A new control system for synthetic genes

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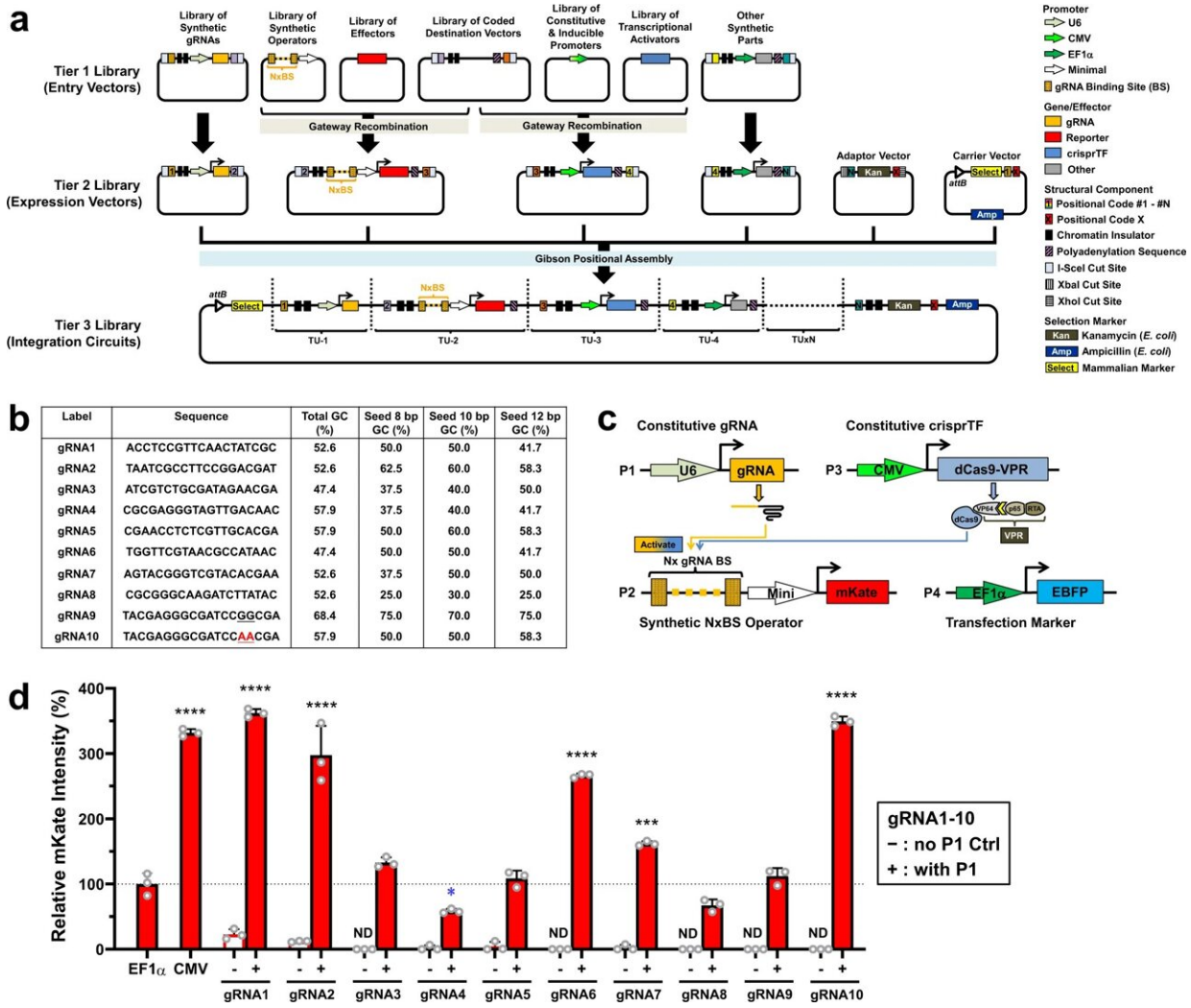

Label	Sequence	Total GC (%)	Seed 8 bp GC (%)	Seed 10 bp GC (%)	Seed 12 bp GC (%)
gRNA1	ACCTCCGTTCAACTATCGC	52.6	50.0	50.0	41.7
gRNA2	TAATCGCCTTCCGGACGAT	52.6	62.5	60.0	58.3
gRNA3	ATCGTCTGCGATAGAACGA	47.4	37.5	40.0	50.0
gRNA4	CGCGAGGGTAGTTGACAAC	57.9	37.5	40.0	41.7
gRNA5	CGAACCTCTCGTTGCACGA	57.9	50.0	60.0	58.3
gRNA6	TGGTTCGTAACGCCATAAC	47.4	50.0	50.0	41.7
gRNA7	AGTACGGGTCGTACACGAA	52.6	37.5	50.0	50.0
gRNA8	CGCGGGCAAGATCTTATAC	52.6	25.0	30.0	25.0
gRNA9	TACGAGGGCGATCCGGCGA	68.4	75.0	70.0	75.0
gRNA10	TACGAGGGCGATCCAACGA	57.9	50.0	50.0	58.3

Design and development of the crisprTF promoter system. **a** Schematic illustration of the programmable and modular design of the crisprTF-based transcription system. To increase its programmability, this platform was modularly divided into three tiers of libraries constructed with the Gateway-Gibson cloning approach. The Tier 1 library was composed of entry vector

modules separately encoding gRNAs, synthetic operators with gRNA binding sites (BS) upstream of a minimal promoter, effector genes, crisprTFs and their associated promoters, and other transcriptional control elements. Tier 1 library units were assembled into positional expression vectors with pre-defined orders by Gateway cloning, forming the Tier 2 library. Positional assembly by Gibson cloning was performed to connect independent transcriptional units (TUs), derived from positional expression vectors in the Tier 2 library by I-SceI restriction digestion, into complete gene circuits. The Tier 3 library comprised integration circuits enabling precision control of the target gene(s) when integrated into a landing pad (i.e., a designated chromosomal safe harbor) with BxB1 integrase-mediated, site-specific chromosomal integration. **b** Ten gRNAs (gRNA1-10) orthogonal to the CHO genome were screened for expression. gRNA10 was modified from gRNA9 with GG-to-AA mutations to reduce the GC content of the seed and the entire sequences. **c** To evaluate episomal gene expression levels, CHO-K1 cells were transiently transfected with four plasmids: plasmid #1 (P1) constitutively expressing gRNA; plasmid #2 (P2) encoding the synthetic operator with some number (x) of gRNA BS to drive mKate expression; plasmid #3 (P3) constitutively expressing a crisprTF; and plasmid #4 (P4) constitutively expressing the transfection marker (EBFP). mKate signals were assessed at 48 hours post-transfection. **d** For the gRNA screening, each gRNA was paired with a matching synthetic operator containing 8× gRNA BS to control mKate expression. EF1α and CMV promoters driving mKate expression served as positive controls. Experimental groups (+), represented by red solid bars, were transfected with four plasmids (P1–P4). Control groups (−), represented by red hollow bars, to detect baseline operator leakage were transfected without P1 (P2–P4). Data were normalized to the EF1α control and are presented as relative median mKate intensity (%). gRNA1 and gRNA2 operators exhibited notable leakage without gRNA, suggesting non-specific transcriptional activities that were not associated with targeted crisprTF binding (CMV, gRNA1, gRNA2, gRNA4, gRNA6, gRNA7, and gRNA10 P1 + vs. EF1α: p