}* and *gfp-sad-6* transgenes are numerous, and they include differences in coding sequences, 3' UTRs, termination sequences, and locations in the genome. At this point, it is unclear which, if any, of these dissimilar factors are the primary cause of the poor expression of *gfp-cas9^{Hs}* in *N. crassa*.

To gain insight into which regions of the *cas9^{Hs}* coding sequence may negatively influence its expression, we systematically removed 5' segments of increasing length from the *cas9^{Hs}* coding sequence with a series of *gfp-cas9^{HsC#}* transgenes. With this approach, we found it was necessary to remove nearly all the *cas9^{Hs}* coding sequence from a *gfp-cas9^{HsC#}* transgene

before it would express GFP at detectable levels. One possibility is that mRNAs from transgenes with shorter *cas9^{Hs}* coding sequences are found at higher levels in *N. crassa* than are mRNAs with longer *cas9^{Hs}* coding sequences. If true, this could be due to differences in transcriptional efficiency or transcript stability. However, while we have not measured *gfp-cas9^{Hs}* mRNA levels in this study, we did measure GFP levels in strains carrying *gfp-stop-cas9^{Hs}* transgenes and we found that all *gfp-stop-cas9^{Hs}* transgenes express GFP at detectable levels, even if the transgene contained the full length *cas9^{Hs}* coding sequence. These findings suggest that *gfp-cas9^{Hs}* expression problems are encountered after *N. crassa* ribosomes finish translating *gfp* sequence and begin translating *cas9^{Hs}* sequences.

Codon optimization has long been considered a useful technique for improving the expression of heterologous sequences in transgenic organisms. In this study, we sought to improve the expression of *gfp-cas9^{HsC213}* and *gfp-cas9^{HsC60}* transgenes by increasing the codon adaptation index (CAI) (Sharp and Li 1987) of each transgene. This codon optimization strategy involves calculating relative adaptiveness (RA) values for each of the 64 possible codons from a set of species-specific high abundance mRNAs and exchanging codons with low RA values for synonymous codons with high RA values. We focused our efforts on *gfp-cas9^{HsC213}* and *gfp-cas9^{HsC60}* because the *cas9^{Hs}* coding sequences in both transgenes are relatively short, and even though *gfp-cas9^{HsC60}* expresses GFP, it does so at a level that is an order of magnitude below that of the shortest transgene examined in this study (i.e., *gfp-cas9^{HsC4}*). Therefore, there appears to be room to improve the expression of both *gfp-cas9^{HsC213}* and *gfp-cas9^{HsC60}* transgenes. Interestingly, the *N. crassa* optimized transgenes *gfp-cas9^{NcC213}* and *gfp-cas9^{NcC60}* did not express GFP at higher levels than their human optimized counterparts (Figure 4). This finding suggests

that *N. crassa* codon preferences are neither the major factor preventing expression of *gfp-cas9^{HsC213}* at detectable levels, nor the major factor limiting expression of *gfp-cas9^{HsC60}*.

A possible explanation of the results observed in this study is that the *N. crassa* translational machinery stalls within the *cas9^{Hs}* segments of *cas9^{Hs}*-containing mRNAs. To shed light on this possibility, we placed stop codons between *gfp* and *cas9^{Hs}* sequences, effectively placing *cas9^{Hs}* “coding” sequences in the 3' UTR of each transgene-derived mRNA. The presence of *cas9^{Hs}* coding sequences within the 3' UTRs of *gfp-stop-cas9^{Hs}* transgene derived mRNAs influenced expression in unexpected ways. For example, while transgene expression was detected for all *gfp-stop-cas9^{HsC#}* transgenes, it was highest for transgenes containing the smallest number of *cas9^{Hs}* codons in the 3' UTR. This finding is consistent with the faux 3' UTR model (Amrani *et al.* 2006; Nicholson *et al.* 2010; Zhang and Sachs 2015), where abnormally long 3' UTRs are thought to trigger degradation of the mRNA by nonsense mediated decay. However, our observations are not completely consistent with the faux 3' UTR model because transgene levels were not strictly directly proportional to the length of the 3' UTR. For example, transgene expression levels were higher when the 3' UTR contained between 796 and 612 *cas9^{Hs}* codons than when the 3' UTR contained between 535 and 213 codons. Currently, we do not have an explanation for this phenomenon. More importantly, we performed this set of experiments to determine if halting translation of *gfp-cas9^{Hs}* transgenes before ribosomes entered the *cas9^{Hs}* regions of an mRNA would improve their expression, and indeed, we found that it does. GFP production was higher from every *gfp-stop-cas9^{HsC#}* transgene than from the equivalent *gfp-cas9^{Hs}* counterpart, suggesting that the primary obstacle to *cas9^{Hs}* expression in *N. crassa* occurs during translation of *cas9^{Hs}* sequences.

Our investigation of *cas9^{Hs}* expression in *N. crassa* evolved from a desire to study *cas9*-based synthetic gene drivers in filamentous fungi, an area of research that complements our primary focus on fungal meiotic drive elements (Hammond *et al.* 2012; Harvey *et al.* 2014; Pyle *et al.* 2016; Svedberg *et al.* 2018; Rhoades *et al.* 2019a). Although our current findings suggest that *cas9^{Hs}* sequences are poorly expressed in *N. crassa*, other researchers have successfully used *cas9^{Hs}* to edit genes in *N. crassa* (Matsu-Ura *et al.* 2015). Additionally, Cas9-based technologies have been used with success in dozens of filamentous fungi (Song *et al.* 2019). Still, identifying the mechanistic basis of *cas9^{Hs}*'s poor expression in *N. crassa* could help increase the usefulness of Cas9-based technologies in this organism.

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FIGURE LEGENDS

Figure 1 Transgene illustrations. (A) Diagram of the *tef-1(P)::cas9^{Hs}* transgene. The *cas9^{Hs}* transgene contains the *N. crassa tef-1* promoter (*tef-1[P]*), the *cas9^{Hs}*-coding sequence, an SV40 nuclear localization signal (*nls*), an *S. cerevisiae cyc1* terminator (*cyc1[T]*), and a nourseothricin resistance cassette (*nat*). (B) Diagram of the *N. crassa his-3* locus. The orange horizontal bar marks the DNA interval that was deleted and replaced with the *tef-1(P)::cas9^{Hs}* transgene. The diagram is drawn to scale, and the orange bar represents a length of 919 bp. The *his-3* coding region is depicted by the white rectangle. (C) Diagram of the *ccg-1(P)::gfp-(GA)₅* construct used to construct *gfp-cas9^{Hs}* transgenes. Note that the *gfp* sequence in every *gfp*-containing transgene examined in this study is immediately followed by the coding sequence for a ten amino acid glycine-alanine linker ([GA]₅). (D) Diagram showing the DNA intervals of *tef-1(P)::cas9^{Hs}* that were deleted and replaced with *ccg-1(P)::gfp*. The orange vertical dashed lines mark the two *tef-1(P)::cas9^{Hs}* transgene borders. The orange horizontal bars mark the intervals that were deleted and replaced by *ccg-1(P)::gfp* with various transformation vectors (e.g., v76, v95, etc.). (E) Diagram of the *ccg-1(P)::gfp-cas9^{Hs}* transgene obtained by transformation of strain ISU-4145 with transformation vector v76. (F) Diagram of the *ccg-1(P)::gfp-cas9^{HsC318}* transgene obtained by transformation of strain ISU-4145 with transformation vector v103.

Figure 2 Confocal microscopy-based analysis of transgene expression. (A–D) Conidia from a *tef-1(P)-cas9^{Hs}* strain (ISU-4145), a *gfp-cas9^{Hs}* strain (ISU-3994), a *gfp-cas9^{HsC4}* strain (ISU-4231), and a *gfp-cas9^{HsC25}* strain (ISU-4236) were examined by confocal microscopy. Upper panels, transmitted light image; middle panels, GFP signal; lower panels, overlay of transmitted and GFP images. The white horizontal bar represents 10 μ m.

Figure 3 Flow cytometry-based analysis of transgene expression. (A) Conidia from *gfp-cas9^{Hs}* and *gfp-cas9^{HsC#}* strains were analyzed for the presence of GFP by flow cytometry. Mean fluorescence intensity (MFI) values are plotted on the Y axis relative to the *cas9^{Hs}* codon number to which *gfp* is fused in each transgene. Note that *cas9^{Hs}* contains 1380 codons, including seven codons for the SV40 NLS and one codon for the translational stop signal). The gray shaded region of the chart marks the range of MFI values (+/- standard deviation) obtained for negative control strains. The pink shaded region marks the MFI value (+/- standard deviation) obtained for the *gfp-sad-6* positive control strain. MFI values are averages obtained from two assays that were completed one day apart. Error bars are standard deviation values. The data used to construct the chart are provided in Table 2. Raw data is provided in Table S5. (B) Conidia from *gfp-stop-cas9^{Hs}* and *gfp-stop-cas9^{HsC#}* strains were analyzed for the presence of GFP by flow cytometry as in panel A. MFI values are averages of values obtained from two assays that were completed one day apart. The data used to construct the chart is provided in Table 3. Raw data is provided in Table S5.

Figure 4 Expression profiles of similar *gfp-cas9^{HsC#}* and *gfp-cas9^{NcC#}* transgenes. Conidia from *gfp-cas9^{HsC213}*, *gfp-cas9^{NcC213}*, *gfp-cas9^{HsC60}*, and *gfp-cas9^{NcC60}* were analyzed for the presence of GFP by flow cytometry. The gray shaded region of the chart marks the range of MFI values (+/- standard deviation) obtained for negative control strains. The pink shaded region denotes the MFI values and standard deviation obtained for the *gfp-sad-6* positive control strain. The data used to construct the chart is provided in Table 2. Raw data is provided in Table S5.

Figure S1 *cas9^{NcC213}* sequence. The sequence of *cas9^{NcC213}* was ordered as a gBlock® (Integrated DNA technologies) and inserted into pJET1.2 to create plasmid pNR177.2. The *cas9* coding sequence is depicted in red font. There are 221 codons: 213 codons for the *cas9* C terminal end, seven codons for the SV40 NLS, and a single stop codon. The remainder of the sequence includes the *S. cerevisiae cyc-1* terminator (black font) and a pTH1150.1 plasmid sequence to facilitate transformation vector construction (blue font).

Figure S2 Conidia sampling method. VMM slants were inoculated with *N. crassa* by qualitative transfer of conidia. After a 7–8 day incubation period (see methods), an approximately 2.5 mm path of conidia, from points “A” to “B” in the diagram, were transferred to tubes containing 1× PBS.

Figure S3 Fluorescence microscopy-based analysis of transgene expression. (A) Conidia from a control strain (ISU-4233), a *gfp-cas9^{Hs}* strain (ISU-3994), a *gfp-cas9^{HsC4}* strain (ISU-4231), and a *gfp-sad-6* strain (ISU-3121) were examined by fluorescence microscopy with a Leica DMBRE microscope and imaging system.

Figure S4 Comparison of *cas9^{HsC213}* and *cas9^{NcC213}* sequences. A sequence alignment of *cas9^{HsC213}* and *cas9^{NcC213}* is shown. A total of 120 codons in *cas9^{HsC213}* were changed to synonymous codons that are found at higher frequencies in highly expressed *N. crassa* genes. The first codons in the *cas9^{Hs60}* and *cas9^{Nc60}* are marked with a blue horizontal bar.

Figure 1

Figure 2

Figure 3

Figure 4

Figure S1

>*cas9*^{NcC213}

GTCAAGGAGCTCCTCGGTATCACTATCATGGAGCGTTCTTCCTTCGAGAAGAACCCTATCGATTT
CCTCGAGGCTAAGGGTTACAAGGAGGTTAAGAAGGATCTCATCATCAAGCTCCCTAAGTACTCTC
TCTTCGAGCTCGAGAACGGTCGTAAGCGTATGCTCGCTTCCGCTGGTGAGCTCCAGAAGGGTAAC
GAGCTCGCTCTCCCTTCTAAGTACGTAACTTCCTCTACCTCGCTTCCCCTACGAGAAGCTCAA
GGGTTCTCCTGAGGATAACGAGCAGAAGCAGCTCTTCGTTGAGCAGCACAAGCACTACCTCGATG
AGATCATCGAGCAGATCTCCGAGTTCTCTAAGCGTGTTATCCTCGCTGATGCTAACCTCGATAAG
GTCCTCTCTGCTTACAACAAGCACCGTGATAAGCCTATCCGTGAGCAGGCTGAGAACATCATCCA
CCTCTTCACTCTCACCAACCTCGGTGCTCCTGCTGCTTTCAAGTACTTCGATACTACTATCGATC
GTAAGCGTTACACCTCTACTAAGGAGGTCCTCGATGCTACTCTCATCCACCAGTCTATCACCGGT
CTCTACGAGACTCGTATCGATCTCTCTCAGCTCGGTGGTGATTCCCGTGCTGATCCTAAGAAGAA
GCGTAAGGTTTAAATCTCTTCTCGAGTCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTCC
CCCCACATCCGCTCTAACCGAAAAGGAAGGAGTTAGACAACCTGAAGTCTAGGTCCCTATTTATT
TTTTTATAGTTATGTTAGTATTAAGAACGTTATTTATATTTCAAATTTTCTTTTTTTTTCTGTAC
AGACGCGTGTACGCATGTAAACATTATACTGAAAACCTTGCTTGAGAAGGTTTTGGGACGCTCGAA
GGCTTTAATTTGCGGCCGGTACCCAATTGCCCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGC
CGTCGTTTTACAACGTGCTGACTGGGAAAACCTGGCGTTACCCAACCTAATCGCCTTGCAGCAC
ATCCCGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGCCCTTAACATATGCC
GCACAGGTGTTGTCCTCTGAGGACATAAAATACACACCGAGATTCATCAACTCATTGCTGGAGTT
AGCATATCTACAATTGGGTGAAATGGGGAGCGATTTGCAGGCATTTGCTCGGCATGCCGGTAGAG
GTGTGGTCAATAA

Figure S2

Figure S3

Figure S4

	V K E L L G I T I M E R S S F E K N P I D F L	
CAS9-HS	GTCAAGGA ACT GCTGGG CAT CAC AAT CATGGAGCG ATC AAGCTTCGAA AAA AAACCC CAT CGA CTT CTCG	3547
CAS9-NC	GTCAAGGAGCT CCT CGG TAT CAC TAT CATGGAGCG TTC TTCCTTCGAA GAA GAACCC TAT CGA TTT CTCG	3547
	E A K G Y K E V K K D L I I K L P K Y S L F E L	
CAS9-HS	AGGCG AAA AGG ATAT AAAGAGGT C AAAAAG AGC CTCATCAT TAA GCT TCC CAAGTACTCTCTCT TT GAGCT	3617
CAS9-NC	AGGCT TAA GGG TTA CAAGAGGT TAA GAAG GAT CTCATCAT CA AGCT CCT TAAAGTACTCTCTCT TC GAGCT	3617
	E N G R K R M L A S A G E L Q K G N E L A L P	
CAS9-HS	T GAA AA CGG C CGG AAA CG AAT GCTCGCT AGT GCGGG C GAGCTG CAG AAAGGTAACGAGCT G GCA CT GCCC	3687
CAS9-NC	CG GA AA CGG T CG TAA GC TAT GCTCGCT TCC GCTGGT T GAGCT C CAGAA G GGTAACGAGCT CG CT CC CT	3687
	S K Y V N F L Y L A S H Y E K L K G S P E D N	
CAS9-HS	TCTAA A TACGTTAA T TTCT TT GTAT CT GGC CAG CCACTAT TGA AAAGCTCAA AGG CTCTCC CGA AGATAATG	3757
CAS9-NC	TCTAAG TAC GTTAA CTT CT CT TAC CT CGCT TCC ACTAC CGA AGCTCAA AGG TCTCTCC TG AGGATAACG	3757
	E Q K Q L F V E Q H K H Y L D E I I E Q I S E F	
CAS9-HS	AGCAGAAGCAGCTG TT CGTG GAA C AA CACAA AG CACTACCT T GATGAGATCATCGAGCA AAT AAGCGGA ATT	3827
CAS9-NC	AGCAGAAGCAGCT CT TCGT T GAG CA CACAAGCACTACCT CG ATGAGATCATCGAGCAGAT CTC CGAGTT	3827
	S K R V I L A D A N L D K V L S A Y N K H R D	
CAS9-HS	CTC CAA AG AG TGATCCTCGC CGAC CGCTAACCTCGATAAGGTGCT T TCTGCTTACAA T AAGCAC AGG GAT	3897
CAS9-NC	CTC TAA GC CT GT TAT CCTCGCT TGAT GCTAACCTCGATAAGGT CCT CTCTGCTTACAA CA AGCAC CGT GAT	3897
	K P I R E Q A E N I I H L F T L T N L G A P A	
CAS9-HS	AAGCC CAT CA GG GAGCAGGC AGAA AACAT TAT CCACT TG TTTACTCTGACCAACT TT GGG CGC CCCTGC AG	3967
CAS9-NC	AAGCC TAT C GT GAGCAGGC TGAG AACAT CAT CCACT CT TTTACTCT CT ACCAACT CT CGGT GCT CCCTGC TG	3967
	A F K Y F D T T I D R K R Y T S T K E V L D A T	
CAS9-HS	C TT CAAGTACTTCGAC CAC CAC CAT AGA CAG AAAGCGGTACACCTCTAC AA AGGAGGTCC TG AC GC CCAC	4037
CAS9-NC	C TT CAAGTACTTCGAT TAC TAC TAT CGA TCG TAAGCGTTACACCTCTAC TA AGGAGGTCC CT GA TG CTAC	4037
	L I H Q S I T G L Y E T R I D L S Q L G G D S	
CAS9-HS	ACT GAT T CAT T CAGTCAAT TAC GGGCTCTAT TGA AC AG AAATCGA CT CTCTCTCAGCTCGGTGG AGA CAGC	4107
CAS9-NC	TCT CAT C CAC CAG TC TAT CAC CGG TCCTAC CGA GAC TCG TATCGAT CT CTCTCTCAGCTCGGTGG TGAT TCC	4107
	R A D P K K K R K V *	
CAS9-HS	AGG GCTGA CCC CAAGAAGAAG AGG AAGGTGTGA	4140
CAS9-NC	CGT GCTGAT CC TAAAGAAGAAG CGT AAGGT TT AA	4140
	nls and stop codon	

Table 1 Strains used in this study

Strain name (alias)	Strain genotype ^a
F2-23 (RTH1005.1)	<i>rid; fl A</i>
F2-26 (RTH1005.2)	<i>rid; fl a</i>
ISU-3121 (RTH61.3.1.2)	<i>rid his-3; gfp-sad-6-hph a</i>
ISU-3866 (RAB1.8)	<i>rid A</i>
ISU-3994 (RAB22.4)	<i>rid his-3⁺::gfp-cas^{9Hs}-hph; mus-51^Δ::bar A</i>
ISU-4092 (TAB38.1)	<i>rid his-3⁺::gfp-cas^{9HsC991}; mus-51^Δ::bar a</i> (ISU-4145 het)
ISU-4107 (TAB44.1)	<i>rid his-3⁺::gfp-cas^{9HsC318}; mus-51^Δ::bar a</i> (ISU-4145 het)
ISU-4145 (HAB11.53.2)	<i>rid his-3⁺::tef1(p)-cas^{9Hs}; mus-51^Δ::bar a</i>
ISU-4231 (RAB20.1)	<i>rid his-3⁺::gfp-cas^{9HsC4}-hph a</i>
ISU-4232 (HAB45.1.1)	<i>rid his-3⁺::gfp-cas^{9HsC213}; mus-51^Δ::bar a</i>
ISU-4233 (RAB1.7)	<i>rid a</i>
ISU-4234 (HPM32.14.2)	<i>rid his-3⁺::gfp-cas^{9HsC135}; mus-51^Δ::bar a</i>
ISU-4235 (HPM33.11.2)	<i>rid his-3⁺::gfp-cas^{9HsC70} a</i>
ISU-4236 (HPM34.1.1)	<i>rid his-3⁺::gfp-cas^{9HsC25} a</i>
ISU-4522 (RAY25.1.4)	<i>rid his-3⁺::gfp-cas^{9HsC62} a</i>
ISU-4618 (RAY11.13)	<i>rid his-3⁺::gfp-cas^{9HsC60}; mus-51^Δ::bar a</i>
ISU-4624 (RAY13.1)	<i>rid his-3⁺::gfp-cas^{9HsC40} a</i>
ISU-4875 (CTH293.3.3)	<i>rid his-3⁺::gfp-stop-cas^{9HsC213}; mus-51^Δ::bar a</i>
ISU-4884 (CNR307.3.1)	<i>rid his-3⁺::gfp-cas^{9NcC213}; mus-52^Δ::bar A</i>
ISU-4886 (CNR308.4.1)	<i>rid his-3⁺::gfp-cas^{9NcC60}; mus-52^Δ::bar A</i>
ISU-4888 (RABOB.36.4.1)	<i>rid his-3⁺::gfp-cas^{9HsC1209}; mus-51^Δ::bar a</i>
ISU-4889 (RABLG.37.1.2)	<i>rid his-3⁺::gfp-cas^{9HsC1078}; mus-51^Δ::bar a</i>
ISU-4892 (RABBL.39.1.1)	<i>rid his-3⁺::gfp-cas^{9HsC888}; mus-51^Δ::bar a</i>
ISU-4894 (RABJE.41.1.1)	<i>rid his-3⁺::gfp-cas^{9HsC656}; mus-51^Δ::bar a</i>
ISU-4895 (RABTrH.42.1.1)	<i>rid his-3⁺::gfp-cas^{9HsC535}; mat a</i>
ISU-4897 (RAB.XL.43.2.2)	<i>rid his-3⁺::gfp-cas^{9HsC443}; mat a</i>
ISU-4933 (CNR326.2.1/76.2.1)	<i>rid his-3⁺::gfp-stop-cas^{9Hs}; mus-51^Δ::bar a</i>
ISU-4934 (CNR327.1.1/77.1.1)	<i>rid his-3⁺::gfp-stop-cas^{9HsC4}; mus-51^Δ::bar a</i>
ISU-4935 (CNR336.1.1/104.1.1)	<i>rid his-3⁺::gfp-stop-cas^{9HsC213}; mus-51^Δ::bar a</i>
ISU-4936 (CNR337.1.1/124.1.1)	<i>rid his-3⁺::gfp-stop-cas^{9HsC135}; mus-51^Δ::bar a</i>
ISU-4937 (CNR339.1.1/126.1.1)	<i>rid his-3⁺::gfp-stop-cas^{9HsC25}; mus-51^Δ::bar a</i>
ISU-4938 (CNR347.1.5/v193h.5)	<i>rid his-3⁺::gfp-stop-cas^{9HsC62}; mus-51^Δ::bar a</i>
ISU-4939 (CNR340.2.1/161.2.1)	<i>rid his-3⁺::gfp-stop-cas^{9HsC60}; mus-51^Δ::bar a</i>

ISU-4941 (CNR341.1.2 /163.1.2)	<i>rid his-3⁺::gfp-stop-cas9^{HsC40}; mus-51^Δ::bar a</i>
ISU-4942 (CNR328.1.1/95.1.1)	<i>rid his-3⁺::gfp-stop-cas9^{HsC1209}; mus-51^Δ::bar a</i>
ISU-4943 (CNR329.1.1/96.1.1)	<i>rid his-3⁺::gfp-stop-cas9^{HsC1078}; mus-51^Δ::bar a</i>
ISU-4944 (CNR330.1.1/97.1.1)	<i>rid his-3⁺::gfp-stop-cas9^{HsC991}; mus-51^Δ::bar a</i>
ISU-4945 (CNR331.1.1/98.1.1)	<i>rid his-3⁺::gfp-stop-cas9^{HsC888}; mus-51^Δ::bar a</i>
ISU-4946 (CNR332.1.1/v100.1.1)	<i>rid his-3⁺::gfp-stop-cas9^{HsC656}; mus-51^Δ::bar a</i>
ISU-4947 (CNR333.2.1/101.2.1)	<i>rid his-3⁺::gfp-stop-cas9^{HsC535}; mus-51^Δ::bar a</i>
ISU-4948 (CNR334.1.2/102.1.2)	<i>rid his-3⁺::gfp-stop-cas9^{HsC443}; mus-51^Δ::bar a</i>
ISU-4949 (CNR335.2.1/103.2.1)	<i>rid his-3⁺::gfp-stop-cas9^{HsC318}; mus-51^Δ::bar a</i>
ISU-4951 (CNR348.1.7/307.1.7)	<i>rid his-3⁺::gfp-stop-cas9^{HsC796}; mus-51^Δ::bar a</i>
ISU-4952 (CNR349.1.2/308.1.2)	<i>rid his-3⁺::gfp-stop-cas9^{HsC714}; mus-51^Δ::bar a</i>
ISU-4953 (CNR350.2.8/309.2.8)	<i>rid his-3⁺::gfp-stop-cas9^{HsC612}; mus-51^Δ::bar a</i>
P8-42	<i>rid his-3; mus-51^Δ::bar a</i>
P8-43	<i>rid his-3; mus-52^Δ::bar A</i>

^aThe symbols *cas9^{Hs}* and *cas9^{Nc}* are used for human and *N. crassa* optimized Cas9 coding sequences, respectively.

Table 2 *gfp-cas9^{Hs}* expression levels

Strain name	Transgene name	vector	<i>gfp</i> seq ^a	<i>cas9</i> seq ^b	Average MFI ^c	stdev
Control strains						
ISU-4233	<i>none</i>	na	na	na	107.30	6.78
ISU-3866	<i>none</i>	na	na	na	101.15	5.32
ISU-4145	<i>cas9^{Hs}</i>	na	na	no ^d	113.12	28.33
ISU-3121	<i>gfp-sad-6</i>	na	yes	na	445.07	13.30
Test strains						
ISU-3994	<i>gfp-cas9^{Hs}</i>	v76	no	no	107.22	21.42
ISU-4888	<i>gfp-cas9^{Hs}C1209</i>	v95	yes	no	107.82	0.56
ISU-4889	<i>gfp-cas9^{Hs}C1078</i>	v96	yes	no	102.49	7.25
ISU-4092	<i>gfp-cas9^{Hs}C991</i>	v97	no	no	103.45	14.27
ISU-4892	<i>gfp-cas9^{Hs}C888</i>	v98	yes	no	113.74	13.53
ISU-4894	<i>gfp-cas9^{Hs}C656</i>	v100	yes	no	112.94	19.64
ISU-4895	<i>gfp-cas9^{Hs}C535</i>	v101	yes	no	116.87	19.95
ISU-4897	<i>gfp-cas9^{Hs}C443</i>	v102	yes	no	101.96	21.30
ISU-4107	<i>gfp-cas9^{Hs}C318</i>	v103	no	no	104.77	15.48
ISU-4232	<i>gfp-cas9^{Hs}C213</i>	v104	yes	yes	116.96	11.79
ISU-4234	<i>gfp-cas9^{Hs}C135</i>	v124	yes	yes	155.74	21.98
ISU-4235	<i>gfp-cas9^{Hs}C70</i>	v125	yes	yes	227.29	10.30
ISU-4522	<i>gfp-cas9^{Hs}C62</i>	v193	yes	yes	440.88	99.47
ISU-4618	<i>gfp-cas9^{Hs}C60</i>	v161	yes	yes	1686.60	799.05
ISU-4624	<i>gfp-cas9^{Hs}C40</i>	v163	yes	yes	4674.03	783.60
ISU-4236	<i>gfp-cas9^{Hs}C25</i>	v126	yes	yes	13973.04	2898.18
ISU-4231	<i>gfp-cas9^{Hs}C4</i>	v77	yes	yes	21681.28	3130.25
ISU-4884	<i>gfp-cas9^{Nc}C213</i>	v293	yes	yes	97.65	4.33
ISU-4886	<i>gfp-cas9^{Nc}C60</i>	v294	yes	yes	519.11	116.86

^a “yes” indicates that the *gfp* coding sequence and the ten amino acid GAGAGAGAGA linker of the *gfp-cas9* transgene were confirmed to be free of mutations in the indicated strain by Sanger sequencing. “no” means the sequence is assumed to be correct but it was not confirmed by Sanger sequencing.

^b “yes” indicates that the *cas9* coding sequence was confirmed to be free of mutations in the indicated strain by Sanger sequencing. “no” means the sequence is assumed to be correct but it was not confirmed by Sanger sequencing.

^c Average mean fluorescence intensity (MFI) values were calculated from MFI measurements of the same strain taken on different days (replicate assays separated in time by one day).

^d The *cas9^{Hs}* coding sequence in plasmid pAH41.1 was confirmed to be free of mutations by Sanger sequencing but the *cas9^{Hs}* coding sequence was not confirmed to be mutation free after integration into the *N. crassa* genome.

Standard deviation values (stdev) are provided. na, not applicable.

Table 3 *gfp-stop-cas9^{Hs}* expression levels

Strain name	Transgene name	vector	<i>gfp</i> seq ^a	<i>cas9</i> seq ^b	Average MFI ^c	stdev
Control strains						
ISU-4233	<i>none</i>	na	na	na	107.30	6.78
ISU-3866	<i>none</i>	na	na	na	101.15	5.32
ISU-4145	<i>cas9^{Hs}</i>	na	na	no ^d	113.12	28.33
ISU-3121	<i>gfp-sad-6</i>	na	yes	na	445.07	13.30
Test strains						
ISU-4933	<i>gfp-stop-cas9^{Hs}</i>	v76-stop	yes	no	229.49	44.12
ISU-4942	<i>gfp-stop-cas9^{HsC1209}</i>	v95-stop	yes	no	249.28	73.12
ISU-4943	<i>gfp-stop-cas9^{HsC1078}</i>	v96-stop	yes	no	184.49	17.75
ISU-4944	<i>gfp-stop-cas9^{HsC991}</i>	v97-stop	yes	no	427.81	19.80
ISU-4945	<i>gfp-stop-cas9^{HsC888}</i>	v98-stop	yes	no	294.55	34.53
ISU-4951	<i>gfp-stop-cas9^{HsC796}</i>	v307-stop	yes	no	718.78	134.25
ISU-4952	<i>gfp-stop-cas9^{HsC714}</i>	v308-stop	yes	no	975.18	405.91
ISU-4946	<i>gfp-stop-cas9^{HsC656}</i>	v100-stop	yes	no	926.00	27.67
ISU-4953	<i>gfp-stop-cas9^{HsC612}</i>	v309-stop	yes	no	1937.17	1077.00
ISU-4947	<i>gfp-stop-cas9^{HsC535}</i>	v101-stop	yes	no	412.50	44.49
ISU-4948	<i>gfp-stop-cas9^{HsC443}</i>	v102-stop	yes	no	353.63	39.77
ISU-4949	<i>gfp-stop-cas9^{HsC318}</i>	v103-stop	yes	no	281.53	92.79
ISU-4875	<i>gfp-stop-cas9^{HsC213}</i>	v296-stop	yes	no	479.87	9.64
ISU-4935	<i>gfp-stop-cas9^{HsC213}</i>	v104-stop	yes	no	464.78	24.76
ISU-4936	<i>gfp-stop-cas9^{HsC135}</i>	v124-stop	yes	no	4370.11	793.21
ISU-4938	<i>gfp-stop-cas9^{HsC62}</i>	v193-stop	yes ^e	no	2611.50	844.40
ISU-4939	<i>gfp-stop-cas9^{HsC60}</i>	v161-stop	yes	no	16852.27	4310.24
ISU-4941	<i>gfp-stop-cas9^{HsC40}</i>	v163-stop	yes	no	16753.66	5419.47
ISU-4937	<i>gfp-stop-cas9^{HsC25}</i>	v126-stop	yes	no	21235.32	5861.72
ISU-4934	<i>gfp-stop-cas9^{HsC4}</i>	v77-stop	yes	no	39746.19	7110.40

^a “yes” indicates that the *gfp* coding sequence, ten amino acid GAGAGAGAGA linker, and stop codon of the *gfp-stop-cas9* transgene were confirmed to be free of mutations in the indicated strain by Sanger sequencing. “no” means the sequence is assumed to be correct but it was not confirmed by Sanger sequencing.

^b “yes” indicates that the *cas9* coding sequence was confirmed to be free of mutations in the indicated strain by Sanger sequencing. “no” means the sequence is assumed to be correct but it was not confirmed by Sanger sequencing.

^c Average MFI values were calculated from measurements of the same strain taken on different days (replicate assays were separated in time by one day).

^dThe *cas9^{Hs}* coding sequence in plasmid pAH41.1 was confirmed to be free of mutations by Sanger sequencing but the *cas9^{Hs}* coding sequence was not confirmed to be mutation free after integration into the *N. crassa* genome.

^eStrain ISU-4938 contains a single nucleotide deletion in the coding sequence for the ten amino acid GAGAGAGAGA linker. The deleted nucleotide causes a frameshift that changes the amino acids at the end of GFP from GAGAGAGAGA to GAGAGRVLERSTCLL. Standard deviation values (stdev) are provided. na, not applicable.

Table S1 Primers used in this study

Primer Name	Sequence (5' > 3')
298	GTTGGTTAGGTGGGAACGCTTGT
550	GCGCGTCCATTCGCCATTCA
551	CGCTCCAGCACCCGCACCCGCTCCT
589	TTTTGCGGCCGCGGGATGTGCTGCAAGGCGATTA
598	CCATTGTCGTTTCGCCTGTGGT
599	TTTTACTAGTTTTGACGGTTGATGTGCTGACTGG
610	TTTTGCGGCCGCTGAAGCTTGTGGGTAGCGTAGGA
624	CCGAGCAAATGCCTGCAAATC
877	TCAGGCTATTGAGGACGAGGTTCA
878	GCAGCCTGAATGGCGAATGGACGCGCTAAAAGACACCATTTCACACTCC
879	CAGGAGCGGGTGCGGGTGCTGGAGCGATGGACAAGAAGTACTCCATTGGGCTCGA
880	CTTGCTCCGCCGTCAATGTATCC
881	TTGAGGACGAGGTTACCGTCAG
882	GGATGCGTTGATCGGGTTCTCTT
888	CAGGAGCGGGTGCGGGTGCTGGAGCGAGCAGGGCTGACCCCAAGAAGAAG
1089	CAGGAGCGGGTGCGGGTGCTGGAGCGTTTCGGGGACACTTCCTCATCG
1090	CCGTCAATGTATCCGGCGTAGC
1091	AGTCTTGGTGGTGCTCATCATA
1092	CAGGAGCGGGTGCGGGTGCTGGAGCGAACCTGTCAGACGCCATTCTGCTG
1093	TCGATGAAGGACTGGGCAGAGG
1094	GTAGGGTATCCGAAATGTGAGG
1095	CAGGAGCGGGTGCGGGTGCTGGAGCGAAAATGGACGGCACCGAGGAG
1096	CCTCCACTCCGCTGATTTCAACA
1097	TTCTCATCCCTTCTGTGACGTA
1098	CAGGAGCGGGTGCGGGTGCTGGAGCGGGGGCCTCTGCCCAGTCCTTCATC
1099	AGTTCCGGTTGGCAAATCCATC
1100	GCGTAAGTTTTCAAGCGTTCTT
1104	CAGGAGCGGGTGCGGGTGCTGGAGCGGGGGACAGTCTTCACGAGCACA
1105	CAGACAGGCCACCTCGTTCAGC
1106	TGAGGGGACGTTATCACTCTT
1107	CAGGAGCGGGTGCGGGTGCTGGAGCGGTGGATCATATCGTGCCCCAGTC
1108	GGTGGCCTTGCCTATTTCTCTGCT
1109	TGATAAGTGCAGTGCCTACCAC
1110	CAGGAGCGGGTGCGGGTGCTGGAGCGCACGTGGCCCAAATTCTCGATTCA
1111	CAGCTTGTGCTGTTCTCTTTTCG
1112	CGGTCTGTACTTCGGTCTTTTTT
1113	CAGGAGCGGGTGCGGGTGCTGGAGCGGGAGAGATTTCGAAGCGACCAC
1114	CGGGAGACCCTTTGAGCTTTTCA
1115	CCGTTTTCAAGCTCAAAGAGAG
1116	CAGGAGCGGGTGCGGGTGCTGGAGCGGTCAAGGAACTGCTGGGCATCAC
1117	CCTGCTGTCTCCACCGAGCTG

1118	GGATAATGTTTTCTGCCTGCTC
1165	CAGGAGCGGGTGCGGGTGCTGGAGCGATTTCATCAGTCAATTACGGGGCTCT
1166	CAGGAGCGGGTGCGGGTGCTGGAGCGAGGGAGCAGGCAGAAAACATTATCCA
1167	CAGGAGCGGGTGCGGGTGCTGGAGCGCTGGCCAGCCACTATGAAAAGCTCA
1387	CAGGAGCGGGTGCGGGTGCTGGAGCGTTTACTCTGACCAACTTGGGCGC
1389	CAGGAGCGGGTGCGGGTGCTGGAGCGAGAAAAGCGGTACACCTCTACAAAGGA
1528	CAGGAGCGGGTGCGGGTGCTGGAGCGCACTTGTTTACTCTGACCAACTTGGGCGC
1980	CAGGAGCGGGTGCGGGTGCTGGAGCGGTCAAGGAGCTCCTCGGTATCACTATCATGG
1983	CAGGAGCGGGTGCGGGTGCTGGAGCGTTCACTCTCACCAACCTCGGTGCT
2021	CAAGACCTGCCTGAAACCGAACTG
2022	CGCTCCAGCACCCGCACCCGCTCCT
2024	AAAAGCGCCGCATGCATAGAAGGAGCAGTCCATCTGCGTGAA
2025	TGATGAATCTCGGTGTGTATTTTATGTCC
2031	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGGTCAAGGAACTGCTGGGCATCAC
2083	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGGACAAGAAGTACTCCATTGGGCTCGA
2084	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGTTTCGGGGACACTTCCTCATCG
2085	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGAACCTGTCAGACGCCATTCTGCTG
2086	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGAAAATGGACGGCACCGAGGAG
2087	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGGGGGCCTCTGCCCAGTCCTTCATC
2088	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGGGGGACAGTCTTCACGAGCACA
2089	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGGTGGATCATATCGTGCCCCAGTC
2090	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGCACGTGGCCCAAATTCTCGATTCA
2091	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGGGAGAGATTCGGAAGCGACCAC
2092	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGGTCAAGGAACTGCTGGGCATCAC
2093	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGCTGGCCAGCCACTATGAAAAGCTCA
2094	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGAGGGAGCAGGCAGAAAACATTATCCA
2095	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGCACTTGTTTACTCTGACCAACTTGGGCGC
2096	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGTTTACTCTGACCAACTTGGGCGC
2097	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGAGAAAGCGGTACACCTCTACAAAGGA
2098	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGATTCATCAGTCAATTACGGGGCTCT
2099	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGAGCAGGGCTGACCCCAAGAAGAAG
2101	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGGTTGAAATCAGCGGAGTGGAGGA
2102	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGGGGCGGCTGTCAAGAAAACCTGAT
2103	CAGGAGCGGGTGCGGGTGCTGGAGCGTAGGAGATGGCCCGAGAGAACCAAAC
2104	TACTTAGCGGTGGCCTTGCTATTT
2105	TTGAGGACGAGGTTACCGTCAG
2106	CCTGCTCAGACTTTGCGATCATTT

Table S2 Primer combinations for transformation vector construction by DJ-PCR

Vector name	Left Fr	Left Rv	Cen Fr	Cen Rv	Right Fr	Right Rv	Nest Fr	Nest Rv
v76	877	878	550	551	879	880	881	882
v76-stop	877	878	550	551	2083	880	881	882
v77	877	878	550	551	888	298	881	624
v77-stop	877	878	550	551	2099	298	881	624
v95	877	878	550	551	1089	1090	881	1091
v95-stop	877	878	550	551	2084	1090	881	1091
v96	877	878	550	551	1092	1093	881	1094
v96-stop	877	878	550	551	2085	1093	881	1094
v97	877	878	550	551	1095	1096	881	1097
v97-stop	877	878	550	551	2086	1096	881	1097
v98	877	878	550	551	1098	1099	881	1100
v98-stop	877	878	550	551	2087	1099	881	1100
v100	877	878	550	551	1104	1105	881	1106
v100-stop	877	878	550	551	2088	1105	881	1106
v101	877	878	550	551	1107	1108	881	1109
v101-stop	877	878	550	551	2089	1108	881	1109
v102	877	878	550	551	1110	1111	881	1112
v102-stop	877	878	550	551	2090	1111	881	1112
v103	877	878	550	551	1113	1114	881	1115
v103-stop	877	878	550	551	2091	1114	881	1115
v104	877	878	550	551	1116	1117	881	1118
v104-stop	877	878	550	551	2092	1117	881	1118
v124	877	878	550	551	1167	298	881	624
v124-stop	877	878	550	551	2093	298	881	624
v125	877	878	550	551	1166	298	881	624
v125-stop	877	878	550	551	2094	298	881	624
v126	877	878	550	551	1165	298	881	624
v126-stop	877	878	550	551	2098	298	881	624
v161	877	878	550	551	1387	298	881	624
v161-stop	877	878	550	551	2096	298	881	624
v163	877	878	550	551	1389	298	881	624
v163-stop	877	878	550	551	2097	298	881	624
v193	877	878	550	551	1528	298	881	624
v193-stop	877	878	550	551	2095	298	881	624

v296-stop	877	878	550	551	2031	1117	881	1118
v307-stop	877	878	550	551	2101	2104	2105	2106
v308-stop	877	878	550	551	2102	2104	2105	2106
v309-stop	877	878	550	551	2103	2104	2105	2106

Construction of transformation vectors by DJ-PCR was performed by amplification of left flanks, center fragments, and right flanks with the primers indicated above. For left and right flanks, genomic DNA from strain ISU-4145 was used as the template. For center fragments, plasmid pTH1117.12 (GenBank JF749202.1) was used as the template. After the flanks and center fragments were fused by PCR, the fusion products were amplified with nested primers. Please see Hammond *et al.* (2011) a more complete description of DJ-PCR. Abbreviations are as follows: Left flank forward primer, Left Fr; Left flank reverse primer, Left Rv; Center fragment forward primer, Cen Fr; Center fragment reverse primer, Cen Rv; Right flank forward primer, Right Fr; Right flank reverse primer, Right Rv; Nested forward primer, Nest Fr; and, Nested reverse primer, Nest Rv.

Table S3 A set of 100 *N. crassa* high abundance mRNAs

Rank	Gene	Rank	Gene	Rank	Gene
1	NCU06110T0	35	NCU05804T0	69	NCU00726T0
2	NCU01528T0	36	NCU03302T0	70	NCU08964T0
3	NCU09345T0	37	NCU03150T0	71	NCU09089T0
4	NCU16635T0	38	NCU00618T0	72	NCU07808T0
5	NCU00315T0	39	NCU05498T0	73	NCU01546T0
6	NCU04553T0	40	NCU04552T0	74	NCU07014T0
7	NCU07562T0	41	NCU00979T0	75	NCU01776T0
8	NCU05667T0	42	NCU00634T0	76	NCU03148T0
9	NCU02003T0	43	NCU02193T0	77	NCU09109T0
10	NCU10042T0	44	NCU03102T0	78	NCU07829T0
11	NCU05599T0	45	NCU01948T0	79	NCU01949T0
12	NCU00294T0	46	NCU06432T0	80	NCU08960T0
13	NCU01418T0	47	NCU06431T0	81	NCU07439T0
14	NCU03988T0	48	NCU10498T0	82	NCU02181T0
15	NCU05561T0	49	NCU09475T0	83	NCU07857T0
16	NCU01754T0	50	NCU06047T0	84	NCU00635T0
17	NCU00971T0	51	NCU08332T0	85	NCU03757T0
18	NCU08389T0	52	NCU00258T0	86	NCU05816T0
19	NCU07817T0	53	NCU03753T0	87	NCU03565T0
20	NCU09476T0	54	NCU07962T0	88	NCU06226T0
21	NCU08963T0	55	NCU07182T0	89	NCU00413T0
22	NCU03738T0	56	NCU01962T0	90	NCU08502T0
23	NCU08627T0	57	NCU05274T0	91	NCU02437T0
24	NCU02250T0	58	NCU04779T0	92	NCU01221T0
25	NCU00464T0	59	NCU03703T0	93	NCU16844T0
26	NCU01317T0	60	NCU02707T0	94	NCU02744T0
27	NCU01827T0	61	NCU01452T0	95	NCU07826T0
28	NCU06661T0	62	NCU06892T0	96	NCU05032T0
29	NCU03806T0	63	NCU05338T0	97	NCU04114T0
30	NCU05554T0	64	NCU09477T0	98	NCU05810T0
31	NCU08990T0	65	NCU10069T0	99	NCU06185T0
32	NCU00475T0	66	NCU07776T0	100	NCU00706T0
33	NCU01552T0	67	NCU06743T0		
34	NCU07830T0	68	NCU02905T0		

The genes were ranked from highest mRNA abundance (rank 1) to lowest abundance (rank 100).

Table S4 Codon RA value estimates for *N. crassa*

Amino acid	Codon	Total ^a	RA	Amino acid	Codon	Total ^a	RA
*	TAA	86	1.00	M	ATG	366	1.00
*	TAG	9	0.10	N	AAC	625	1.00
*	TGA	5	0.06	N	AAT	27	0.04
A	GCC	1044	1.00	P	CCC	576	1.00
A	GCT	582	0.56	P	CCT	168	0.29
A	GCG	79	0.08	P	CCG	46	0.08
A	GCA	22	0.02	P	CCA	24	0.04
C	TGC	205	1.00	Q	CAG	527	1.00
C	TGT	10	0.05	Q	CAA	48	0.09
D	GAC	519	1.00	R	CGC	726	1.00
D	GAT	234	0.45	R	CGT	374	0.52
E	GAG	945	1.00	R	AGA	57	0.08
E	GAA	55	0.06	R	AGG	44	0.06
F	TTC	491	1.00	R	CGG	38	0.05
F	TTT	42	0.09	R	CGA	13	0.02
G	GGC	709	1.00	S	TCC	567	1.00
G	GGT	583	0.82	S	TCT	233	0.41
G	GGA	41	0.06	S	AGC	187	0.33
G	GGG	12	0.02	S	TCG	119	0.21
H	CAC	362	1.00	S	TCA	22	0.04
H	CAT	31	0.09	S	AGT	17	0.03
I	ATC	750	1.00	T	ACC	722	1.00
I	ATT	165	0.22	T	ACT	223	0.31
I	ATA	9	0.01	T	ACG	53	0.07
K	AAG	1563	1.00	T	ACA	22	0.03
K	AAA	34	0.02	V	GTC	953	1.00
L	CTC	852	1.00	V	GTT	285	0.30
L	CTT	249	0.29	V	GTG	96	0.10
L	CTG	125	0.15	V	GTA	19	0.02
L	TTG	92	0.11	W	TGG	158	1.00
L	CTA	7	0.01	Y	TAC	414	1.00
L	TTA	3	0.00	Y	TAT	43	0.10

^aTotal is the total number of occurrences of each codon in the coding sequences of the 100 genes listed in Table S3. Relative adaptiveness (RA) values were calculated according to the method of Sharp and Li (1987).

Table S5 MFI values

Data set ^a	Strain Name	Vector	<i>cas9</i> codons remaining	<i>gfp</i> fusion position on <i>cas9</i>	MFI 7-day culture	MFI 8-day culture
all	ISU-3121	na	na (PC)	na (PC)	435.66	454.47
all	ISU-3886	na	na (NC)	na (NC)	104.91	97.38
all	ISU-4145	na	na (NC)	na (NC)	133.15	93.09
all	ISU-4233	an	na (NC)	na (NC)	112.09	102.5
cas	ISU-3994	v76	1372	1	122.36	92.07
cas	ISU-4888	v95	1209	164	107.42	108.21
cas	ISU-4889	v96	1078	295	107.62	97.36
cas	ISU-4092	v97	991	382	113.54	93.36
cas	ISU-4892	v98	888	485	123.31	104.17
cas	ISU-4894	v100	656	717	126.82	99.05
cas	ISU-4895	v101	535	838	130.97	102.76
cas	ISU-4897	v102	444	930	117.02	86.9
cas	ISU-4107	v103	318	1055	115.71	93.82
cas	ISU-4232	v104	213	1160	125.3	108.62
cas	ISU-4234	v124	135	1238	171.28	140.2
cas	ISU-4235	v125	70	1303	220.01	234.57
cas	ISU-4522	v193	62	1311	511.21	370.54
cas	ISU-4618	v161	60	1313	2251.61	1121.58
cas	ISU-4624	v163	40	1333	5228.12	4119.94
cas	ISU-4236	v126	25	1349	16022.36	11923.71
cas	ISU-4231	v77	4	1369	23894.7	19467.86
opt	ISU-4884	v293	213	1160	94.59	100.71
opt	ISU-4886	v294	60	1313	601.74	436.47
stop	ISU-4933	v76-stop	1372	1	260.68	198.29
stop	ISU-4942	v95-stop	1209	164	300.98	197.57
stop	ISU-4943	v96-stop	1078	295	197.04	171.94
stop	ISU-4944	v97-stop	991	382	413.81	441.81
stop	ISU-4945	v98-stop	888	485	318.96	270.13
stop	ISU-4951	V307-stop	796	577	813.71	623.85
stop	ISU-4952	V308-stop	714	659	1262.2	688.16
stop	ISU-4946	v100-stop	656	717	945.56	906.43
stop	ISU-4953	v309-stop	612	761	2698.72	1175.61
stop	ISU-4947	v101-stop	535	838	381.04	443.96
stop	ISU-4948	v102-stop	443	930	325.51	381.75
stop	ISU-4949	v103-stop	318	1055	347.14	215.91
stop	ISU-4875	v296-stop	213	1160	486.69	473.05
stop	ISU-4935	v104-stop	213	1160	482.28	447.27
stop	ISU-4936	v124-stop	135	1238	4930.99	3809.22
stop	ISU-4938	v193-stop	62	1311	3208.58	2014.42
stop	ISU-4939	v161-stop	60	1313	19900.07	13804.47

stop	ISU-4941	v163-stop	40	1333	20585.8	12921.51
stop	ISU-4937	v126-stop	25	1348	25380.18	17090.45
stop	ISU-4934	v77-stop	4	1369	44774	34718.37

^aThese data were used to construct the charts presented in Figure 3 and Figure 4. Dataset: all, strains provided the positive and negative control values in all charts; cas9, strains provided the values used to produce the chart in Figure 3A and Figure 4; stop, strains provided the values used to produce the chart in Figure 3B. Abbreviations: na, not applicable, PC, positive control, GFP-SAD-6 strain; NC, negative control, no GFP transgenes present.