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CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity

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CRISPR-Cas12a (Cpf1) proteins are RNA-guided enzymes that bind and cut DNA as components of bacterial adaptive immune systems. Like CRISPR-Cas9, Cas12a has been harnessed for genome editing based on its ability to generate targeted, double-stranded DNA (dsDNA) breaks. Here we show that RNA-guided DNA binding unleashes indiscriminate single-stranded DNA (ssDNA) cleavage activity by Cas12a that completely degrades ssDNA molecules. We find that target-activated, non-specific ssDNase cleavage is also a property of other type V CRISPR-Cas12 enzymes. By combining Cas12a ssDNase activation with isothermal amplification, we create a method termed DNA Endonuclease Targeted CRISPR *Trans* Reporter (DETECTR), which achieves attomolar sensitivity for DNA detection. DETECTR enables rapid and specific detection of human papillomavirus in patient samples, thereby providing a simple platform for molecular diagnostics.

CRISPR-Cas adaptive immunity in bacteria and archaea uses RNA-guided nucleases to target and degrade foreign nucleic acids (*1*–*3*). The CRISPR-Cas9 family of proteins has been widely deployed for gene editing applications (*4*, *5*) based on the precision of double-stranded DNA (dsDNA) cleavage induced by two catalytic domains, RuvC and HNH, at sequences complementary to a guide RNA (*6*, *7*). A second family of enzymes, CRISPR-Cas12a (Cpf1), uses a single RuvC catalytic domain for guide RNA-directed dsDNA cleavage (*8*–*13*) (Fig. 1A). Distinct from Cas9, Cas12a enzymes recognize a T-rich protospacer adjacent motif (PAM) (*8*), catalyze their own guide RNA (crRNA) maturation (*14*) and generate a PAM-distal dsDNA break with staggered 5′ and 3′ ends (*8*), features that have attracted interest for gene editing applications (*15*, *16*). However, the substrate specificity and DNA cleavage mechanism of Cas12a remain to be fully elucidated.

While investigating substrate requirements for Cas12a activation, we tested *Lachnospiraceae* bacterium ND2006 Cas12a (LbCas12a) for guide RNA-directed single-stranded DNA (ssDNA) cleavage, a capability of diverse CRISPR-Cas9 orthologs (*17*, *18*). Purified LbCas12a or *Streptococcus pyogenes* Cas9 (SpCas9) proteins (fig. S1) were assembled with guide RNA sequences targeting a circular, single-stranded M13 DNA phage. In contrast to SpCas9, we were surprised to find that LbCas12a induced rapid and complete degradation of M13 by a cleavage mechanism that could not be explained by sequence-specific DNA cutting (Fig. 1B). This ssDNA shredding activity, not observed with a catalytically inactive LbCas12a (D832A), raised the possibility that a target-bound LbCas12a could degrade any ssDNA sequence. Remarkably, LbCas12a also catalyzed M13 degradation in the presence of a different guide RNA and its complementary ssDNA "activator" that has no sequence homology to the M13 phage genome (Fig. 1C). These findings reveal that binding of the LbCas12a-crRNA complex to a guide-complementary ssDNA unleashes robust, non-specific ssDNA *trans*-cleavage activity.

We next investigated the requirements for LbCas12a-catalyzed *trans*-cleavage activity. Using a fluorophore quencher (FQ)-labeled reporter assay (*19*), we assembled LbCas12a with its crRNA and either a complementary ssDNA, dsDNA or single-stranded RNA (ssRNA), and introduced an unrelated ssDNA- or ssRNA-FQ reporter in *trans* (fig. S2). Both the crRNA-complementary ssDNA or dsDNA (the activator) triggered LbCas12a to cleave the ssDNA-FQ reporter substrate (fig. S2A). However, ssRNA was neither capable of activating *trans*-cleavage nor susceptible to degradation by LbCas12a (fig. S2B), confirming that LbCas12a harbors a DNA-activated general DNase activity.

To determine how LbCas12a-catalyzed ssDNA cleavage relates to site-specific dsDNA cutting, we tested the target

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strand (TS) and non-target strand (NTS) requirements for LbCas12a activation. Although TS cutting occurred irrespective of the NTS length (fig. S3A), NTS cleavage occurred only when the TS contained at least 15 nucleotides (nt) of complementarity with the crRNA (fig. S3B). This showed that TS recognition is a prerequisite for NTS cutting. To test whether LbCas12a remains active for non-specific ssDNA cleavage following dsDNA target cleavage, we first cut a dsDNA plasmid with LbCas12a-crRNA, and then added an unrelated dsDNA or ssDNA to the reaction (Fig. 2A). Whereas the non-specific dsDNA substrate remained intact, the ssDNA was rapidly degraded in a RuvC-domain dependent manner (Fig. 2A and figs. S4 and S5). Using truncated activators that are too short to be cleaved, we determined that only target DNA binding is required to activate *trans*-ssDNA cleavage (fig. S6). Together, these results show that RNA-guided DNA binding activates LbCas12a for both site-specific dsDNA cutting and non-specific ssDNA *trans*-cleavage.

The rapid degradation of a *trans* substrate suggested that the kinetics of LbCas12a-catalyzed site-specific dsDNA (*cis*-) cleavage and non-specific ssDNA (*trans*-) cleavage are fundamentally different. Stoichiometric titration experiments showed that *cis*-cleavage is single-turnover (*20*) (Fig. 2B), whereas *trans*-cleavage is multiple-turnover (Fig. 2C). Although the Cas12a-crRNA complex remains bound to the dsDNA target following *cis*-cleavage, the complex releases its PAM-distal cleavage products from the RuvC active site (*20*), enabling ssDNA substrate access and turnover. We found that LbCas12a-crRNA bound to a ssDNA activator molecule catalyzed *trans*-ssDNA cleavage at a rate of ~250 per second and a catalytic efficiency (k_{cat}/K_m) of 5.1×10^8 s^{-1} M^{-1}. When bound to a dsDNA activator, LbCas12a-crRNA catalyzed ~1250 turnovers per second with a catalytic efficiency approaching the rate of diffusion (*21*) with a k_{cat}/K_m of 1.7×10^9 s^{-1} M^{-1} (Fig. 2D and fig. S7). These differences suggest that the NTS of the dsDNA activator helps stabilize the Cas12a complex in an optimal conformation for *trans*-ssDNA cutting.

We next tested the specificity of *trans*-cleavage activation using either a ssDNA or dsDNA activator. We found that the PAM sequence required for dsDNA binding by CRISPR-Cas12a (*20*) is critical for catalytic activation by a crRNA-complementary dsDNA (*9*), but not for a crRNA-complementary ssDNA (Fig. 3A). Mismatches between the crRNA and activator slowed the *trans*-cleavage rate, with PAM mutations or mismatches in PAM-adjacent "seed region" inhibiting *trans*-ssDNA cleavage only for a dsDNA activator (Fig. 3B and fig. S8). Consistent with the mismatch tolerance pattern observed in Cas12a off-target studies (*22*, *23*), these data corroborate PAM-mediated dsDNA target binding and the role of base pairing between the crRNA and target strand to activate *trans*-ssDNA cutting.

We wondered if this *trans*-ssDNA cutting activity might be a property shared by type V CRISPR effector proteins, considering that all Cas12 proteins contain a single RuvC nuclease domain (*3*, *24*). Consistent with this possibility, purified Cas12a orthologs from *Acidaminococcus sp.* (AsCas12a) and *Francisella novicida* (FnCas12a), as well as a Cas12b protein from *Alicyclobacillus acidoterrestris* (AaCas12b), all catalyzed non-specific ssDNA cleavage when assembled with a crRNA and complementary ssDNA activator (Fig. 3C and fig. S9). In contrast, none of the type II CRISPR-Cas9 proteins tested showed evidence for *trans*-cleavage (Fig. 3C and fig. S9). These results reveal the unexpected functional convergence of Cas12 enzymes with the type III CRISPR-Csm/Cmr and type VI CRISPR-Cas13 effectors, which also exhibit target-activated, non-specific ssDNase or ssRNase activity, respectively (*25*, *26*).

We next explored whether LbCas12a could be repurposed as a DNA detection platform. In particular, accurate and rapid detection of human papillomavirus (HPV) is critical for identifying those at risk of HPV-related cancers, with types 16 (HPV16) and 18 (HPV18) accounting for the majority of precancerous lesions (*27*). To test if LbCas12a could distinguish between these two dsDNA viruses, we selected a target sequence next to a TTTA PAM that varied by six base pairs between the two HPV genotypes (fig. S10). HPV16- or HPV18-containing plasmids were incubated with LbCas12a-crRNA targeting either the HPV16 or HPV18 fragment and a ssDNA-FQ reporter, which produced a signal only in the presence of the cognate target (fig. S10). To enhance sensitivity, we coupled isothermal amplification by Recombinase Polymerase Amplification with LbCas12a to develop a one-pot detection method termed DNA Endonuclease Targeted CRISPR *Trans* Reporter (DETECTR) (fig. S11A). When programmed to its cognate plasmid, DETECTR identified targets with attomolar sensitivity (fig. S11B).

To assess whether we could detect HPV in complex mixtures, DNA extracted from cultured human cells infected with HPV types 16 (SiHa), 18 (HeLa), or without HPV (BJAB) was added to LbCas12a-crRNA targeting the hypervariable loop V of the L1 gene within HPV16 or HPV18 (Fig. 4A). Whereas LbCas12a-crRNA alone lacked the sensitivity to detect HPV, DETECTR unambiguously identified HPV types 16 and 18 only in SiHa and HeLa cells, respectively (Fig. 4B and fig. S12, A and B). To investigate the utility of DETECTR on patient samples, we tested crude DNA extractions from 25 human anal swabs previously analyzed by a PCR-based method for HPV infection (fig. S13) (*28*). Within one hour, DETECTR accurately identified HPV16 (25/25 agreement) and HPV18 (23/25 agreement) in patient samples containing a heterogeneous mixture of HPV types, with good correlation between the PCR-based intensity and DETECTR signal (Fig. 4, C and D, and figs. S12, C and D, and S13). These results demonstrate a new platform for CRISPR-based diagnostics, similar to that

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developed for RNA detection using CRISPR-Cas13a (*19*, *29*), and suggest that DETECTR could in principle detect any DNA sequence with high sensitivity and specificity (fig. S14).

Together, these findings support a mechanism of target interference that begins with the Cas12a-guide RNA complex binding to a complementary DNA sequence in a PAM-dependent (dsDNA) or PAM-independent (ssDNA) manner (fig. S15). Within a host bacterium, such enzyme activation could provide simultaneous protection from both dsDNA and ssDNA phages, and could also target ssDNA sequences that arise temporarily during phage replication or transcription (*30*). In a genome-editing context, target-activated ssDNA cutting by Cas12a has the potential to cleave transiently exposed ssDNA at replication forks (*31*), R-loops (*32*) and transcription bubbles (*33*), or ssDNA templates used for homology-directed repair (*34*). Finally, unleashing the ssDNase activity of Cas12 proteins offers a new strategy to improve the speed, sensitivity and specificity of molecular diagnostic applications.

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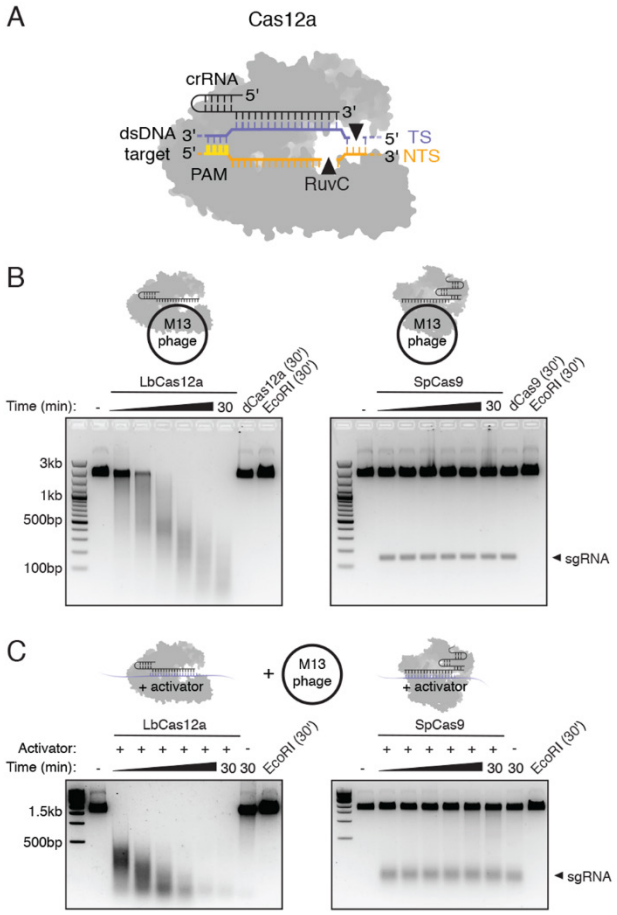
SUPPLEMENTARY MATERIALS

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Fig. 1. Cas12a target recognition activates non-specific single-stranded DNA cleavage. (**A**) Cas12a-crRNA complex binds a dsDNA substrate and generates a 5′ overhang staggered cut using a single RuvC nuclease. (**B** and **C**) Representative M13 ssDNA cleavage timecourses with purified LbCas12a (left) and SpCas9 (right) complexed with a (B) guide RNA complementary to M13 phage or (C) a guide RNA and complementary ssDNA activator with no sequence homology to M13 phage.



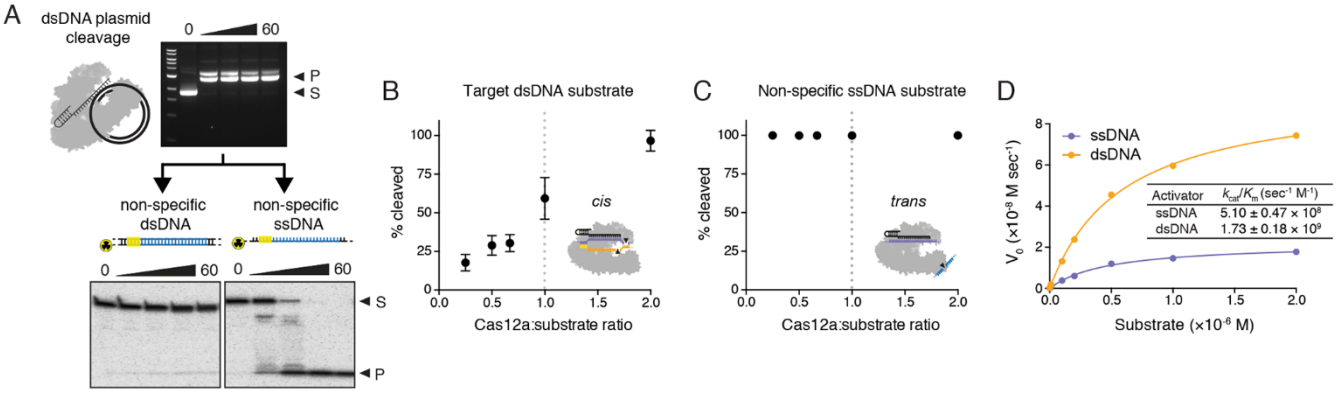


Fig. 2. Kinetics of Cas12a ssDNA *trans*-cleavage. (**A**) Sequence-specific plasmid DNA cleavage reactions by LbCas12a-crRNA (top) were introduce to a separate radiolabeled dsDNA or ssDNA substrate of unrelated sequence (bottom); timecourses represent minutes. (**B**) Target dsDNA or (**C**) non-specific ssDNA incubated with molar ratios of LbCas12a-crRNA as indicated. Each point represents the mean quantified percent cleavage after 30 min at 37°C, at which time the reaction was at completion. Error bars represent the mean ± s.d., where n = 3 replicates. (**D**) Representative Michaelis-Menten plot for LbCas12a-catalyzed ssDNA *trans*-cleavage using a dsDNA or ssDNA activator. Measured k_{cat}/K_m values report mean ± s.d., where n = 3 replicates.

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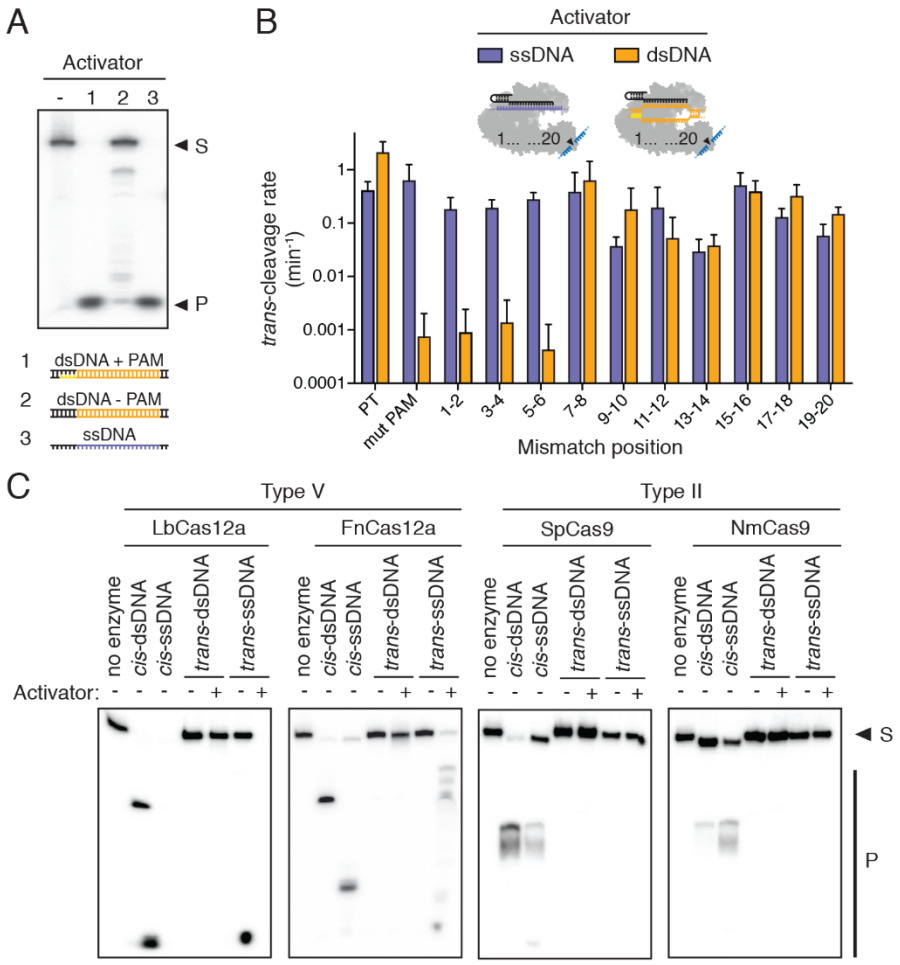
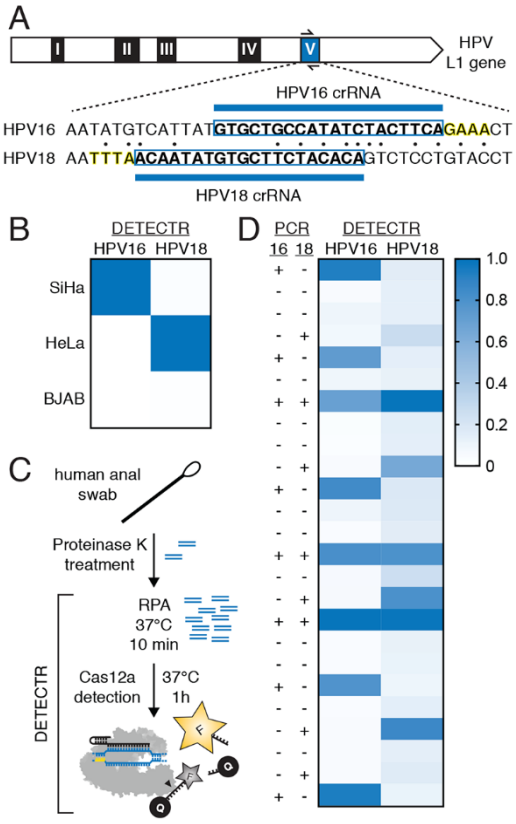


Fig. 3. Specificity and conservation of *trans*-cleavage activation. (**A**) LbCas12a-crRNA in the absence or presence of indicated activator, incubated with a radiolabeled non-specific ssDNA substrate (S) for 30 min at 37°C; products (P) resolved by denaturing PAGE. (**B**) Observed *trans*-cleavage rates for LbCas12a using a ssDNA or dsDNA activator with indicated mismatches; rates represent the average of three different targets measured in triplicate, and error bars represent mean ± s.d., where $n = 9$ (three replicates for three independent targets). (**C**) Radiolabeled *cis* (complementary) or *trans* (non-complementary) substrates were incubated with Cas12a-crRNA or Cas9-sgRNA in the presence or absence of a ssDNA activator for 30 min at 37°C; a *cis*-dsDNA substrate was used in the "no enzyme" lanes. Substrate (S) and nucleotide products (P) were resolved by denaturing PAGE.

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Fig. 4. Rapid identification of HPV types 16 and 18 in human samples by DETECTR. (**A**) Diagram of HPV16 and HPV18 sequences within the hypervariable loop V of the L1 gene targeted by Cas12a; highlighted bases indicate 5′ PAM sequence. (**B**) Heatmap represents normalized mean fluorescence values of HPV types 16 and 18 detected in human cell lines by DETECTR; normalized scale represented in (D). (**C**) Schematic outlining DNA extraction from human anal samples to HPV identification by DETECTR. (**D**) Identification of HPV types 16 and 18 in 25 patient samples by PCR (left) and DETECTR (right); DETECTR heatmap represents normalized mean fluorescence values.



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CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity

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