Original Research Article

Design of artificial small regulatory trans-RNA for gene knockdown in Bacillus subtilis

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A B S T R A C T

Bacillus subtilis as the Gram-positive model bacterium has been widely used in synthetic biology and biotechnology while the regulatory RNA tools for B. subtilis are still not fully explored. Here, a bottom-up approach is proposed for designing artificial trans-acting sRNAs. By engineering the intrinsic sRNA SR6, a minimized core scaffold structure consisting of an 8 bp stem, a 4 nt loop, and a 9 nt polyU tail was generated and proven to be sufficient for constructing sRNAs with strong repression activity (83%). Moreover, we demonstrate this artificial sRNA system functions well in an hfq-independent manner and also achieves strong repression efficiency in Escherichia coli (above 80%). A structure-based sRNA design principle was further developed for the automatic generation of custom sRNAs with this core scaffold but various sequences, which facilitates the manipulation and avoids structure disruption when fusing any base-pairing sequence. By applying these auto-designed sRNAs, we rapidly modified the cell morphology and biofilm formation, and regulated metabolic flux toward acetoin biosynthesis. This sRNA system with cross-species regulatory activities not only enriched the gene regulation toolkit in synthetic biology for B. subtilis and E. coli but also enhanced our understanding of trans-acting sRNAs.

1. Introduction

Non-coding RNAs (ncRNAs) have performed central cellular functions in all three kingdoms of life. Naturally, ncRNAs are widely involved in sensing intracellular and extracellular environmental changes and rapidly responding via altering the expression of other RNA or protein species [1-3]. Because of their versatile functions in a variety of cell processes, ncRNAs have attracted intense studies on their working mechanisms [4] and the standardization as artificial synbio parts [5-8]. Generally, such RNA modules contain antisense RNAs [9], small regulatory RNAs [10], and a variety of 3’ or 5’ untranslated regions [11,12] for controlling mRNA stability, transcription, or translation [4].

Comprehensive studies [4,13] have demonstrated the activation or repression of target genes mainly are ascribed to the conformational changes of target mRNA when binding to metabolites [14-16] or other RNAs [17,18]. Among them, small regulatory RNAs (sRNAs) constitute the main class of posttranscriptional regulators (trans-encoded and cis-encoded RNAs) and act by base-pairing with target mRNAs. The cis-encoded sRNAs are transcribed in the opposite direction to the target gene in the same locus while the trans-encoded sRNAs are transcribed elsewhere and only partially complementary to their target genes [13]. Both types of sRNAs form duplexes when encountering target RNAs and eventually alter their translation or stabilities.

According to this knowledge, native sRNAs have been adopted as gene-knockdown tools in the fields of metabolic engineering and synthetic biology due to their high structural flexibility and function modularity [6,19]. Traditionally, genome engineering strategies such as random mutagenesis [20], gene-knockout [21], and multiplex automated genome engineering (MAGE) [22] are wildly used in manipulating bacteria to improve the yields of valuable chemicals. While these approaches have proven powerful, laborious work and little mechanistic insight limit the efficiency of optimization or redesign of the pathways. Therefore, sRNA has currently drawn considerable attention as an alternative for the effortless remodeling of the metabolic network [23].
Based on canonical sRNA systems, chimeric sRNAs were constructed by replacing their base-pairing regions of the original target genes with those genes of interest [24]. However, only a few naturally occurring sRNAs hold the potential for being used as a synthetic sRNA scaffold allowing sequence swapping, since the disruption of those fragile scaffolds always comes up when connecting to an inapposite base-pairing sequence [25]. Hence, most sRNA studies have mainly focused on screening more native scaffolds [26,27] and developing novel artificial scaffolds [18,28,29] in Gram-negative bacteria *Escherichia coli*. In contrast, although the *E. coli* sRNA-Hfq system has been transplanted into the Gram-positive model bacterium like *Corynebacterium glutamicum* [30], it is uncertain about whether those sRNA design principles can be extrapolated to *B. subtilis*. Therefore, it’s worth exploring the diversity of sRNA regulatory mechanisms among these different species, by developing artificial small regulatory trans-RNAs from naturally occurring sRNA in *B. subtilis*.

As the key Gram-positive model bacterium widely used to produce valuable chemical and protein [31–33], developing handy but stringent gene regulation toolkits are in great demand. At the transcriptional level, promoter engineering such as maltose-induced MATE-ON/OFF system serves as a useful tool for flexible and tunable gene control [34], however at the translational level, the regulatory RNA tools of *B. subtilis* haven’t been fully explored due to the small number of its intrinsic sRNAs with identified targets comparing to *E. coli* to date [9], and most well-studied sRNAs belong to the type I toxin-antitoxin(TA) systems [17]. As antitoxins, sRNAs interact with the mRNAs of their corresponding toxin genes, inhibiting the expression of toxin protein via RNase III mediated mRNA-sRNA complexes degradation or steric hindrance with the ribosome binding site(RBS) [17]. In view of this inherent interaction of sRNA-mRNA, a post-transcriptional regulation system based on the barg/SR4 TA system termed MS-DOS [35] has been recently developed by inserting partial toxin-encoding region to the downstream of the target gene’s open reading frame and expressing the corresponding antitoxin sRNA to promote their RNA complex degradation [7]. Nonetheless, MS-DOS still requires time-consuming genome editing in gene knockdown. Thus, the construction of a more efficient and portable gene knockdown tool is imperative.

Here, we proposed a convenient method for the *de novo* design of sRNAs for strong and rapid regulation in *B. subtilis*. Inspired by the mechanism of the yoyJ/SR6 TA system (Fig. 1A), SR6-based trans-acting sRNA is initially constructed by directly replacing its original base-pairing region to the complementary sequence of any target gene’s start codon and subsequent 21 nucleotides, achieving an 83% repression on *gfp* expression. Further truncation and redesign experiment results suggest a sequence-independent core scaffold structure consisting of an 8 bp stem, a 4 nt loop, and a 9 nt polyU tail is sufficient to confer a strong repression function and display cross-species ability in both *B. subtilis* and *E. coli*. Moreover, a *de novo* design program was developed for the custom design of sRNAs with the above structure constraints. By applying these auto-designed sRNAs, we achieved the rapid modification of the cell morphology and biofilm formation, and regulation of the metabolic flux towards acetoin biosynthesis.
2. Material and methods

2.1. Strains and plasmids construction

*B. subtilis* SCK6 was constructed as described in a previous study [36], and the endogenous RNA chaperone Hfq gene was further knockout to construct the *B. subtilis* SCK6 ΔHfq via CRISPR/Cpf1 [21]. The above strains with *E. coli* BL21(DE3) were used as the host to perform sRNA repression experiments. *E. coli* JM109 was used for plasmid construction, amplification, and stock. All plasmids and primers used in this study are listed in Table S1 and Table S2.

The plasmid pHT01-gfp was constructed by inserting the Psp124BI/MluI expression cassette between the Psp124BI/Mul sites of the plasmid pHTO1, and the plasmid pCOLADuet-gfp was constructed by inserting the P_T7-gfp expression cassette between BgIII/Xhol sites.

The sRNA expression plasmids were constructed using pP42NMK or pTargetF as the template. Briefly, the linear form of sRNA expression plasmid was amplified via polymerase chain reaction (PCR) with a pair of primers whose overlap contained the sRNA sequences at their 3’ ends as homogenous sequences, and the PCR products were self-cyclized after being transformed into *E. coli* JM109 using its endogenous DNA recombinase. The sequences of those recombinant plasmids were confirmed by DNA sequencing.

2.2. Medium and cultivation

Luria-Bertani (LB) medium (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract) was used for routine cell cultivation. And LBGM [37] (10 g/L tryptone, 5 g/L NaCl, and 5 g/L yeast extract, supplemented with 100 μM MnSO₄ and 1% of glycerol) was used for biofilm formation. Solid media were prepared by adding 1.5 g/L agar to LB broth. As for acetoin production, the recombinants were grown in 25 mL of the fermentation medium [21] (20 g/L glucose, 6 g/L tryptone, 6 g/L yeast extract, 6 g/L (NH₄)₂SO₄, 12.5 g/L KH₂PO₄, 2.5 g/L KH₂PO₄, 5 mM MgSO₄). The following antibiotics were supplemented when necessary: chloromycetin, 5 μg/mL; kanamycin, 50 μg/mL; spectinomycin, 50 μg/mL. 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) was used to induce the expression of sRNA and gfp controlled by T7 promoter in *E. coli*. In all shake flask experiments, cells were pre-cultivated in 50 mL tubes containing 5 mL LB medium at 37 °C for 12 h and then transferred to 25 mL designated broth with a starting OD₆₀₀ of 0.1.

2.3. Fluorescence measurement and microscopy

Cell samples were collected at designated time points to measure the GFP fluorescence intensity (excitation, 490 nm; emission, 530 nm) and cell densities by an Infinite 200 PRO plate reader after appropriate dilution. To analyze the cell morphology, the recombinants were grown on LB plates at 37 °C for 36 h. An Eclipse Ni-E microscope (Nikon, Tokyo, Japan) was used to observe the cell shape and green fluorescence under the phase-contrast lens.

2.4. Analysis of biofilm formation

Cells were preculture in 5 mL LB medium to late exponential growth phase (OD₆₀₀ ≈ 1) and 3 μL of culture were transferred to 3 mL LBGM broth in a 6-well polyvinyl plate [37]. The plates were incubated statically at 30 °C for 64 h. The image was taken with a Redmi Note11 Pro camera.

2.5. Measurement of acetoin concentration

Every recombinant was set three replicated and grown in 25 mL fermentation medium in 250 mL shake flasks at 37 °C under 220 rpm for 48 h. The concentrations of acetoin were measured by high-performance liquid chromatography (HPLC) with a Waters ACQUITY Arc HPLC system equipped with an HPX-87H column (Bio-Rad, Hercules, CA) and a refractive index detector. HPLC conditions were as follows [21]: mobile phase, Aqueous H₂SO₄ (10 mM); flow rate, 0.6 mL/min; temperature, 40 °C; injection volume, 5 μL.

3. Results

3.1. Design and construction of SR6-based trans-acting sRNA

*yoyT-yoj/SR6* TA system is encoded on prophage SP6 in the *B. subtilis* genome, which contains two toxin genes transcribed on a polycistronic mRNA and neutralized by the same antitoxin SR6 utilizing two different mechanisms [38] (Fig. 1A). SR6 interacts with yoj mRNA at its 3’ ends and forms RNase III cleaving sites for its degradation, which is similar to bsr/G4 and bsr/SR5 [39] systems. In contrast, the stability of yoj mRNA is barely changed when interacting with SR6 in their 5’ ends. The downregulation of yoj is ascribed to the direct blocking of its translation by masking the RBS region [13,38].

According to the regulation mechanism of the yoj/SR6 TA system, we replaced the sequence that base-pairing with yoj with a sequence that is complementary to the N-terminal coding sequence of gfp mRNA starting from AUG (SR6-anti_gfp), which could block the translation from the beginning [24]. Notably, 24 nucleotides for target binding met the minimal requiring binding energy (∼20 kcal/mol) [24] (Fig. 1B). A control sRNA (SR6-anti_nowhere) was constructed by replacing the yoj/SR6 base-pairing region in the wild-type SR6 with a randomly generated DNA sequence that does not match any region. After expressing SR6-anti_gfp and SR6-anti_nowhere in *B. subtilis* SCK6, it could be observed that the GFP expression was significantly repressed (Figs. 1D and 2B), confirming that SR6 could be used as a scaffold for creating trans-acting sRNAs in *B. subtilis*.

3.2. Identification of the core structure of SR6-based sRNA

As the SR6-based chimera sRNA with 108 nt in length worked well in *B. subtilis*, we further investigated the key elements responsible for sRNA activity. When interacting with gfp mRNA, the spacer between the base-pairing region and hairpin would overhang the target mRNA (Fig. 1C), which might cause the steric-hindrance effect in blocking the initiation of translation. Thus, three step-by-step spacer truncation variants were constructed (Fig. 2A) and we found that mutants with no spacer gave rise to higher sRNA activity (over 88% repression to GFP) compared with the control SR6-anti_gfp, suggesting the nonessential role of the spacer sequence in interaction. Moreover, in consideration of the unstable state of the head loop (Fig. 2A), we further truncated this hairpin and constructed the simplified SR6-scaffold for GFP repression. Impressively, variant 3 with this simplified SR6-scaffold generated almost the same repression efficiency compared to variant 3 (Fig. 2B). This result indicates that a base-pairing region directly connecting to the simplified SR6-scaffold is fully competent for engineering an effective functional sRNA (Fig. 2A). Additionally, we also found that after 8 h cultivation, the repression rates achieved the maximum values, confirming the stable repression effect during cultivation.

3.3. Investigation of the elements that affect SR6-based sRNA performance

After identifying the simplified SR6-scaffold, we further investigated the relationship between sequence, structure, and repression activity. Intriguingly, SR6-based sRNA scaffolds are also considered as the terminator of sRNA, and further remodeling and mutation of SR6 scaffolds should meet the requirements of general terminators in *B. subtilis*. First, we analyzed 425 *B. subtilis* endogenous rho-independent terminators [40] and found that the majority of these canonical rho-independent terminators are T-stretch followed hairpins, which are composed of a 7–12 bp stem and 3–6 nt loop with ΔG ranging from ~10
to \(-20\) kcal/mol, indicating such structure is adequate for strong transcriptional termination. (Fig. 3A, Supplementary Table S4). Hence, by applying the above structural and thermodynamic constraints, three random sRNA scaffolds with the same hairpin structure composed of different sequences were designed via NUPACK v4.0.0.23 (Fig. 3B) [41]. At the same time, 6 variants with different stem lengths from 12 to 7 bp were also designed and constructed (Fig. 3B). As shown in Fig. 3C, all the scaffold variants with different sequences but similar structures conferred an over 80% repression on GFP, suggesting that SR6-based sRNA functions in a sequence-independent manner. Moreover, when adjusting the stem length of the simplified SR6 scaffold from 12 to 7 bp, the 8 bp-stem simplified SR6 scaffold was the minimalized scaffold for maintaining a high repression rate (Fig. 3C). Therefore, a normalized sRNA structure with high activity was designated to an 8 bp-stem 4 nt-loop hairpin preceded by a 24 nt base-pairing region and followed by a polyU9 tail (Fig. 3D).

### 3.4. The artificial sRNA is active across B. subtilis and E. coli

The E. coli sRNA-hfq system has been developed [8,24] and transplanted into other species including C. glutamicum [30], Shewanella oneidensis [42], Clostridium acetobutylicum [43] and Halomonas bluephagenesis [44] with simultaneous overexpression of E. coli Hfq. Here, the dependency of the artificial sRNA on endogenous Hfq was investigated. As shown in Fig. 4A, it could be noted that the inactivation of Hfq in B. subtilis generated a slightly decreased repression rate, which was consistent with the previous study that it’s unlike that Hfq plays a central role in RNA transactions in B. subtilis [45,46]. In addition, we also evaluated its cross-species regulation potential. As expected, by only expressing the anti_gfp sRNA, this artificial sRNA system also achieved about 84% repression rate in E. coli (Fig. 4B).

### 3.5. Development of de novo sRNA design program

 Arbitrarily fusing some base-pairing sequences to the same sRNA scaffold might cause a final structure defect with unknown

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**Fig. 2. Determine the core structure of SR6-based sRNA.** A. The predicted secondary structure of SR6 variant sRNAs. The structure details were marked by different colors: the base-pairing region to gfp, the spacer, and the simplified SR6 scaffold are shown in green, grey, and violet, respectively. The inset is the predicted secondary structure of anti_gfp sRNA with a simplified SR6 scaffold. B. The repression efficiency of SR6 variants to gfp. Cells were collected at designated time points to measure the fluorescence intensity and cell density (OD_{600}). All the data are expressed as the mean ± S.D. from three biologically independent replicates.

**Fig. 3. Resolve the elements that affect sRNA performance.** A. Analysis of distribution of the hairpin features of rho-independent terminators in B. subtilis. B. The predicted secondary structure of SR6-based variants. The sequence mutants are shown in blue while the stem truncation variants are shown in violet. C. Variants’ repression rates to gfp. Samples were collected at time point 12 to measure cell density (OD_{600}) and fluorescence intensity. Fluorescence intensity/OD_{600}, Flu/OD_{600}. All the data are expressed as the mean ± S.D. from three biologically independent replicates. D. Schematic diagram of minimalized sRNA core scaffold.
consequences, it is critical to rationally design and assign different scaffolds according to the designated target gene. Hence, a de novo sRNA design program was developed according to the above findings, allowing the custom design of sRNA scaffolds matching the genes of interest (Fig. 5A). Briefly, when the target gene sequence is provided, the scaffold sequences will be randomly generated and assigned to combine with the target base-pairing sequence for outputting sRNA candidates. After being filtered by overall structural analysis via NUPACK, the sRNA candidates meeting the standard structure requirements will be documented as custom designed sRNA for this target. This procedure will be repeatedly performed until the desired sRNA is generated. To test this sRNA design program, we utilized it to generate sRNAs targeting \textit{ftsZ} and \textit{comER}. The defect in \textit{ftsZ} expression commonly leads to apparent cell morphology change [47] while the \textit{ΔcomER} mutant of \textit{B. subtilis} exhibits a biofilm defect [37]. As shown in Fig. 5B, when expressing the anti-\textit{comER} sRNA, a significant defect in pellicle biofilm formation was observed. In contrast, no changes were detected when expressing anti-nowhere sRNA and anti-\textit{ftsZ} sRNA. In parallel, when expressing anti-\textit{ftsZ} sRNA, the short rod-shaped \textit{B. subtilis} cells were reformed to filamentous morphology while no filamentary shape was observed when expressing anti-nowhere sRNA and anti-\textit{comER} sRNA. The results demonstrate the effectiveness of the de novo designed sRNAs.

3.6. Automatically designed sRNA for engineering acetoin biosynthesis pathway

Rational metabolic engineering has been considered a frequently-used approach for manipulating the cell factory to produce desired chemicals of high yield. As a proof of concept, the sRNA strategy was applied for adjusting the biosynthesis of acetoin, a common flavor compound and native overflowed metabolite of \textit{B. subtilis} [48]. As a consequence, the sRNAs targeting the genes \textit{ldh} (encoding lactate dehydrogenase), \textit{pta} (encoding phosphotransacetylase), \textit{bdhA} (encoding (R,R)-butanediol dehydrogenase) and \textit{acoA} (encoding 2,6-dichlorophenolindophenol oxidoreductase subunit alpha) for by-products synthesis and acetoin consumption, and \textit{alsS} (encoding acetolactate synthase), \textit{alsD} (encoding alpha-acetolactate decarboxylase) and \textit{alsR} (encoding a transcriptional activation factor for \textit{alsS} and \textit{alsD}) for acetoin synthesis (Fig. 6A) were automatically designed and constructed via de novo sRNA design program (Supplementary Table S3). As expected, individual

Fig. 4. Explore the SR6-based sRNA function properties. A. Analysis of the SR6-8 stem-anti\textunderscore gfp sRNA performance in \textit{B. subtilis} Δhfq, and B. in \textit{E. coli} BL21(DE3). Cells were collected at designated time points to measure the fluorescence intensity and cell density(OD\textsubscript{600}). All the data are expressed as the mean ± S.D. from three biologically independent replicates.

Fig. 5. Development of de novo design sRNA program. A. The work pipeline for de novo design of SR6-based sRNA. The aforementioned structural and thermodynamic constraints were applied to generate SR6-based scaffold candidates, which would further be connected to the base-pairing sequence of the target gene and filtered by overall structure analysis to make a standard SR6-like sRNA. B. Analysis of morphology change and biofilm formation of \textit{B. subtilis} SCK6 containing de novo designed anti-\textit{comER} sRNA, anti-\textit{ftsZ} sRNA, and anti-nowhere. Statically cultivated for 36–64 h, images of cell shape and biofilm were taken.
repression of ldh, pta, acoA, or bdhA resulted in the increase of acetoin titers while repression of alsS or alsD lead to a significant decrease of acetoin synthesis. This is probably because the decreased level of expression of alsR was still enough for activating the expression of alsS and alsD, which are required for acetoin biosynthesis. Interestingly, downregulating pta expression resulted in higher acetoin yield and cell density but lower per cell production. It’s suggested that the down-regulation of pta should not only drive more metabolic flux to the acetoin synthesis pathway but also the TCA cycle. More contribution of the TCA cycle in carbon metabolism would bring about increased biomass (higher cell density), which resulted in lower acetoin production per cell. The results demonstrate the remarkable efficiency and convenience of these de novo designed sRNA for rapidly regulating genes of interest in B. subtilis.

4. Discussion

As versatile and robust regulators of gene expression, the native sRNAs are identified and classified as trans-acting and cis-acting sRNAs, which have inspired the top-down and bottom-up design of novel RNA devices. Due to the high programmability of sRNA structures and functions [49], synthetic sRNAs have been created to repress [24] or activate [50] the expression of genes of interest while these available sRNA tools without genome-editing requirements are mainly developed in Gram-negative bacteria E. coli. Here, we firstly developed artificial sRNAs for B. subtilis on the basis of investigating its native sRNA SR6, and achieved the efficient knockdown of genes of interest (Figs. 2B and 5B). Obviously, the engineered sRNA system here is more convenient compared to the previously engineered RNA based regulatory systems such as MS-DOS, toehold switch, etc. [7,29,50,51], since no pre-editing of the flank sequences of target genes would be needed.

Moreover, we find that the synthetic sRNAs with an 8 bp-stem 4 nt-loop hairpin with a 9 nt polyU tail still function well in the Δhfq mutant, and also possess a remarkable repression rate (over 85%) in E. coli. In contrast, only with co-overexpression of E. coli RNA chaperone Hfq, the sRNAs from the MicC scaffold can function in Gram-positive bacteria such as Corynebacterium glutamicum [30] and Clostridium acetobutylicum [43]. The results confirmed the previous speculation that the RNA chaperone Hfq in B. subtilis is nonessential for most sRNAs function (Fig. 4A). Also, the developed sRNA system with the cross-species regulation ability and low metabolic burden (no co-overexpression of accessory proteins, for instance, Hfq or dCas protein) should have more promising applications.

In addition, we find that the sRNA scaffold consists of an 8 bp-stem 4 nt-loop hairpin with a 9 nt polyU tail also fits into the general pattern of canonical rho-independent terminator in B. subtilis (Fig. 3A). Thus, after integrating this knowledge and bioinformatics, a de novo sRNA design program (Fig. 5A) for automatically generating custom sRNAs with the standard structure but random sequences was developed, which should be favorable to regulating multiple genes with the reduced recombinant probability of sRNAs. By applying the automatically designed sRNAs, we achieved the rapid modulation of morphology (Fig. 5B) and acetoin synthesis (Fig. 6B). Consequently, the developed sRNA system for posttranscriptional regulation would be powerful in facilitating the characterization of gene function or the rapid identification of key regulatory nodes of the biosynthesis pathways of interest.

Overall, by investigating the endogenous sRNA SR6, a novel sRNA system for posttranscriptional regulation was developed in B. subtilis, which achieved efficient repression with the expression of only a customized sRNA. To facilitate the manipulation and avoid potential recombination between sRNAs with the same scaffold, a structure-based sRNA design principle was further proposed, enabling the automatic generation of custom sRNAs with the standard structure (a 24 nt base-pairing region and an 8 bp-stem 4 nt-loop hairpin with a 9 nt polyU tail) but random sequences. This sRNA system with cross-species activity not only enriched the gene regulation toolkit in synthetic biology for B. subtilis and E. coli but also enhanced our understanding of trans-acting sRNAs. In addition, by coupling with the advances in promoter engineering [34], protein-based regulators [52], CRISPR/Cas systems [53], RNA-based regulators [54,55], increasingly sophisticated forms of gene circuits for highly programmable and dynamic biological functions should be constructed in near future.

CRediT authorship contribution statement

Guobin Yin: Conceptualization, Investigation, Formal analysis, Software, Writing – original draft. Anqi Peng: Investigation. Luyao Zhang: Investigation. Yang Wang: Reviewing. Guocheng Du: Reviewing. Jian Chen: Reviewing. Zhen Kang: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sysbio.2022.11.003.
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