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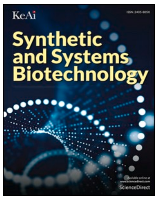

Original software publication

CRISPy-web 3.0: A unified platform for multi-modal guide RNA design for CRISPR and TnpB genome editing applications

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ABSTRACT

CRISPy-web is an interactive web-based platform designed for rational guide RNA design in CRISPR-Cas9-based genome editing systems. Here, we present CRISPy-web 3.0 (https://crispy.secondarymetabolites.org/), an upgraded and versatile version that extends its functionality beyond classical Cas9-based systems. This version integrates support for diverse genome editing systems, including Cas9, CRISPR interference (CRISPRi), and TnpB/ωRNA. The redesigned interface enables users to toggle between multiple editing modes, select target regions such as ORFs or 5′ UTRs, and visualize strand orientation, off-targets, and predicted mutation outcomes. It also incorporates scoring systems that evaluate guide RNA efficiency and specificity based on mismatch tolerance, positional context, and PAM requirements for CRISPR-Cas9-based applications. With an improved user interface design, enhanced backend scalability, and modular support for customizable inputs, CRISPy-web 3.0 provides a comprehensive and extensible platform for guide RNA design, enabling genome editing across a broader range of prokaryotic systems, including both CRISPR and TnpB.

1. Introduction

CRISPR technologies have revolutionized genome engineering in both model and non-model organisms by enabling programmable, sequence-specific manipulation of DNA and RNA. The foundational CRISPy tool was originally introduced as a Python-based utility to identify sgRNA targets in Chinese hamster ovary (CHO) cells, with built-in off-target prediction tailored to the CHO–K1 genome [1]. Recognizing the need for broader accessibility and flexibility, CRISPy-web 1.0 was subsequently launched as an online service, enabling researchers to perform sgRNA searches on user-provided microbial genomes in GenBank format [2]. It featured interactive selection interfaces, genome visualization, and compatibility with outputs from antiSMASH [3], addressing a major gap for the natural product research communities. Building on this, CRISPy-web 2.0 [4] introduced support for CRISPR-BEST (Base Editing SysTem) [5], which enabled single-nucleotide resolution editing using engineered cytidine and adenosine deaminases in *Streptomyces* species.

The past few years have witnessed an expansion in genome editing, including prime editing [6], INTEGRATE [7], Cas12n [8], STITCHR [9], TIGR-Tas [10], CASCADE-Cas3 [11], and novel compact nucleases such as TnpB [16] and Fanzor [12]. Among these, TnpB-based systems, small RNA-guided endonucleases derived from IS200/IS605-family transposons, have emerged as evolutionarily ancient precursors to class 2 CRISPR-Cas12 [13–15]. Functioning with a distinct ωRNA rather than canonical sgRNA, TnpB effectors offer programmable DNA targeting with minimal protein size, facilitating delivery in challenging *in vivo* contexts and expanding the programmable editing toolbox for synthetic biology. Parallel to this, transcriptional interference (CRISPRi) [16] has also gained traction as a non-destructive method for conditional gene repression, widely adopted in functional genomics and metabolic rewiring studies [17,18].

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Table 1
Feature comparison of CRISPy-web 3.0 and selected CRISPR guide design tools.

Tool	Cas9	CRISPRi	BE	TnpB	Advanced Parameters	Off-target	User Genome	Non-model organisms	Last Update	Reference
CRISPy-web 1.0	✓	×	×	×	Basic	Yes	Yes	Yes	2016	[2]
CRISPy-web 2.0	✓	×	✓	×	Basic	Yes	Yes	Yes	2020	[4]
CRISPy-web 3.0	✓	✓	✓	✓	**Advanced**	**Yes**	**Yes**	**Yes**	**2025**	**This work**
CHOPCHOP	✓	✓	✓	×	Moderate	Yes	Yes	Yes	2023	[25]
BE-Designer	✓	×	✓	×	Moderate	Yes	Limited	Limited	2021	[26]
GuideScan	✓	✓	✓	×	Advanced	Yes	Yes	Yes	2022	[27]
CRISPOR	✓	×	✓	×	Moderate	Yes	Yes	Yes	2022	[28]

√ = supported,×= not supported. “Advanced Parameters” includes expert mode for PAM/TAM, spacer length, editing window, etc. “BE” means base editing.

The CRISPR-Cas9 system, still the most widely applied genome editing tool, faces challenges with off-target effects and variable editing efficiency. To address this, recent research has introduced empirical and machine learning-based scoring models to evaluate guide RNA quality, including CFD and DeepCRISPR scores [19,20]. These models assess factors such as mismatch position, thermodynamic stability, nucleotide composition, and chromatin accessibility. However, many online tools only provide basic rule-based filtering or are not compatible with non-model genomes. CRISPy-web 3.0 incorporates a flexible scoring logic [21] tailored to prokaryotic systems, highlighting guide RNAs based on mismatch type and location (especially within the 8–20 bp window upstream of PAM), and integrating region-specific heuristics to optimize guide selection for Cas9 and CRISPRi applications.

Despite the increasing adoption of these modalities, most sgRNA design tools remain restricted to Cas9-based editing or lack support for custom genomes and emerging effector systems. To bridge this gap, we developed CRISPy-web 3.0, a unified, modular platform supporting guide RNA design for Cas9, CRISPRi, and ωRNA-based TnpB system STAGE [22], complete with scoring logic, mismatch-aware specificity filters, and compatibility with user-provided microbial genomes. This comprehensive feature set positions CRISPy-web 3.0 as one of the most flexible and forward-compatible solutions for microbial genome editing (Table 1).

2. Design and implementation

2.1. Support for CRISPRi design

CRISPy-web 3.0 supports sgRNA design for CRISPR interference by identifying target sites in promoter regions, 5′ untranslated regions (5′ UTRs), and early coding sequences. In prokaryotic systems, the choice of DNA strand targeted by dCas9 or dCpf1 is a critical determinant of CRISPRi efficiency: targeting the non-template (coding) strand is generally required for effective transcriptional repression, as dCas9 binding to the template strand typically does not block elongating RNA polymerase in bacteria [17,18]. CRISPy-web 3.0 explicitly incorporates this principle by allowing users to select either the template or non-template DNA strands and visually indicates strand orientation on the gene map and results table. The platform automatically prioritizes and annotates guides on the non-template strand for bacterial CRISPRi applications but also provides full flexibility for experimental designs involving eukaryotes or non-canonical systems. Each sgRNA is scored based on positional effectiveness [21] and categorized into “within-ORF” or “upstream-of-ORF” (including promoter and 5′ UTR) groups. Additionally, the interface highlights the distance of each guide to the translation start codon, allowing users to prioritize sgRNAs expected to mediate stronger transcriptional repression. This integrated consideration of strand orientation, prokaryotic transcript structure, and functional annotation allows CRISPy-web 3.0 to deliver more precise, biologically informed, and strand-aware CRISPRi designs for prokaryotic genomes.

2.2. Integration of TnpB and ωRNA design

The platform now supports the design of ωRNAs compatible with TnpB, TnpBi, and TnpB-BEST, expanding CRISPy-web beyond traditional CRISPR-Cas systems. For each genomic locus, the tool first identifies the appropriate target-adjacent motifs (TAMs) required for TnpB-mediated DNA recognition. The surrounding regions are then scanned for candidate ωRNA target sites, prioritizing sequences with minimal off-target potential (up to 2 mismatches) and favorable context for cleavage. To simulate the TnpB-BEST strategy, which combines programmable ωRNA targeting with precise base editing via recruited deaminases, the platform can annotate ωRNA designs that align with editable cytidines or adenines within a defined editing window. The design logic incorporates both the sequence position relative to TAM and the distribution of editable bases to maximize base editing success rates while minimizing nonspecific cuts. This integrated workflow allows users to explore next-generation programmable base editing in compact and delivery-friendly formats.

2.3. Guide RNA scoring system

CRISPy-web 3.0 incorporates a modular scoring framework that balances intrinsic guide RNA efficiency with mismatch-aware specificity penalties. For Cas9-based applications, candidate guides are first evaluated for their sequence-intrinsic activity using position- and dinucleotide-dependent features identified in prior empirical studies. Off-target potential is then quantified by applying a mismatch-sensitive penalty matrix, with stronger penalties assigned to mismatches within the PAM-proximal seed region. The combined raw score is normalized via a sigmoid transformation, yielding an intuitive range between 0 (low confidence) and 1 (high confidence). For CRISPR interference (CRISPRi), the system employs a position-weighted model that accounts for strand orientation and proximity to the start codon, providing scores reflective of transcriptional repression efficiency. At present, ωRNA-based TnpB designs are reported without scoring due to limited benchmarking data, but future updates will extend this framework as experimental datasets become available.

2.4. Genome input and annotation support

CRISPy-web 3.0 accepts both FASTA and GenBank formats, with preference for annotated sequences to enable gene name, locus tag, and protein ID queries. For unannotated genomes, users are advised to use annotation tools such as BAKTA [23], RAST [24], and optionally preprocess the genome with antiSMASH [3] for BGC targeting.

2.5. Visualization and export

All candidate guide RNAs are visualized with interactive zoom and filter functionalities. Guide RNAs can be sorted by off-target count, position, or score, and downloaded in CSV format. Annotations indicate whether a guide RNA can be used for conventional DSB-based genome editing, base editing, or interference.

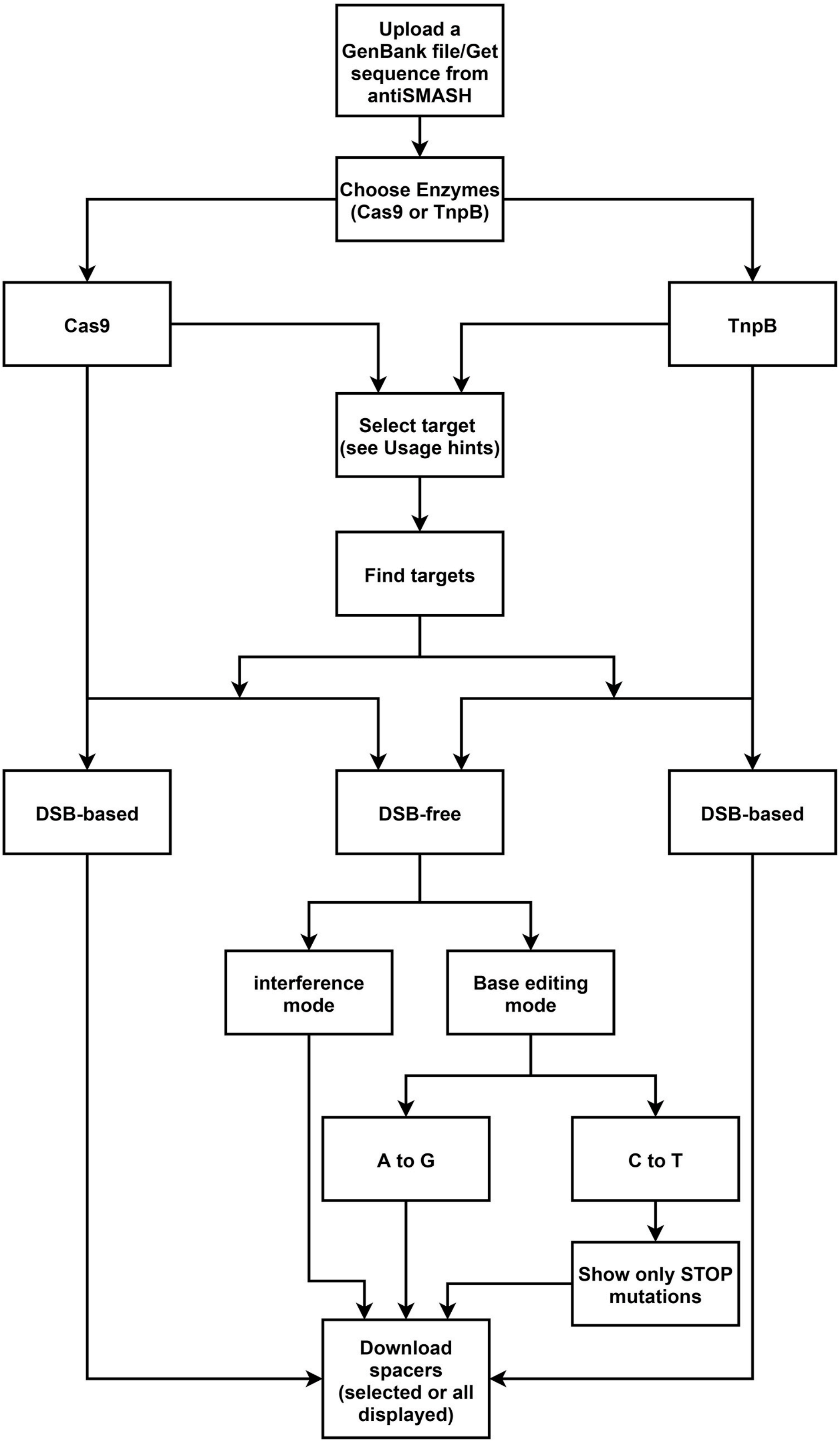
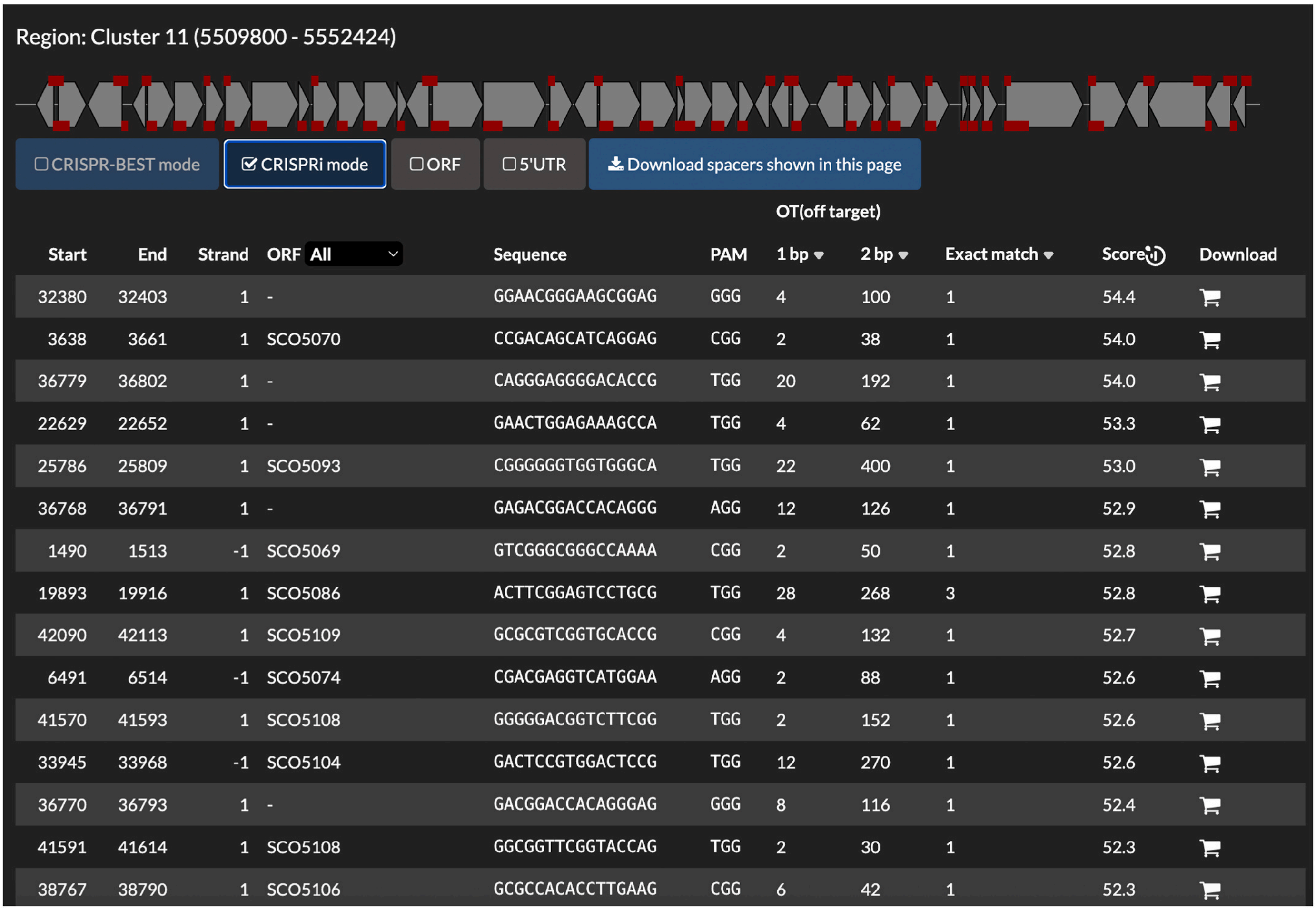


Fig. 1. Workflow of CRISPy-web 3.0. Users can upload annotated genomes (GenBank files) or directly obtain sequences from antiSMASH job ID, choose between Cas9 and TnpB modes, and identify target sites. The platform supports both DSB-based and DSB-free strategies, including interference (CRISPRi and TnpBi) and base editing modes (A→G, C→T in CRISPR-BEST and TnpB-BEST). All selected or displayed spacers can be exported for downstream applications.



Region: Cluster 11 (5509800 - 5552424)

ORF: All

Start	End	Strand	ORF	Sequence	PAM	OT(off target)			Score	Download
						1 bp ▾	2 bp ▾	Exact match ▾		
32380	32403	1	-	GGAACGGGAAGCGGAG	GGG	4	100	1	54.4	🛒
3638	3661	1	SCO5070	CCGACAGCATCAGGAG	CGG	2	38	1	54.0	🛒
36779	36802	1	-	CAGGGAGGGGACACCG	TGG	20	192	1	54.0	🛒
22629	22652	1	-	GAACTGGAGAAAGCCA	TGG	4	62	1	53.3	🛒
25786	25809	1	SCO5093	CGGGGGGTGGTGGGCA	TGG	22	400	1	53.0	🛒
36768	36791	1	-	GAGACGGACCACAGGG	AGG	12	126	1	52.9	🛒
1490	1513	-1	SCO5069	GTCGGGCGGGCCAAAA	CGG	2	50	1	52.8	🛒
19893	19916	1	SCO5086	ACTTCGGAGTCCTGCG	TGG	28	268	3	52.8	🛒
42090	42113	1	SCO5109	GCGCGTCGGTGCACCG	CGG	4	132	1	52.7	🛒
6491	6514	-1	SCO5074	CGACGAGGTCATGGAA	AGG	2	88	1	52.6	🛒
41570	41593	1	SCO5108	GGGGGACGGTCTTCGG	TGG	2	152	1	52.6	🛒
33945	33968	-1	SCO5104	GACTCCGTGGACTCCG	TGG	12	270	1	52.6	🛒
36770	36793	1	-	GACGGACCACAGGGAG	GGG	8	116	1	52.4	🛒
41591	41614	1	SCO5108	GGCGGTTCGGTACCAG	TGG	2	30	1	52.3	🛒
38767	38790	1	SCO5106	GCGCCACACCTTGAAG	CGG	6	42	1	52.3	🛒

Fig. 2. Visualization of candidate sgRNAs designed for CRISPRi targeting of the actinorhodin cluster in *Streptomyces coelicolor*. Each guide is displayed with genomic location, strand orientation, associated gene, PAM, and a comprehensive off-target profile. The scoring system integrates both efficiency and specificity to help users identify optimal sgRNA candidates.

3. Case studies: planning versatile genome editing of the actinorhodin cluster in *Streptomyces coelicolor*

To comprehensively demonstrate the capabilities of CRISPy-web 3.0, we conducted a multi-modal genome editing case study targeting the actinorhodin biosynthetic gene cluster (region 11: 5,509,800–5,552,424 bp) in *Streptomyces coelicolor* (RefSeq: NC_003888.3).

3.1. CRISPRi, CRISPR-BEST, and CRISPR-Cas9 applications

Upon uploading the annotated GenBank file or get sequence from antiSMASH [3], users are presented with an interactive visualization, where different enzymes (editing systems) with customized parameters and all regions are clearly displayed. A workflow is shown in Fig. 1.

Switching to CRISPRi mode, the platform identifies all possible sgRNA targets within the selected cluster, with real-time visualization of each guide's position and strand orientation. In prokaryotic genomes, effective CRISPRi relies on targeting the non-template (coding) strand; this is clearly annotated in the results table and cluster map (Fig. 2). Users can filter candidate guides by region (ORF, 5′ UTR) and sort by distance to translation start or scores, leveraging evidence that targeting near the start codon maximizes transcriptional repression efficiency. Each guide is annotated with positional effectiveness, functional region, and predicted impact with off-targets and scores.

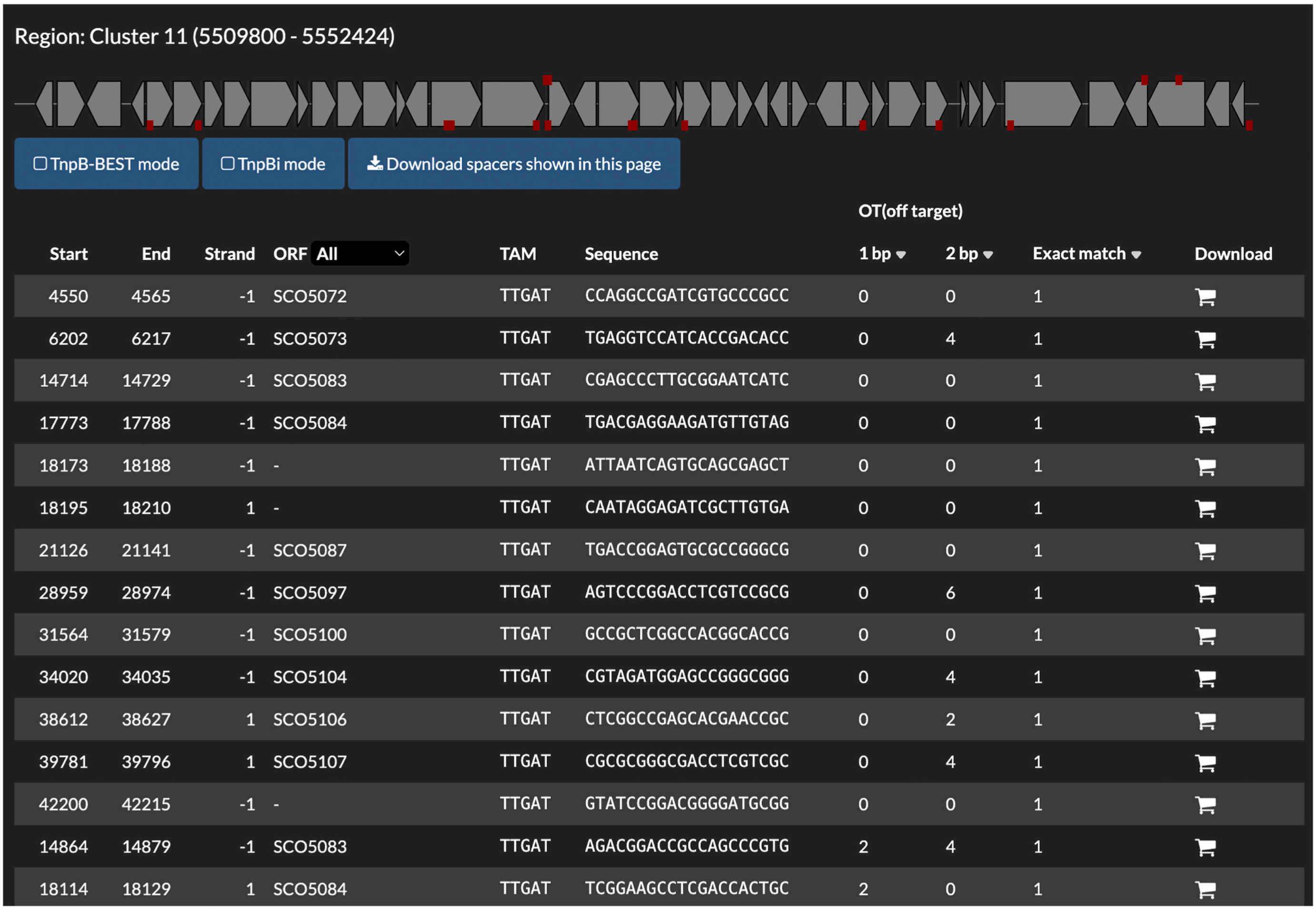
Switching to CRISPR-BEST mode, users can screen for sgRNAs capable of introducing STOP codons or precise point mutations. The results table includes mutation prediction at both DNA and amino acid level (e.g., W57*, G15K), and a "STOP-only" filter streamlines selection for gene knockout studies. Comprehensive off-target profiling (exact match, 1 bp, 2 bp mismatch) and scoring are provided for each sgRNA. All results shown in the page or customer-chosen spacers can be downloaded for synthesis or cloning.

3.2. ωRNA design for TnpB-related applications

In TnpB, TnpBi, or TnpB-BEST mode, users access the expert settings panel to specify the TnpB subtype, TAM motif, spacer length, and editable window parameters, reflecting the diversity of TnpB systems recently uncovered in actinobacteria. The platform automatically scans for all potential ωRNA targets adjacent to the defined TAM within the actinorhodin cluster (Fig. 3). For each candidate ωRNA, the editing window is visualized on the gene map and in the table, indicating which bases fall within range of the base editor. Using similar logic, CRISPy-web 3.0 provides predicted ωRNA for TnpBi applications; amino acid changes, highlights newly introduced STOP codons, and performs genome-wide off-target analysis (Fig. 3). This streamlined process enables precise, compact, and programmable base editing in non-model hosts.

3.3. Cross-mode comparison and workflow optimization

A distinctive feature of CRISPy-web 3.0 is the rapid toggling between CRISPR-Cas9/CRISPRi and TnpB design modes for the same cluster or gene region. Users can directly compare the density, distribution, and



Region: Cluster 11 (5509800 - 5552424)

						OT(off target)			
Start	End	Strand	ORF All	TAM	Sequence	1 bp ▾	2 bp ▾	Exact match ▾	Download
4550	4565	-1	SCO5072	TTGAT	CCAGGCCGATCGTGCCCGCC	0	0	1	🛒
6202	6217	-1	SCO5073	TTGAT	TGAGGTCCATCACCGACACC	0	4	1	🛒
14714	14729	-1	SCO5083	TTGAT	CGAGCCCTTGCGGAATCATC	0	0	1	🛒
17773	17788	-1	SCO5084	TTGAT	TGACGAGGAAGATGTTGTAG	0	0	1	🛒
18173	18188	-1	-	TTGAT	ATTAATCAGTGCAGCGAGCT	0	0	1	🛒
18195	18210	1	-	TTGAT	CAATAGGAGATCGCTTGTGA	0	0	1	🛒
21126	21141	-1	SCO5087	TTGAT	TGACCGGAGTGCGCCGGGCG	0	0	1	🛒
28959	28974	-1	SCO5097	TTGAT	AGTCCCGGACCTCGTCCGCG	0	6	1	🛒
31564	31579	-1	SCO5100	TTGAT	GCCGCTCGGCCACGGCACCG	0	0	1	🛒
34020	34035	-1	SCO5104	TTGAT	CGTAGATGGAGCCGGGCGGG	0	4	1	🛒
38612	38627	1	SCO5106	TTGAT	CTCGGCCGAGCACGAACCGC	0	2	1	🛒
39781	39796	1	SCO5107	TTGAT	CGCGCGGGCGACCTCGTCGC	0	4	1	🛒
42200	42215	-1	-	TTGAT	GTATCCGGACGGGGATGCGG	0	0	1	🛒
14864	14879	-1	SCO5083	TTGAT	AGACGGACCGCCAGCCCGTG	2	4	1	🛒
18114	18129	1	SCO5084	TTGAT	TCGGAAGCCTCGACCACTGC	2	0	1	🛒

Fig. 3. Identification of ωRNA guides for TnpB and TnpB-BEST applications within the actinorhodin cluster. Results include all suitable target sites with relevant sequence context, predicted editing windows, and off-target analysis, enabling precise design of compact base editing tools for microbial genomes.

predicted efficiency of sgRNA versus ωRNA targets within the cluster. For synthetic biologists, this facilitates parallel design and experimental planning, choosing the optimal nuclease system, region, and guide set for targeted editing, activation, or repression. All tables, visualizations, and off-target reports can be exported for documentation, synthesis, or integration into downstream pipelines.

Through this workflow, users gain a comprehensive view of all possible editing strategies: traditional knockout, CRISPRi repression, or advanced TnpB-driven base editing, tailored to the structure and regulatory landscape of the actinorhodin cluster. The high degree of interactivity, region and strand awareness, mutational prediction, and expert configuration demonstrated CRISPy-web 3.0's unique value for microbial genome engineering and natural product pathway optimization.

4. Discussion

CRISPy-web 3.0 represents a substantial advance in the landscape of guide RNA design tools for microbial genome engineering and synthetic biology. While numerous platforms exist for Cas9-based guide design, such as CHOPCHOP [25], BE-Designer [26], GuideScan [27], and CRISPOR [28], these are often limited in their flexibility, extensibility, or compatibility with emerging editing modalities and non-model microbial genomes. In contrast to general-purpose off-target search engines such as Cas-OFFinder, CRISPy-web 3.0 is not intended as a direct replacement but rather as a complementary platform. It integrates target discovery, multi-modal design (Cas9, CRISPRi, TnpB/ωRNA), and pathway-level visualization into a unified workflow specifically optimized for non-model microbial genomes. Importantly, this unified design environment not only ensures flexibility for non-model organisms and newly sequenced strains but also introduces support for TnpB/ωRNA systems, which remains uncommon among existing sgRNA design platforms.

A key differentiator of CRISPy-web 3.0 is its modular, multi-modal architecture. It uniquely integrates support for Cas9, CRISPRi, and the recently discovered TnpB and TnpB-related systems like TnpB-BEST and TnpBi, alongside base editing, within a unified, interactive web interface. The platform enables seamless switching between editing modes, region-aware design (e.g., ORF, 5′UTR, promoters), and strand-aware targeting, which is essential for effective CRISPRi applications in prokaryotes. This level of customization is rarely available in competing platforms and addresses critical needs for pathway engineering, genome mining, and high-throughput functional genomics.

One of the most notable features is the "Expert mode", which allows advanced users to specify enzyme variants, PAM/TAM motifs, spacer lengths, and editing windows—capabilities particularly relevant for the rapidly expanding CRISPR and TnpB toolbox.

To provide an objective comparison with other mainstream guide RNA design tools, Table 1 summarizes the key features, genome compatibility, off-target prediction capabilities, and update status for CRISPy-web 3.0, previous CRISPy versions, and several popular alternatives. Notably, only CRISPy-web 3.0 supports all the following in a single platform: multi-mode design (Cas9, CRISPRi, TnpB/ωRNA), advanced parameter customization, off-target analysis for both Cas9 and ωRNA-based systems, high-throughput guide export, and flexible support for user-uploaded or non-model genomes (Table 1).

The ongoing evolution of CRISPR and TnpB technologies is driving the need for ever more adaptable and scalable computational tools. CRISPy-web 3.0 is designed with this in mind—its backend architecture supports genome-scale design, large batch processing, and rapid integration of new editing enzymes and target motifs. Looking ahead, planned updates include support for prime editing, library-scale design for pooled screens, and expanded enzyme/target annotation, ensuring that the platform remains at the forefront of CRISPR-based microbial genome engineering.

CRediT authorship contribution statement

Sihan Yang: Methodology, Data curation. **Zupeng Cai:** Methodology, Data curation. **Natalie Chia:** Validation. **Jing Luo:** Validation. **Tilmann Weber:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Kai Blin:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis, Conceptualization. **Yaojun Tong:** Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors Tilmann Weber and Yaojun Tong are members of the Editorial Board of *Synthetic and Systems Biotechnology* and were not involved in the editorial review or the decision to publish this article. Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2025.10.003.

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