Rationally designed Spot 42 RNAs with an inhibition/toxicity profile advantageous for engineering *E. coli*

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Abstract

Bacterial regulatory small RNAs (sRNAs) have shown promise for gene knock-down studies and metabolic engineering. However, some mRNAs might be difficult to target due to poor binding by the Hfq chaperone, individual synthetic sRNAs can have off-target effects, potential sRNA toxicities have not been studied globally, and a consensus on optimal sRNA design has yet to emerge. Here, Spot 42 sRNA is validated as an excellent scaffold by showing that its over-expression minimally affects the growth rate of *Escherichia coli*, and that inhibition is reliably achieved for all eight tested protein targets by designing antisense to target the first few codons. Two related sRNAs that could not be cloned, possibly due to lethality of the encoded sRNAs, became clonable when an eight-nucleotide sequence was inserted directly upstream of the antisense region. Global fitness costs for *E. coli* of the designer sRNAs were measured and found to be variable but tolerable. Importantly for utility, there was no correlation between target inhibition and cellular toxicity. As a proof of concept for applications, suppression of the UAG stop codon was improved by knock down of translation release factor 1 (RF1).

**KEYWORDS**

fitness cost, small RNA, Spot 42, translation release factor 1

1 | INTRODUCTION

Antisense strategies are appealing for regulating gene expression because specific mRNA binding and inhibition can be designed, in principle, by using base-pairing rules. Antisense nucleic acids also have the advantage of acting directly without requiring their translation. Yet, despite a plethora of antisense methods, there are many problems such as delivery, inhibition efficiency, target specificity, and stability. Thus, in prokaryotes, inhibition of gene expression has been engineered mostly by recombination knock-out technology. But finer tools than knock outs are needed for studying or
targeting essential genes\textsuperscript{4} and for metabolic engineering, where total inhibition of an mRNA can be lethal or toxic, so it is desirable to test various levels of partial inhibition (knock down). This has led to a renaissance of interest in bacterial regulatory small RNAs (sRNAs), now numbering in the hundreds\textsuperscript{5} as they allow fine tuning of target expression.\textsuperscript{6-8}

In \textit{Escherichia coli} and other Gram-negative bacteria, the Hfq chaperone protein has many functions including protecting sRNAs against enzymatic degradation and facilitating sRNA-mRNA base pairing.\textsuperscript{9,10} The mechanism is that the sRNA binds to one face of the hexameric Hfq via a U-rich sequence while the target mRNA binds simultaneously on the other Hfq face if it has at least three repeats of ARN.\textsuperscript{11} One region of the mRNA targeted by natural and engineered sRNAs\textsuperscript{12,13} is the ribosome binding site (RBS; Figure S1A) which contains an ARN-rich Shine-Dalgarno sequence (SD). Actions of sRNAs include inhibition of translation, activation of translation and promotion of mRNA degradation (Figure S1).\textsuperscript{5}

mRNA inhibitors have been selected from libraries containing random sequences replacing the mRNA-binding domain of Spot 42 sRNA, an sRNA that regulates carbon metabolism (Figure 1A). The selections yielded mostly binders to the SD of the target mRNA, but also some binders to the amino-terminal coding region.\textsuperscript{15,16} sRNAs with complementarity to the SD were found to be cross-reactive with other mRNAs,\textsuperscript{15} which is unsurprising given the similarity shared between the SD of many mRNAs. Na et al\textsuperscript{18} and Kim et al\textsuperscript{17} instead targeted amino-terminal coding regions selectively using the MicC sRNA scaffold, and Hoynes-O’Connor and Moon\textsuperscript{7} targeted upstream, SD or amino-terminal regions of an mRNA using five different sRNA scaffolds. Na et al minimized cross-reactivity at their three tested nontarget genes by shortening the antisense sequences, while Hoynes-O’Connor and Moon found highest cross-reactivity on their two tested nontarget genes by the Spot 42 scaffold, leading them to focus on the MicF scaffold with mutated hairpin structures termed M7.4. Yet Spot 42 also exhibited the highest target inhibitions of the several scaffolds tested (Reference 7; confirmed in an artificial tag-targeting system by Reference 6). It also should be borne in mind that off-target effects of an over-expressed sRNA are caused not only by base pairing with off-target mRNAs, but also by competition with endogenous sRNAs for binding to Hfq. This is because Hfq is generally limiting for mRNA regulation by sRNAs.\textsuperscript{18} Thus, off-target toxicity may be minimized by choosing for engineering an sRNA scaffold whose over-expression has minimal effects on regulation by other sRNAs. In this respect, over-expression of Spot 42 had less effects on sRNA regulation than over-expression of MicC or other individual sRNAs.\textsuperscript{18} Mindful that off-target effects of mRNA-targeting Spot 42 derivatives had not been studied globally, we thus reasoned that Spot 42 might yet prove an ideal sRNA scaffold for engineering, provided we could demonstrate an excellent inhibition/toxicity profile.

2 | EXPERIMENTAL PROCEDURES

2.1 | Construction of plasmids and strains

\textbf{Strains.} This study used \textit{E. coli} DH5\textalpha, MG1655 and BL21(DE3). Plasmid cloning was in DH5\textalpha using BioBrick genetic parts and plasmid vectors (http://parts.igem.org/Main_Page).
**Antisense constructs.** The native Spot 42 sequence from *E. coli* MG1655 (*spf*) was cloned by PCR adjacent to a constitutive promoter (BBa_J23101) in the high copy (pMB1 ori) pSB1C3 (chloramphenicol R) and deposited in the Registry of Biological Parts as BioBrick no. BBa_K864440 (http://parts.igem.org/Main_Page; a kind gift of Uppsala iGEM 2012). The mRNA-binding domain of Spot 42 (Figure 1A) was replaced by 10-, 15-, and 20-mer sequences (Figure S3) complementary to the exact N-terminal sequences of (a) aeBlue, asCP, YFP and BFP by inverse PCR mutagenesis\(^1\) or (b) amilGFP and amajLime (in addition to ACTCGAG directly upstream of the antisense) by custom mutagenesis (GenScript). Alternative versions of these inserts for targeting BFP were created by total synthesis (oligos from Integrated DNA Technologies) and hybridization as for RF1 in Figure S14 (with GGGGAAAA directly upstream of the antisense).

**Targets.** Plasmids were assembled in medium copy (p15A ori) pSB3K3 plasmid vectors (kanamycin R) using a constitutive promoter (BBa_J23110), a ribosomal binding site containing a strong SD sequence (BBa_B0034) and the respective chromoprotein or fluorescent protein: aeBlue\(^2\) (BBa_K864401, also known as aeCP597), asCP\(^2\) (BBa_K1033927, also known as asFP595 and asPink), SYFP\(^2\) (BBa_K864100) abbreviated as YFP, mTagBFP\(^2\) (as in BBa_K592100, but with A520G and T521C) abbreviated as BFP, amilGFP\(^2\) (BBa_K592010) and amajLime\(^2\) (BBa_K1033916, also known as amajCFP and amFP486). For *E. coli* codon-optimized sequences of eukaryotic aeBlue, asCP and amajLime see Reference 26. For the N-terminal codons of chromosomal RF1 see Figure S14. For the aminoglycoside acetyltransferase AAC(6\(^\prime\))Ib-cr antibiotic resistance target see References 27,28 and http://2012.igem.org/Team:Uppsala_University/Translational.

**β-galactosidase-α-peptide(UAG) plasmid.** The lacZ-α peptide gene (BBa_I732020) was coupled to constitutive promoter BBa_J23110 in medium copy pSB3K3 (kanamycin R). Based on the rationale of Xu et al.,\(^2\) a UAG codon was inserted in frame between codons 6 and 7 by inverse PCR mutagenesis.\(^1\) In cells transformed with two different plasmids, agarose gel electrophoresis was used to confirm that the relative copy numbers of the plasmids were roughly as expected.

### 2.2 β-galactosidase assay

Over-night cultures with/without β-gal-α-UAG and/or anti-RF1 sRNA plasmids were inoculated into LB media at 1:100 dilutions with chloramphenicol (36 μg/mL) and/or kanamycin (36 μg/mL) and 0.5 mM IPTG. After 24 hours growth, cells were permeabilized and β-gal activity in hydrolyzing ONPG was measured by spectrophotometer.\(^3\) Activity was calculated by the following formula, where A=absorbance, T=time (min.) and V=volume of cells (mL):

\[
\text{Miller units} = \frac{1000 \times (A_{420} - (1.75 \times A_{550}))}{T \times V \times A_{600}}.
\]

### 2.3 Anti-RF1 western blots

The method was based on Korkmaz et al.\(^3\) Cells were harvested at 0.5 OD\(_{600}\) and each sample containing 1.25 × 10\(^8\) cells was resuspended in 5 μL TMK buffer (10 mM Tris-HCl pH 7.5, 5 mM MgCl\(_2\), 95 mM KCl) and 5 μL 2× Laemmli buffer (4% SDS, 20% glycerol, 0.125 M Tris-HCl pH 6.8, 10% 2-mercaptoethanol, 0.004% bromphenol blue), heated at 95°C for 10 minutes and stored at −20°C. These total cells were loaded on a 12% SDS PAGE gel, and purified His-tagged RF1 (2 and 5 pmol) was used as a quantitative standard. Gel-separated proteins were transferred to nitrocellulose (Semi-Dry Trans-Blot Cell, Bio-Rad) for 45 minutes at 12 V in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol). Proteins transferred to the membrane were stained with Ponceau S (0.1% [w/v] in 5% acetic acid) and imaged by the ChemiDoc MP System (Bio-Rad). The Ponceau S was washed from the membrane with wash buffer (optimized to 100 mM Tris, pH 7.4, 100 mM NaCl, 0.1% Tween 20 to reduce loss of RF1 from the membrane). Then the membrane was blocked over-night with blocking buffer (5% nonfat milk in wash buffer). The membrane was incubated with the rabbit polyclonal primary antibody against RF1 (1:5000 dilution) for 1 hour at room temperature, washed in wash buffer for 30 minutes and further incubated with secondary antibody (Peroxidase-labelled goat-anti-rabbit-IgG, #A27036, Invitrogen) at 1:20000 dilution for 1 hour at room temp. The membrane was washed for 30 minutes in wash buffer and then incubated with ECL solution (Clarity Western ECL Substrate, Bio-Rad) for 15 minutes at room temp. Bound antibodies were detected by the ChemiDoc MP System (Bio-Rad). Band intensities were quantified by the Image LabTM Version 6.0.1 (Bio-Rad). The chemiluminescent blot intensity values were adjusted for variation in the protein loading between different lanes detected by Ponceau S staining.
2.4 | Fluorescent protein assays

Cells were grown in LB media at 37°C with chloramphenicol (36 μg/mL) and/or kanamycin (30 μg/mL) for maintenance of plasmids. Single colonies were inoculated into 4 mL in 50 mL Erlenmeyer flasks and grown with shaking at 220 rpm for 18 hours. As oxygen is necessary for fluorophore maturation, growth conditions were designed for optimal aeration. Cell fluorescence was measured with a flow cytometer (BD FACSaria IIu) using FITC settings (488 nm excitation, 530/30 filter) for YFP and amILGFP, DAPI settings (405 nm excitation, 450/50 filter) for BFP, and Alexa Fluor 430 settings (405 nm excitation, 530/30 filter) for amajLime.

2.5 | Chromoprotein assays

Single colonies were inoculated into 7 mL in 100 mL Erlenmeyer flasks and grown with shaking at 220 rpm for 20 hours. Cell pellets from 4 mL of culture were washed in PBS buffer and resuspended in 700 μL lysis buffer (10 mM Tris, 100 mM NaCl, 1 mM sodium EDTA, 3% Triton X-100). Lysozyme (1.75 × 10^5 Units, Fluka) was added and the samples were incubated with slow shaking at 37°C for 1 hour. Samples were then frozen in −80°C, heated to 70°C for 3 minutes, and subjected to two further freeze-thaw cycles at −80°C and 37°C. Cell debris and precipitated proteins were pelleted, and the absorbance of the supernatant was measured. To calculate each sample’s absorbance (A) by taking into account the different wavelengths needed for the chromoprotein colors and culture density measurements and allowing for subtraction of the baseline absorbance, we used this equation:

\[ A = \frac{A_{\text{peak}} \cdot A_{430} \cdot \text{Control}}{A_{430} \cdot \text{Control}} \]

where Control is a negative control strain, and wavelengths were 468 nm for asCP peak, 597 nm for aeBlue peak and 430 nm for a baseline with negligible absorbance of asCP and aeBlue chromoproteins.

These corrected A measurements were then normalized (Figure 2B, left).

2.6 | Growth rate assays

Growth rates at 37°C were measured using a Bioscreen C (Growth Curves Oy, Helsinki, Finland) and an Infinite M200 PRO (TECAN Nordic AB, Stockholm, Sweden). Over-night cultures were inoculated in wells at 1:1000 dilutions. OD 600 was measured every 4 minutes during 16 hours. Measurements with OD 600 between 0.02 and 0.1 were considered to be in early log phase and were fitted to a logarithmic function using MATLAB.

3 | RESULTS

3.1 | Over-expression of Spot 42 sRNA minimally affects E. coli growth rate

For global toxicity measurement, the standard assay is the effect on growth rate under conditions relevant for metabolic engineering. In comparison with an empty plasmid control, Spot 42 over-expression exhibited a growth defect of only 3 ± 1% (Figure 1B), demonstrating minimal cellular toxicity (in apparent contrast with Reference 33).

3.2 | Target inhibition by screening randomized Spot 42-based RNAs has disadvantages

To test the generality of the published Spot 42 method for target inhibition,15,16 Uppsala University’s international Genetically Engineered Machine (iGEM) student team targeted a different 5′ untranslated/N-terminal coding region, one from the antibiotic resistance gene aminoglycoside acetyltransferase AAC(6′)Ib-cr,27 fused to a similar fluorescent protein reporter. A very similar approach to that of Sharma et al15,16 was used, randomizing the same region of
FIGURE 2  Expression of target chromoproteins and fluorescent proteins using different antisense lengths in the Spot 42 scaffold. A. Example of sRNA (top) complementary to mRNA (bottom), while also bound by Hfq (green). The complementarity (first 10 nucleotides common to all three antisense variants in dark brown, next five nucleotides red and final five nucleotides light brown) stretches from the start codon (blue) of the chromoprotein aerBlue (see also Figure S4). The Shine-Dalgarno is in yellow. Taken with permission from Liljerumh et al. 32 B. Expression of target chromoproteins and fluorescent proteins in Escherichia coli MG1655 in presence of plasmids expressing different antisense lengths in the Spot 42 scaffold. U, unclonable antisense plasmids. C to E. Plots of properties of sRNAs against their repression efficiencies in (B) using the same colors. ▲ 10 nucleotides, ■ 15 nucleotides, ◆ 20 nucleotides antisense. F. Expression of BFP target in DH5α using different antisense lengths containing a GGGAGAAA insertion (*) directly upstream of the antisense regions in the Spot 42 scaffold. The left 10mer antisense lacks the insertion (same construct as used in (B)). Expression is normalized to Spot 42 and error bars are SEs

Spot 42, except that screening by manual inspection of agar plates was substituted by fluorescence-activated cell sorting to increase throughput (Figure S2), and the classical restriction-enzyme cloning method was simplified by using BioBrick cloning. 34 Like in Sharma et al, several sRNAs with partial inhibitory activities against the fused mRNA were identified (http://2012.igem.org/Team:Uppsala_University/Translational). Also in agreement with Sharma et al, computational searching for complementarity suggested that the majority of the inhibitory sRNAs acted by partial hybridization to the SD and/or the first few codons of the fused mRNA (http://2012.igem.org/Team:Uppsala_University/Modelling). However, this screening approach (a) produced many false positives, (b) required further validation with bona fide nonfused targets, and (c) theoretically would target the SD of many nontarget mRNAs to cause significant off-target effects.

3.3 Rational design of inhibitor sRNAs

We envisioned circumventing problems (a) to (c) above by rationally designing Spot 42 sRNAs (Figure S3) to act through perfect complementarity with the first few codons of several nonfused targets (eg, Figure 2A), though natural sRNAs tend to bind with mismatches and bulges. Although each mRNA target might require a good Hfq binding site of at least three repeats of ARN11 near the start codon, essentially generalizable mRNA targeting is theoretically possible because the upstream SD sequence is ARN-rich, ARN occurs frequently in random sequences and the sRNA would be over-expressed. This approach is analogous to the method developed independently by Na et al using 24 straight complementary nucleotides within the MicC scaffold, but potentially might avoid the toxicity associated with the MicC scaffold. 18
In order to address potential toxicity issues, we thus opted for testing three different-lengthed antisense sequences for each target, 10, 15, and 20 nucleotides (Figure S3; complementarity starts at the initiator AUG codon) and measuring growth rates in addition to target inhibitions.

### 3.4 Generalizable inhibition of target protein expression by designer sRNAs

In order to test the generality of our design, we chose six different target proteins related to the green fluorescent protein (GFP) reporter: the chromoproteins aeBlue (Figure 2A) and asCP, and the fluorescent proteins SYFP2 (YFP), mTagBFP2 (BFP), amilGFP, and amajLime (middle structures of Figures S4-S9). The targets were encoded on different plasmids than the sRNAs (see Experimental Procedures). Use of the two chromoproteins enabled rapid, “low-tech” preliminary tests based on simple visual inspection of the color intensities of cell pellets, where clear inhibition of expression was observed for both aeBlue and asCP (Figure S10). sRNA inhibitions were quantitated by cell lysis, removal of cell debris, and spectrophotometric analysis of the supernatants (Figure 2B, left). Inhibitions of the four fluorescent proteins were analyzed by standard flow cytometry (Figure 2B, middle and right). Inhibition of all six targets was obtained by at least one of the three sRNAs designed against each target. Thus, while Na et al. recommended using a 24-nucleotide antisense region in MicC, for Spot 42 we recommend also testing shorter antisense sequences. Na et al. found that repression efficiencies in engineered MicC RNAs were largely dependent on the calculated binding energy between the sRNA and the target mRNA, but a consensus on optimal sRNA design is yet to emerge. Among the sRNAs in our study, potential correlations between variation in repression and binding energies, guanine and cytosine (GC) contents or length of antisense sequences had low significance (Figure 2C-E; $R^2 = 0.070$, 0.23, and 0.014, respectively). Computed secondary structures, although hypothetical, can be informative. However, such predicted structures had substantially altered structures of the Hfq-binding domain and apparently did not correlate with repressions (Figures S4-S9).

Interestingly, both 15- and 20-nucleotide complementarities to BFP were possibly lethal in DH5α. This conclusion was based upon (a) many sRNA cloning attempts, each giving few or no colonies, (b) all sequenced colonies containing nondesigned large insertions in the sRNAs (Tables SI and II), and (c) the possibility that these two antisense regions hybridized efficiently to one or more crucial off-target RNAs. However, upon trying a new cloning strategy (Figure S14), we discovered fortuitously that the problem could be circumvented by an eight-nucleotide, non-antisense insertion directly upstream of the antisense regions (Figure 2F; see Figure S14 legend for explanation of the sequence choice). The eight-nucleotide insertion likely did not decrease the transcription rate because initiation is more efficient with purines than pyrimidines at +1.55 However, it does likely affect the structure of the immediately adjacent antisense region which in turn may decrease the efficiency of target hybridization. Consistent with this idea, the six sRNAs with a non-antisense insertion directly upstream of the antisense in Figure 2B, that is the amilGFP and amajLime antisense sRNAs with seven-nucleotide ACTCGAG insertions, had generally lower repression efficiencies compared with the others in Figure 2B (and see also the larger insertions in the bottom three sequences in Table S1).

### 3.5 Many designer sRNAs have global fitness costs

Given the possible lethality of some of the longer anti-BFP sRNAs, we hypothesized that there may be significant global fitness costs due to over-expression of our other artificial sRNAs. We therefore extended our control growth rate experiments of Figure 1B to all of our engineered sRNAs in the absence of plasmids encoding the target mRNAs (Figures 3A,C). Interestingly, while over-expression of native sRNA Spot 42 minimally affected growth rate, sRNAs only differing in their short antisense sequences had widely differing effects. Sequence-specific effects are clearly demonstrated by the varied toxicities within each length of antisense. Statistical analyses grouping the growth rates of the antisense sRNAs together independent of their target classes showed that any apparent correlations between variation in growth rate and binding energies, GC contents or length of antisense sequences had very low significance (Figure S11; $R^2 = 0.072$, 0.18 and 0.003, respectively). There was no correlation between target protein repression and growth rate (Figure 3B; $R^2 = 0.12$), which is beneficial for adjusting target levels. This contrasts somewhat with the results of Hoynes-O’Connor and Moon,7 where off-target inhibition of two genes increased with increasing target repression.
Knock down of release factor 1 to improve suppression of UAG codons

As a proof of concept, we turned to an application that has long resisted a simple solution: increasing suppression of the UAG stop codon in protein synthesis. UAG is the codon of choice for the ribosomal incorporation of unnatural amino acids in many applications that rely on expanding the genetic code. The advantages of decoding UAG by an unnaturally-charged suppressor tRNA\textsubscript{CUA}, both in vitro extracts and in vivo, are that (a) competition from natural aminoacyl-tRNAs is lower than at all other 63 sense and nonsense codons and (b) that UAG is much rarer than the other two nonsense codons in \textit{E. coli}. However, competition by prokaryotic translation release factor 1 (RF1) at UAG (its cognate termination codon, together with UAA) is unfortunately efficient, which stimulated many studies aiming to knock out RF1. But the genetic knock out of RF1 in \textit{E. coli} by recombination has proved impossible in K12-derived strains unless many other genes terminated by UAG are either knocked out or recoded, and has proved difficult to reproduce when using permissive B-derived strains; even when successful, RF1 knock out resulted in a 3.5× slower growth rate.

We thus envisioned that RF1 knock down by Spot 42 would be a simpler technology to adapt to one's strain of interest and would also be less toxic than a knock out.

Reduction of RF1 protein levels in DH5\textsubscript{α} (a K12-derived strain) was feasible using all three designed sRNAs, as shown by western blots (Figure 4A and Figure S12). Growth rate measurements indicated only a 1.5× slower growth rate (Figure 3D). This was presumably due to inhibition of the RF1 target rather than off-target toxicity because minimal growth rate inhibition was observed from the sRNAs in a B-derived strain, BL21 (which better tolerated reduced RF1 as seen in Figure 3E), despite incurring similar reductions in RF1 (Figure S13). Finally, to test for increased
suppression of the UAG stop codon, a plasmid encoding a β-galactosidase α-peptide containing a premature in-frame stop codon was constructed for complementation of DH5α’s chromosomally encoded, inactive β-galactosidase ω mutant (Figure 4B, top). Then we measured the effect of the anti-RF1 Spot 42 RNAs on α-complementation (Figure 4B, bottom). Results demonstrated 9 to 14× increased suppression of the UAG stop codon, consistent with our desired functional effect of RF1 knock down. Furthermore, construction of our RF1 knock down strains was much simpler (Figure S14) than prior RF1 reduction methods.36,37

4 | CONCLUSION

By rational design of artificial Spot 42 RNAs against eight targets in E. coli, we achieved the desired tailoring of inhibition and fitness costs. There was negligible toxicity from over-expression of Spot 42, implying that sRNA toxicity was not due to Hfq sequestration. Alternatively, it has been argued over the years that longer antisense sequences may either be less specific (more toxic) due to containing more potentially cross-hybridizing sequences or more specific (less toxic) due to higher target affinity. Our study did not favor either argument with high statistical significance. Although the reasons for toxicities of sRNAs other than sequestration of Hfq are not understood and very difficult to ascertain, our possible lethality of the 15- and 20-nucleotide antisense sequences to BFP was nevertheless overcome by inserting a short sequence adjacent to the antisense sequence. As all targets attempted were inhibited, the method is generalizable to date.

Of the several natural sRNA scaffolds engineered by other workers for targeting, Spot 42 gave the best inhibitions, had the lowest effects on sRNA regulation when over-expressed, yet was not favored because of off-target effects on very few model targets (see Introduction). Now that we have shown low global toxicity of Spot 42 constructs, we argue that the advantages of Spot 42 should make it the sRNA scaffold of choice.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.
AUTHOR CONTRIBUTIONS
C.V. contributed to data curation, formal analysis, funding acquisition, methodology, and validation. A.G. contributed to conceptualization, data curation, formal analysis, methodology, validation, writing-original draft. J.Y. contributed to data curation, formal analysis, methodology, validation. L.B. contributed to data curation, formal analysis, methodology, and validation. J.L. contributed to formal analysis, methodology, and supervision. A.F. contributed to conceptualization, formal analysis, funding acquisition, project administration, resources, supervision, validation, writing-original draft, writing-review and editing.

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**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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