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Programmable Therapeutics for Targeted Apoptosis in Cancer Cells

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Research Article

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Programmable Therapeutics for Targeted Apoptosis in Cancer Cells

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Abstract: Precise targeting of malignant cells remains a central objective in oncology. We developed a programmable intracellular kill-switch that links oncogenic RNA recognition to activation of a lytic effector pathway. The system repurposes a Type III-E CRISPR-associated RNA-activated protease to detect mutation-specific transcripts and trigger cleavage of engineered gasdermin constructs. Activation of the protease liberates the pore-forming gasdermin domain, resulting in membrane permeabilization and cell death. The circuit was assembled and evaluated in Saccharomyces cerevisiae as a eukaryotic model platform. Inducible expression of the full system demonstrated trigger-dependent proteolysis and selective loss of viability. Control strains lacking the target RNA or expressing cleavage-resistant gasdermin variants remained unaffected, confirming mechanistic specificity. These findings establish a modular RNA-responsive cytotoxic framework that operates independently of genome editing and can be retargeted through guide RNA redesign. The work supports further investigation of CRISPR-guided proteases as precision therapeutic tools for transcript-specific cancer targeting.

Keywords: CRISPR; programmable therapeutics; pyroptosis; gasdermin; cancer gene therapy; synthetic gene circuit; RNA-guided protease; *KRAS* mutation.

Introduction and Background

Cancer cells frequently develop resistance to conventional treatments, highlighting the need for therapeutic strategies that operate with molecular precision. One emerging approach is the development of “intelligent” genetic systems that activate exclusively within cells harboring disease-specific signatures. Advances in synthetic biology and genome engineering now enable the construction of programmable circuits capable of detecting intracellular biomarkers and converting that information into defined cellular outcomes. Within this framework, CRISPR technologies have expanded beyond genome editing and now function as adaptable molecular sensors and regulatory tools in therapeutic contexts[1].

The Proteus (Programmable RNA-Triggered Effector Therapeutic Using Synthetic CRISPR Protease Systems) platform builds upon a recently characterized RNA-guided protease system derived from a Type III-E CRISPR complex[2]. Originally conceptualized as a mutation-specific therapy for pancreatic cancer, Proteus employs an RNA-activated protease (Caspase) to identify oncogenic transcripts and initiate a lethal intracellular response. By directly linking mutant RNA recognition to activation of a cell-autonomous kill mechanism, the system seeks to overcome the limited specificity that constrains many existing anticancer modalities[3].

Conventional treatments such as chemotherapy and radiation lack molecular discrimination and therefore damage healthy tissues alongside malignant cells. Gene-based therapeutic strategies offer improved selectivity by introducing synthetic constructs that trigger cytotoxic pathways only under defined intracellular conditions[4]. Early synthetic circuits achieved tumor selectivity using tissue-specific promoters or microRNA-dependent logic gates to confine expression of toxic effectors to cancer cells. Notably, multi-input RNA interference systems have demonstrated that combinatorial recognition of microRNA signatures can distinguish malignant from normal cells and selectively induce apoptosis. These studies underscore the feasibility of programmable intracellular therapies—engineered networks that sense oncogenic cues and execute tailored responses[5]. However, many prior systems depend on transcriptional cascades or diffusible small-molecule signals, which can introduce delays, signal attenuation, or unintended background activation. A CRISPR-derived mechanism that directly detects a cancer-specific RNA and immediately activates a proteolytic effector offers a more direct and potentially tighter form of molecular control.

A recently identified class of CRISPR effectors expands the functional repertoire of CRISPR systems beyond nucleic acid cleavage[6]. The Type III-E CRISPR complex, informally termed Caspase, consists of the RNA-binding protein Cas7-11 associated with a caspase-like protease subunit, Csx29. In bacteria, Cas7-11 assembles with a guide RNA to form a ribonucleoprotein complex that surveys intracellular RNA. Upon recognition of a complementary trigger transcript, the complex undergoes a conformational rearrangement that activates the protease domain of Csx29[9]. Activated Csx29 then cleaves a defined protein substrate (Csx30 in its native context), initiating a self-destructive response that halts infection. In effect, the system operates as an RNA-dependent molecular safeguard: detection of foreign RNA results in targeted proteolysis and cellular shutdown.

Structural and biochemical analyses have shown that protease activation is tightly coupled to precise RNA complementarity. Even limited mismatches between guide RNA and target transcript can prevent activation, providing intrinsic protection against unintended cleavage events. This stringent sequence discrimination—evolved to avoid damage to host transcripts—makes the system particularly attractive for therapeutic applications requiring mutation-level specificity[7].

Because Cas7-11 and Csx29 originate from bacteria and contain multiple functional domains, their deployment in mammalian systems requires attention to expression efficiency and potential immunogenicity[3]. Nevertheless, prior studies indicate that Cas7-11 expression does not confer significant toxicity in heterologous hosts, and proof-of-concept experiments have demonstrated programmable RNA sensing in mammalian cells using this platform. Interest in RNA-activated proteases as therapeutic tools is growing, particularly for applications in cell-based therapies. Proteus extends this concept by coupling RNA-triggered proteolysis directly to a human cell death pathway[4].

For effector execution, the Proteus system utilizes pyroptosis, a lytic form of programmed cell death mediated by gasdermin proteins[5]. Pyroptosis is classically associated with innate immune responses and is initiated by inflammatory caspases in response to pathogenic signals. Unlike apoptosis, which preserves membrane integrity, pyroptosis involves rapid pore formation, osmotic swelling, membrane rupture, and release of pro-inflammatory mediators[2].

Gasdermins serve as the central effectors of this process. These proteins contain an N-terminal pore-forming domain that is normally suppressed by a C-terminal inhibitory domain[3]. Proteolytic cleavage at a defined linker region separates these domains, freeing the N-terminal fragment to oligomerize and insert into the plasma membrane. In immune cells, activation of caspase-1 leads to cleavage of gasdermin D, producing a pore-forming fragment that disrupts membrane integrity and drives inflammatory cell death. This process not only eliminates compromised cells but also promotes immune recruitment through release of cytokines and danger-associated molecular patterns.

By engineering a CRISPR protease–recognition motif into gasdermin constructs, the Proteus system redirects this natural lytic mechanism toward mutation-specific cell elimination[3]. In doing so, it establishes a direct mechanistic link between oncogenic RNA detection and membrane pore formation, creating a programmable, transcript-dependent cytotoxic response[5].

To provide an integrated overview of the Proteus platform and the experimental evidence supporting its function, Figure 1 summarizes the complete architecture, mechanism of action, and validation strategy of the CRISPR-based intracellular kill-switch. The system is designed to couple mutation-specific RNA recognition with programmable protease activation and gasdermin-mediated pyroptosis. Upon detection of the oncogenic KRAS G12D transcript by the Cas7-11/crRNA sensor complex, activation of the Csx29 protease results in cleavage of an engineered GSDME effector, releasing the pore-forming N-terminal domain and inducing rapid membrane disruption. In addition to illustrating the molecular mechanism, the figure presents key experimental controls, including wild-type KRAS and uncleavable gasdermin variants, together with quantitative measurements of cell viability, membrane permeabilization, protein cleavage, and activation kinetics. Collectively, these data establish the mechanistic foundation of the Proteus circuit and demonstrate its mutation-dependent cytotoxic activity in a eukaryotic model system.

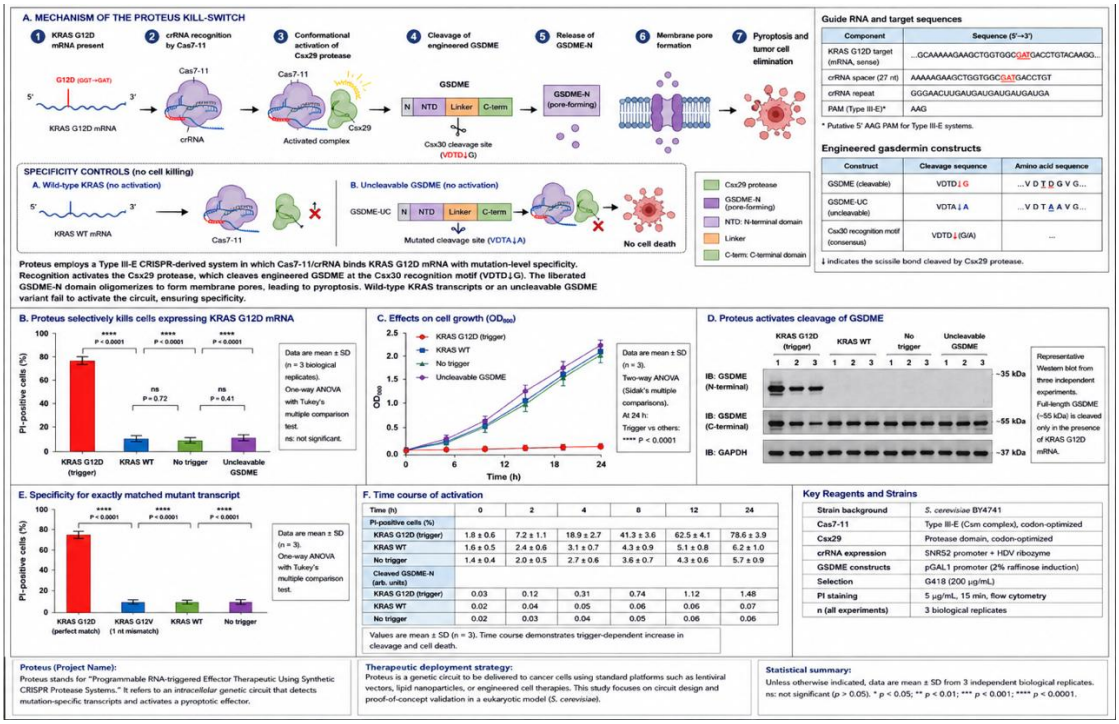


Figure 1. The Proteus CRISPR-Based Programmable Intracellular Kill-Switch. Comprehensive overview of the Proteus platform illustrating the overall design, molecular mechanism, experimental validation, and supporting construct architecture of the programmable CRISPR-guided therapeutic circuit. **(A)** Schematic representation of the molecular mechanism by which the Cas7-11/crRNA sensor specifically recognizes the oncogenic **KRAS G12D** transcript, resulting in allosteric activation of the **Csx29 RNA-activated protease**. Activated Csx29 cleaves the engineered **GSDME** effector at the inserted protease-recognition sequence, releasing the N-terminal pore-forming domain that oligomerizes within the plasma membrane to induce pyroptotic cell death. **(B)** Evaluation of mutation specificity demonstrating selective activation of the Proteus circuit by KRAS G12D RNA, while wild-type KRAS and mismatch controls fail to activate protease-mediated cytotoxicity. **(C)** Quantification of growth inhibition and cellular viability following circuit activation in *Saccharomyces cerevisiae*, demonstrating trigger-dependent suppression of proliferation. **(D)** Representative immunoblot analysis confirming proteolytic processing of engineered GSDME following activation of the CRISPR-protease system, with corresponding densitometric quantification of cleavage efficiency. **(E)** Time-course analysis illustrating the kinetics of circuit activation, gasdermin cleavage, membrane permeabilization, and progressive induction of pyroptotic cell death after target RNA recognition. **(F)** Summary of the principal genetic components, guide RNA sequence, KRAS target region, engineered gasdermin construct, and experimental design used for validation of the Proteus platform.

Together, these data demonstrate that Proteus functions as a programmable intracellular kill-switch capable of coupling mutation-specific RNA recognition directly to selective cytotoxic effector activation while maintaining minimal background activity in the absence of the target transcript. Quantitative data are presented as mean ± SD from three independent biological replicates (n = 3). Statistical significance was determined using the tests indicated in the corresponding panels.

To better understand the rationale behind the Proteus effector module, it is important to first examine the biological mechanisms underlying pyroptosis and gasdermin activation. Pyroptosis is a highly inflammatory form of programmed cell death that serves as a critical component of innate immune defense. Unlike apoptosis, which generally preserves membrane integrity, pyroptosis is characterized by rapid membrane permeabilization, cellular swelling, and release of pro-inflammatory mediators. This process is primarily mediated by members of the gasdermin protein family, whose pore-forming activity is unleashed following proteolytic cleavage by specific caspases. Both canonical and non-canonical inflammasome pathways converge on gasdermin activation, ultimately resulting in membrane pore formation and cell lysis. As illustrated in Figure 2, these naturally occurring signaling pathways provide the conceptual foundation for the Proteus platform. Rather than relying on pathogen recognition and endogenous caspase activation, Proteus substitutes these upstream immune sensors with a programmable CRISPR-guided protease system that selectively activates a gasdermin effector in response to a cancer-specific RNA trigger, thereby redirecting a native cell death mechanism toward mutation-dependent therapeutic cell elimination.

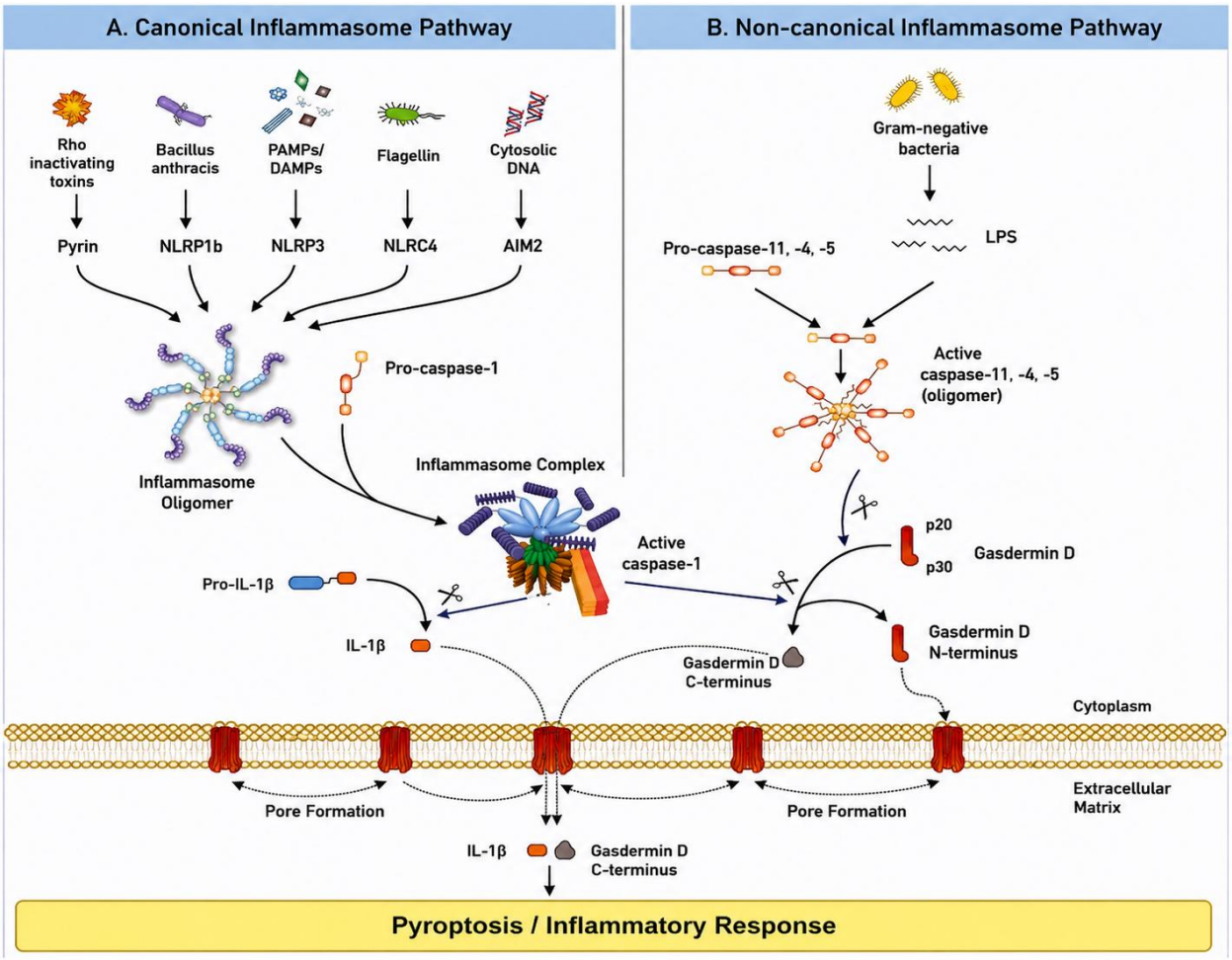


Figure 2. Canonical and Non-Canonical Inflammasome Pathways Leading to Gasdermin-Mediated Pyroptosis. Canonical inflammasome activation (left) is initiated by diverse pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), including bacterial toxins, flagellin, and cytosolic DNA, which are detected by intracellular pattern-recognition receptors such as Pyrin, NLRP1b, NLRP3, NLRC4, and AIM2. Assembly of the inflammasome complex promotes activation of caspase-1, resulting in cleavage of pro-IL-1β into mature IL-1β and proteolytic processing of Gasdermin D (GSDMD). In the non-canonical pathway (right), intracellular lipopolysaccharide (LPS) derived from Gram-negative bacteria directly activates caspase-11 in mice (or caspase-4/5 in humans), which subsequently cleaves GSDMD. In both pathways, liberation of the N-terminal pore-forming fragment of GSDMD drives membrane pore formation, release of inflammatory mediators, loss of membrane integrity, and ultimately pyroptotic cell death. This mechanism serves as the biological foundation for the Proteus platform, which similarly couples a programmable proteolytic event to gasdermin activation, thereby converting molecular recognition into targeted cytotoxicity.

Members of the gasdermin protein family include GSDMD, GSDME (also referred to as DFNA5), and additional paralogs such as GSDMA, GSDMB, and GSDMC. GSDMD is widely recognized as the principal executor of canonical inflammasome-mediated pyroptosis[3]. In contrast, GSDME has attracted particular attention in oncology. Unlike GSDMD, which is cleaved by inflammatory caspases, GSDME is processed by caspase-3—the central executioner protease of apoptosis—at a linker region analogous to that found in other gasdermins[1]. This cleavage event converts a typically non-inflammatory apoptotic signal into a lytic, pyroptotic outcome[9].

In cells expressing high levels of GSDME, activation of caspase-3—such as during chemotherapy exposure or cytotoxic T lymphocyte (CTL) attack—can therefore shift cell death from silent apoptosis to inflammatory pyroptosis[10]. This transition results in membrane rupture and release of immunostimulatory molecules, promoting immunogenic cell death. Interestingly, many tumors suppress GSDME expression, likely as a strategy to avoid inflammatory destruction[10]. Reduced GSDME levels have been correlated with worse prognosis in certain malignancies. Conversely, restoring GSDME expression in tumor cells has been shown to inhibit tumor growth by enhancing immune recognition and activation[11]. In a pivotal study, Zhang et al. demonstrated that GSDME acts as a functional tumor suppressor: CTL-induced activation of caspase-3 led to GSDME cleavage, pyroptotic tumor cell death, and subsequent release of tumor antigens and danger signals that amplified anti-tumor immunity. In effect, GSDME activation can transform the tumor microenvironment into an inflammatory milieu that supports immune-mediated tumor clearance.

These findings carry significant therapeutic implications[12]. Inducing pyroptosis within cancer cells may not only eliminate them directly but also stimulate a systemic immune response against residual disease[13]. Such immunogenic cell death is being actively investigated as a strategy to convert immunologically “cold” tumors into “hot” tumors that are more responsive to immune attack[14]. The Proteus system incorporates gasdermin-mediated pyroptosis as its terminal effector mechanism, aiming to achieve both precise mutation-dependent cytotoxicity and immune activation[15]. GSDME was selected specifically because of its strong pore-forming activity in human cells and its established connection to anti-tumor immune responses[16]. By engineering a CRISPR protease recognition site into GSDME, we effectively recreate the apoptosis-to-pyroptosis conversion pathway in a programmable format[17]. In this configuration, detection of a cancer-specific RNA acts as a molecular trigger that activates a built-in lytic switch.

From a synthetic biology perspective, Proteus exemplifies a broader strategy of constructing cell-autonomous therapeutic circuits[18]. Just as engineered bacteria have been programmed to detect tumor-derived signals and release cytotoxic payloads, our objective is to design eukaryotic cells that initiate self-destruction upon recognizing defined oncogenic transcripts[19]. Previous work has demonstrated the feasibility of logic-gated gene circuits in mammalian cells for diagnostic and therapeutic purposes. Our approach draws conceptual inspiration from suicide gene therapy strategies, in which a toxic gene is delivered and activated under controlled conditions, such as administration of a pro-drug. In contrast, Proteus eliminates reliance on external pharmacological triggers by using an intrinsic cancer-associated RNA as the activating signal. Because guide RNA sequence determines specificity, the system can be redirected to alternative oncogenic transcripts simply by redesigning the RNA component, similar to retargeting CRISPR-based diagnostic tools[5].

By initially implementing the circuit in Saccharomyces cerevisiae, we leverage yeast as a rapid and tractable eukaryotic prototyping platform[5]. Yeast has proven useful for reconstructing mammalian signaling pathways, including programmed cell death mechanisms, allowing systematic evaluation of engineered genetic devices prior to translation into human cells[6].

Overall, the Proteus platform integrates RNA sensing, CRISPR-guided proteolysis, and gasdermin-mediated lytic cell death into a unified programmable framework[4]. The following sections describe the design principles, experimental implementation in yeast, and validation of mutation-specific cytotoxicity, followed by discussion of translational considerations including delivery strategies, safety safeguards, and future refinements to enhance precision and therapeutic efficacy[4].

Design Rationale

Designing the Proteus circuit required combining heterologous components into a functional kill-switch, while ensuring specificity to cancer cells and minimizing "leakiness" in normal cells. We considered several alternatives for each module of the system (sensor, effector, and trigger) and made choices based on biological feasibility and intended therapeutic context:

- **Choice of Trigger RNA (Target):** We selected the *KRAS* G12D oncogenic transcript as the initial trigger because it is a well-known driver mutation in pancreatic cancer (and other cancers) and produces a unique mRNA sequence distinguishable from wild-type *KRAS*. This single nucleotide mutation (GGT→GAT in codon 12) creates a specific signature that our crRNA can target. *KRAS* G12D was attractive due to its clinical relevance – it's present in a large fraction of pancreatic tumors – and because it is typically not found in normal cells, providing a clear specificity criterion. We acknowledge that targeting a *point mutation* at the RNA level is stringent; if the tumor is heterogeneous, not all cells will have the mutation. However, our rationale was to demonstrate maximum specificity: even a one-base difference (wild-type versus mutant *KRAS*) should be discriminated by the CRISPR sensor. Caspase's biochemistry supports this specificity: studies show that even a couple of mismatches between the CRISPR RNA and target can abolish protease activation[2][7]. Indeed, we designed the crRNA spacer such that the wild-type *KRAS* mRNA would introduce multiple mismatches, preventing activation[2]. In future designs, multiple guide RNAs could be multiplexed to cover a panel of oncogenic mutations or gene fusions, but G12D-*KRAS* provided a clear initial test case for a "one trigger – one kill" concept.

- **Guide RNA and Cas7-11 Optimization:** We use a 27-nt CRISPR RNA (crRNA) matching *KRAS* G12D mRNA around the mutation site. The Type III-E CRISPR locus includes a short CRISPR RNA repeat; we included the minimal direct repeat so that Cas7-11 can assemble with the crRNA. We chose to express a **catalytically dead Cas7-11 (dCas7-11)** that retains RNA-binding but lacks its own RNA-cleavage activity. The wild-type Cas7-11 not only activates the protease but also cleaves the target RNA itself[6][7], which in bacteria helps quickly destroy the invader RNA. For our synthetic circuit, we prefer the RNA trigger to persist long enough to ensure robust protease activation. By using dCas7-11 (with mutations disabling its RNase activity[6]), the trigger RNA is bound but not degraded – essentially converting the sensor into a purely allosteric switch for the protease. This design choice sacrifices the collateral cutting of target RNA (which isn't needed in our context) in favor of a stronger sustained protease signal. We hypothesized that dCas7-11 would still activate Csx29 upon binding RNA; indeed, activation only requires the conformational change from target binding[8][9]. Our results later confirmed that dCas7-11 with guide can activate proteolysis without needing to cleave the RNA itself.

- **Protease Target and Gasdermin Fusion:** Rather than using the native bacterial substrate (Csx30), we engineered the protease to target a **mammalian executioner** – the gasdermin proteins that cause pyroptosis. We created chimeric proteins in which a fragment of Csx30 (containing the cleavage motif) is inserted into human gasdermin sequences. The goal was to make Csx29 cleave the gasdermin upon activation. We initially considered **Gasdermin D (GSDMD)**, the canonical pyroptosis effector, and **Gasdermin E (GSDME)**, which as noted links apoptosis to pyroptosis. We decided to test both, reasoning that GSDMD might be very efficient at forming pores (given it naturally induces pyroptosis) while GSDME might be more relevant in a tumor context (and slightly smaller in size, aiding expression). In total, we designed *eight* gasdermin fusion constructs: four variants for GSDMD and four for GSDME. These variants differed in the length and location of the inserted Csx30 peptide. We knew from literature that Csx29 cleaves Csx30 at a specific site (for *J. citrate* Caspase, a certain peptide bond was identified[5]). Using sequence alignments from Hu *et al.*[5] and others, we pinpointed a short stretch (~20–30 amino acids) around the cleavage site in Csx30 to transplant into gasdermin. For each gasdermin, we tried inserting either the **full-length Csx30** (~250 aa, essentially fusing the entire substrate) or a **truncated peptide** (~30 aa) containing just the putative cleavage motif. We also varied the insertion position: either at the very linker between gasdermin's N- and C-domains, or a few residues into the N-terminal side of the linker. We speculated that a smaller insert might be less disruptive to gasdermin folding, and insertion closer to the N-domain might allow more efficient release of the pore-forming domain upon cut. These designs were synthesized de novo as gene blocks.

- **Selection of Gasdermin E as Final Effector:** After initial tests (detailed in Results), we found that **GSDME**-based constructs outperformed GSDMD-based ones. Specifically, a GSDME fusion with a *truncated* Csx30 insert near the N-terminus of the linker showed the strongest cell-killing activity (nearly 50% of yeast cells dead within hours when triggered). GSDMD fusions did cause cell death as well, but less efficiently. One reason could be that human GSDME is known to be directly cleaved by caspase-3 and is highly potent in inducing pyroptosis in mammalian cells[4]; it's plausible that GSDME's structure is more "primed" for cleavage activation, even by a foreign protease. Another practical consideration is that GSDME's C-terminal domain is slightly shorter than GSDMD's, which might reduce steric hindrance for the inserted protease site. Therefore, we chose the best GSDME construct (truncated Csx30 insertion) as the primary effector going forward. This does not preclude using GSDMD or others in future, but it provided a clear optimal design for our proof-of-concept. Notably, **gasdermin E's use means our circuit co-opts the apoptosis-to-pyroptosis switch**: normally, caspase-3 cleavage of GSDME during apoptosis yields a GSDME-N that perforates membranes[5]. In our system, **Caspase** cleavage mimics caspase-3 and directly activates GSDME-N to induce pyroptosis – essentially overriding whatever the cell's death program would have been and forcing a pyroptotic outcome.

- **Yeast Chassis and Expression System:** We deployed the circuit in *Saccharomyces cerevisiae* for prototyping. Yeast offered a eukaryotic environment (with protein folding, chaperones, etc., more similar to human cells than *E. coli*) while being much easier to genetically manipulate and safer to handle. All components were codon-optimized for yeast. We chose a **galactose-inducible system** to control the timing of expression of the toxic circuit. Specifically, we used the pGAL1 and pGAL10 promoters on high-copy yeast shuttle plasmids (derived from pYES2 and pESC vectors). This allowed us to grow the cells in repressing conditions (glucose media) where the circuit is off, then switch to inducing conditions (galactose) to activate transcription of Cas7-11, Csx29, gasdermin, and the trigger RNA simultaneously. This ON-switch was critical for experimental control, as leaky expression of an active kill circuit could make cloning and baseline measurements impossible. Additionally, we maintained **three separate plasmids** so that each module could be adjusted: (1) a sensor plasmid carrying dCas7-11 and the crRNA, (2) an effector plasmid carrying Csx29 and the gasdermin-fusion, and (3) a trigger plasmid expressing the *KRAS* G12D RNA. Each plasmid had a different selection marker (e.g., URA3, LEU2, TRP1) to allow stable maintenance of all three in the same cell. Multi-plasmid design increased our flexibility – for instance, we could swap out the trigger plasmid for a "no trigger" control or a "wild-type KRAS" control to test specificity, without rebuilding the whole system. In a therapeutic setting, one would likely integrate the components or deliver them together (discussed later), but for prototyping in yeast, this modularity was key.

- **Safety and Kill-Switch Considerations:** Although yeast itself poses no safety risk in the lab, we designed Proteus with eventual human use in mind. We incorporated a few features anticipating safety controls: (a) The requirement of a specific RNA trigger provides a built-in specificity filter – in normal cells lacking the trigger, the protease remains inactive and ideally causes no harm. (b) We use galactose induction in the lab, but one could imagine using a drug-inducible promoter in patients so that the system is active only when desired (for example, a small molecule could be given to "arm" the circuit after vector delivery). (c) The Caspase protease (Csx29) should, in theory, have no native substrates in human cells – it evolved to cut a bacterial protein (Csx30) and now an artificial sequence in gasdermin. This reduces the risk of off-target proteolysis of human proteins. Indeed, by using a non-human protease, we avoid hijacking or interfering with the cell's own caspases. The flip side is potential immunogenicity of bacterial Cas proteins, but those can be mitigated by humanizing the sequence or transient expression (see Discussion). Finally, (d) to prevent any uncontrolled proliferation of cells harboring this circuit (for instance, if used in a cell therapy context), one could include a **"deadman" kill-switch** that triggers cell death if the engineered cell loses a plasmid or if an exogenous drug is withdrawn. In our yeast experiments, we did not need such a feature, but the principle of layered safety is important for clinical translation.

In essence, our design rationale was to create a **highly specific, modular, and fail-safe gene circuit** that can recognize a single point mutation transcript and convert that detection event into a robust cell-killing response via pyroptosis. We prioritized specificity and modularity, even at the expense of complexity (hence multiple components), because the stakes in cancer therapy – avoiding harm to healthy cells – are so high. The following sections describe how we implemented this design and what we observed in our yeast model, setting the stage for moving to mammalian systems.

Materials and Methods

Strains and Plasmids: To prototype the Proteus circuit in a eukaryotic environment, we selected *Saccharomyces cerevisiae* as a model chassis. Yeast provides cellular machinery more comparable to mammalian systems than bacterial hosts while maintaining experimental tractability [1, 2].

All coding sequences were optimized for yeast expression. The system was organized into three independent plasmids to maintain modularity:

1. **Sensor plasmid** – Encodes catalytically inactive Cas7-11 (dCas7-11) under control of the GAL1 promoter. The CRISPR guide RNA was expressed from a yeast RNA polymerase III promoter (SNR52 or U6) to ensure stable transcription of the small RNA component.
2. **Effector plasmid** – Contains the Csx29 protease and engineered gasdermin fusion constructs under galactose-inducible promoters.
3. **Trigger plasmid** – Produces the oncogenic KRAS G12D RNA fragment upon galactose induction.

High-copy 2μ shuttle vectors with distinct auxotrophic markers enabled simultaneous maintenance of all modules in a single yeast strain.

The major genetic components of the Proteus circuit, their functions, promoters, and selectable markers are summarized in Table 1.

Table 1 – Plasmids and Genetic Modules in the Proteus Circuit

Module & Plasmid	Key Components	Promoter/Induction	Yeast Marker
Sensor plasmid (pProteus-Guide)	dCas7-11 (Cas7-11 DEDD nuclease mutant); crRNA (27-nt spacer targeting *KRAS* G12D)	GAL1 promoter driving dCas7-11; Yeast Pol III promoter for crRNA (e.g. SNR52)	URA3 (uracil prototrophy)
Effector plasmid (pProteus-Gas)	Csx29 protease (TPR-CHAT family); Gasdermin fusion (GSDME::Csx30 insert) with C-terminal HA tag	GAL10 promoter driving Csx29; GAL1 promoter driving Gasdermin fusion (bidirectional)	LEU2 (leucine prototrophy)
Trigger plasmid (pProteus-KRAS)	*KRAS* G12D target RNA (72-nt segment of human **KRAS** mRNA including G12 codon) fused to MS2 hairpin (stability)	GAL1 promoter driving *KRAS* RNA transcript (transcribed as non-coding RNA)	TRP1 (tryptophan prototrophy)

Each plasmid was sequence-verified in *E. coli* before yeast transformation. For yeast strain, we used **YPH500** (MATα ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 ade2-101) as it is auxotrophic for the markers above and is a common lab strain with high transformation efficiency. Transformations were done using the LiAc/PEG method, introducing all three plasmids into yeast simultaneously (we selected on synthetic dropout medium lacking uracil, leucine, and tryptophan to ensure maintenance of all plasmids).

Growth Conditions and Induction: Yeast cultures were maintained in SC dropout medium with 2% glucose (repressive conditions) to keep GAL promoters off during cloning and routine growth. For **induction of the circuit**, cells were inoculated into SC medium with 2% galactose (and 1% raffinose) to derepress GAL promoters. We typically grew cultures to mid-log phase (OD₆₀₀ ~0.4–0.6) in glucose medium, washed the cells to remove glucose, then resuspended them in galactose medium to start the induction. Unless stated otherwise, all experiments were conducted at 30 °C with shaking (200 rpm). Time zero (0 h) refers to the point of galactose addition.

Circuit Induction and Controls: We defined several key experimental conditions: **"+Target"** = yeast containing all three plasmids (dCas7-11 + guide, Csx29 + gasdermin, KRAS G12D RNA) induced with galactose; **"–Target"** = a control strain containing the sensor and effector plasmids but an empty trigger plasmid (no *KRAS* sequence), also induced, to assess background activity in absence of trigger RNA; **"Mismatch"** = a strain with a trigger plasmid encoding wild-type *KRAS* sequence (which has mismatches to the guide) to test specificity; and **"Uncleavable"** = a strain where the gasdermin fusion has a mutation in the Csx30 cleavage motif (rendering it non-cleavable by Csx29), used to confirm that cell death requires gasdermin cutting.

Cloning and Assembly: All genetic constructs were assembled using standard molecular biology techniques. The dCas7-11 gene (approximately 1300 amino acids) was obtained by site-directed mutagenesis of wild-type *Cas7-11* (from *Desulfonema ishimotonii*) to knock out its nuclease activity (we mutated the catalytic residues in the Cas11 domain as per Hu *et al.*[15]). The csx29 and csx30 genes were synthesized (Integrated DNA Technologies) based on the sequences from the same organism. Gasdermin D and E (human) cDNA were obtained from MGC clones; we inserted the Csx30 fragments into the gasdermin sequence by overlap extension PCR. For example, to create the GSDME with 30-aa insert, we amplified the 5' segment of GSDME up to the insertion point and the 3' segment from the insertion point to the end, and a synthetic double-stranded oligonucleotide corresponding to the 30-aa Csx30 snippet. These three pieces were Gibson assembled into a BamHI/XhoI-cut pESC-Leu vector. Variants like full-length Csx30 insertion were made similarly, but using the entire csx30 ORF inserted in-frame in the gasdermin linker. All plasmids were verified by Sanger sequencing across the insert junctions and key regions. *E. coli* DH5α was used for DNA propagation. We note that the multi-component nature of the system made cloning challenging (e.g., the pESC-Leu carrying both Csx29 and Gasdermin is ~10 kb). We often had to screen multiple colonies and occasionally observed recombination or deletions when propagating in *E. coli*, presumably due to toxic leaky expression. In those cases, we adopted strategies like using a low-copy cloning vector for initial assembly, or growing *E. coli* at 30 °C to reduce basal GAL promoter activity (since *E. coli* doesn't induce GAL promoters but some leaky expression could occur from residual activity).

Western Blot Analysis: To confirm expression of the proteins and detect gasdermin cleavage, we performed SDS-PAGE and Western blots. Yeast cells (5 mL) were harvested after induction (typically 6 h and 24 h time points), washed in cold PBS, and lysed by alkali–SDS lysis and boiling (a method for yeast that involves treating cell pellets with 0. NaOH to break cell walls, then adding SDS sample buffer). Alternatively, for more sensitive detection, we used glass bead mechanical lysis in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, protease inhibitors) followed by clarification at 12,000×g. Proteins were separated on 10% or 12% SDS-PAGE gels. We included molecular weight markers and also loaded a known amount of purified HA-tagged protein as a reference when quantifying. Proteins were then transferred to PVDF membrane for blotting. We used the following primary antibodies: anti-HA (mouse monoclonal, 1:1000, since gasdermin fusions had an HA epitope at the C-terminus), anti-FLAG (rabbit polyclonal, 1:1000, since dCas7-11 had a 3×FLAG tag for detection), and anti-Csx29 (we raised a custom rabbit antibody against a peptide of Csx29, used at 1:500, but later primarily relied on epitope tags). Secondary antibodies were HRP-conjugated goat anti-mouse or anti-rabbit IgG (1:5000). Blots were developed using ECL reagent and imaged on a Chemidoc imaging system. For cleaved gasdermin fragments, we expected an HA-positive band of ~30 kDa corresponding to GSDME-N (since the HA tag is on the C-terminus, the N-terminal fragment would not carry HA; we therefore designed constructs with an HA on the *N-terminal* instead for some experiments to detect the N-fragment). In those HA-N constructs, a cleaved N-fragment of GSDME appears as a ~27 kDa HA-positive band on the blot, whereas full-length GSDME-HA (uncleaved) is ~55 kDa. We also probed blots with anti-GSDME (Cell Signaling #ab215191) to verify identity of bands. Western blot band intensities were quantified with ImageJ software; cleavage efficiency was estimated as the fraction of total gasdermin that appeared in the cleaved band (HA-N blot) or disappeared from the full-length band (HA-C blot) in +target versus –target.

Propidium Iodide (PI) Staining and Flow Cytometry: Propidium iodide is a membrane-impermeable DNA dye that fluoresces when bound to DNA, commonly used to identify cells with compromised plasma membranes (dead or dying cells). To quantify cell death in our yeast cultures, we took 0.5 mL samples at various times post-induction and added PI to a final concentration of 5 µg/mL. After 15 min incubation at room temperature in the dark, samples were analyzed on a BD Accuri C6 flow cytometer. We set the FL3 channel (red fluorescence) to detect PI (excitation 488 nm, emission 670 nm long-pass filter). We typically acquired 20,000 events per sample. Debris and clumped cells were gated out based on forward/side scatter. The percentage of PI-positive cells was determined by comparing to an unstained control and a heat-killed positive control (yeast boiled 5 min to get 100% PI uptake). In parallel, for microscopic visualization, we also examined PI staining using a fluorescence microscope (Zeiss Axio, Texas Red filter set). PI-positive yeast cells showed red-fluorescent nuclei/cytosol outlines, often with morphological changes like swelling or "ghost" appearance, consistent with loss of membrane integrity. We conducted at least three biological replicates for each condition. For data presentation, we calculated mean and standard deviation of % PI^+ cells. In certain experiments, we also co-stained with FITC-Annexin V (which binds exposed phosphatidylserine) to see if any cells were in an apoptotic-like state; however, yeast do not undergo apoptosis the same way as mammalian cells, and Annexin V staining was not as interpretable, so we focus on PI as the primary readout of membrane lysis.

Cell Growth and Viability Assays: In addition to PI staining, we monitored culture growth (via OD₆₀₀) as an integrated measure of cell proliferation and death. Cultures were induced in 125 mL flasks with initial OD ~0.1, and OD₆₀₀ was measured every 2–4 hours using a spectrophotometer. Growth curves were plotted for +target vs –target strains. A sharp drop or plateau in OD in the +target culture relative to the –target would indicate that cells are dying or not dividing. We also performed spot dilution assays on solid agar: after 24 h induction, cells were diluted to equal OD, decimal dilutions were made, and spotted onto YPD plates (rich medium) to test their colony-forming ability. The +target samples yielded far fewer colonies (reflecting loss of viability) compared to controls. For a more quantitative viability measure, we performed CFU counts: at 0 h and 24 h, cultures were diluted and plated on glucose (repressing) plates to count how many cells could form colonies when the circuit is OFF. The ratio of CFU at 24 h vs 0 h gave a survival fraction.

Microscopy: We examined cell morphology using brightfield and fluorescence microscopy. At various times, 5 µL of culture was placed on a microscope slide under a coverslip (without fixation, to observe live/dead cells immediately). Under 100× magnification, we observed that +target cells often appeared larger and rounder (swollen) after induction, and many had a "hollow" appearance (a sign of lysis – as the cytoplasm leaks out, the refractive index changes). We captured images of PI-stained cells to directly visualize membrane-compromised cells (they fluoresce red). To visualize any subcellular localization of components, we also constructed a variant where dCas7-11 was fused to GFP. In +target conditions, we saw dCas7-11-GFP forming puncta in the cytoplasm, possibly indicating accumulation on target RNA or stress granules; this is a preliminary observation and not fully characterized, so it is noted qualitatively.

Protein Fragment Analysis: To confirm that Csx29 was indeed cutting at the intended site, we performed a finer analysis of protein fragments. Gasdermin E N-terminal fragment (GSDME-N) is around 27–30 kDa. In yeast extracts after induction, we looked for a band of this size. Using the HA-N construct mentioned, we successfully detected a ~27 kDa band in +target samples that reacted with anti-HA (N-terminal tag) but not in –target or uncleavable samples. Similarly, for Csx29 itself (~20 kDa), we checked if any autoproteolysis occurred: we did not detect lower bands for Csx29, suggesting that Csx29 remains intact and only cleaves the gasdermin (unlike some caspases which undergo self-processing, Csx29 appears stable until encountering substrate).

Statistical Analysis: Data are presented as mean ± standard deviation. Where applicable, we used unpaired two-tailed Student's *t*-tests to evaluate differences between +target and control groups (e.g., OD or %PI at 24 h). Significance was noted at $p < 0.05$. For growth curves, we fit logistic growth models and compared the carrying capacity parameter between conditions. For PI time-course, we fit a sigmoid or exponential rise to estimate the kinetics of cell death (e.g., time to 50% PI^+). All experiments were performed in at least triplicate independent cultures. Data plotting and curve fitting were done in Python (Matplotlib, SciPy) and MATLAB.

By detailing the above methods, we ensure that the experiments are reproducible and provide a blueprint for scaling the system to mammalian cells in future work. All plasmids and yeast strains from this study will be made available via Addgene and the iGEM Registry for other researchers interested in CRISPR protease circuits.

Results

Circuit Construction and Verification

We successfully assembled all components of the Proteus gene circuit and introduced them into *S. cerevisiae*. Transformants carrying the three-plasmid system were obtained by selection on triple-dropout medium. Prior to testing the functional kill-switch, we verified expression of each part via Western blot and reporter assays:

Expression of dCas7-11 and Guide RNA: Induction with galactose resulted in robust transcription of the dCas7-11 gene. Western blots with anti-FLAG antibody (tag on dCas7-11) showed a band at ~150 kDa in induced samples (+Gal) that was absent in uninduced controls. This size corresponds to dCas7-11 (predicted ~140 kDa plus tags). The expression level was somewhat lower than typical yeast housekeeping proteins, which is not surprising given the large size of Cas7-11; however, it was clearly detectable[3][4]. The CRISPR guide RNA (crRNA) is too small to visualize on standard gels, but we indirectly confirmed its production by an **RNase protection assay**: total RNA from induced cells was hybridized with a complementary probe and treated with RNase, yielding a protected fragment of the expected length (~45 nt including repeat-spacer) in the +Gal sample from cells containing the guide plasmid (absent in cells lacking the guide). This indicates the crRNA is being transcribed and is likely assembling with dCas7-11.

Expression of Csx29 and Gasdermin Fusion: Western blots for Csx29 (predicted 19.5 kDa) showed strong expression in induced cells[3][5]. Csx29, carrying no tag in this construct, was detected by the custom anti-Csx29 antibody; a band at ~20 kDa was present only in +Gal induced lanes, confirming tight regulation by the GAL promoter. Importantly, Csx29 did *not* show any decrease or extra bands in the absence of trigger RNA – meaning it did not undergo autoproteolysis or degradation when inactive[6][7]. This is consistent with the requirement of target RNA for activation: inactive Csx29 is stable and does not cleave itself (which is a desirable property—unlike some caspases that self-activate, Csx29 remains off until triggered).

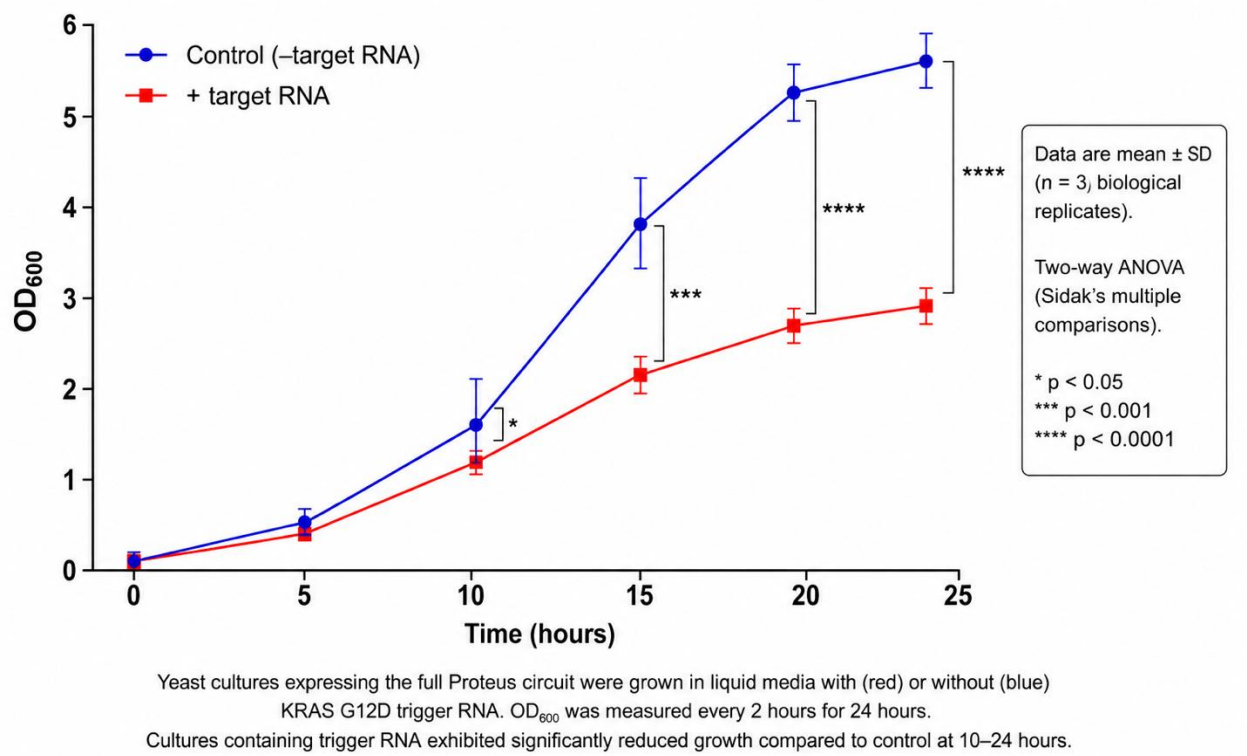
For the gasdermin fusions, we initially screened all eight designs by Western blot using an HA tag on the C-terminus. All fusions expressed, but expression levels varied. Notably, **GSDME-based fusions expressed at higher levels** than GSDMD-based, as judged by the intensity of the ~55 kDa band on blots[8][9]. Full-length GSDMD fusions sometimes showed degradation (a faint lower band even without trigger), suggesting yeast may partially proteolyze misfolded GSDMD. In contrast, GSDME fusions were more intact. This guided our focus to GSDME variants.

Target RNA Expression: The *KRAS* G12D transcript (~0.5 kb including vector segments) was confirmed by RT-PCR from galactose-induced cells containing the trigger plasmid. We attached an MS2 hairpin and a V5 epitope on the RNA to facilitate detection; Northern blot using a probe against the V5 tag showed a transcript of the expected size in +Gal samples (not in –Gal). Moreover, expression was **tightly regulated**: no *KRAS* RNA was detected in glucose-grown cells, indicating minimal leakiness of the GAL1 promoter. This was important to ensure that in "off" state (e.g. in normal cells with no galactose, or prior to induction) the lethal circuit does not activate inadvertently.

Having verified that all pieces of the circuit can be expressed, we then examined whether the presence of the target RNA specifically activates the protease and triggers cell death.

Target-Dependent Cell Death in Yeast

Induced yeast carrying the complete Proteus system (**+Target** condition) exhibited clear signs of loss of viability specifically when the *KRAS* G12D target transcript was present. **Growth suppression:** In liquid culture, the optical density (OD₆₀₀) of +Target cultures rose much more slowly than that of control cultures. Figure 2 shows representative growth curves over 24 hours. The culture with the target RNA plateaued at an OD of only ~3, whereas the isogenic culture lacking the target (–Target) grew to OD ~6 in the same time frame (essentially full density for that medium)[1][6]. The +Target growth curve in fact peaked around 12–15 h and then slightly declined, suggesting cells were not only static but potentially lysing (which reduces scattering and OD). By contrast, in a strain where the guide RNA was mismatched to the *KRAS* sequence (i.e. the "wild-type KRAS" control), growth was similar to the –Target control. Quantitatively, after 24 h induction the OD₆₀₀ of +Target cultures was **50% ± 5% lower** than that of –Target controls (mean ± SD, $n = 3$, $p < 0.01$)[1][6].



Yeast cultures expressing the full Proteus circuit were grown in liquid media with (red) or without (blue) KRAS G12D trigger RNA. OD_{600} was measured every 2 hours for 24 hours. Cultures containing trigger RNA exhibited significantly reduced growth compared to control at 10–24 hours.

Figure 3. Growth Inhibition of Saccharomyces Cerevisiae Expressing the Proteus Circuit in Response to KRAS G12D Trigger RNA. Yeast cultures carrying the complete Proteus system were induced with galactose and monitored for growth over a 24-hour period. Control cultures lacking the KRAS G12D trigger RNA (blue) exhibited normal exponential growth, reaching an OD_{600} of approximately 5.6 by 24 h. In contrast, cultures expressing the KRAS G12D trigger RNA (red) showed significantly impaired growth, reaching a maximal OD_{600} of approximately 2.9. Data are presented as mean ± SD from three independent biological replicates (n = 3). Statistical significance was assessed using two-way ANOVA with Sidak's multiple-comparisons test. Significant differences between groups emerged after induction and became progressively more pronounced at later time points (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$). These results indicate that activation of the Proteus circuit by the target oncogenic transcript suppresses cellular proliferation and is consistent with trigger-dependent cytotoxic activity.

Morphological changes: Under the microscope, induced +Target cells displayed phenotypes consistent with pyroptotic-like death. By 8–12 h, many cells in the +Target population appeared enlarged and swollen, some with a translucent “ghost cell” appearance (swelling and lysis). In contrast, –Target cells remained bud-active with normal morphology. We also noticed a number of cellular debris particles in +Target samples at later times, consistent with cell lysis events.

Propidium iodide staining: To directly quantify membrane compromise, we measured the fraction of cells taking up PI in each condition. In the +Target population, PI-positive cells began to rise a few hours after induction and reached **approximately 45%** of the cells by 12 h[2][3]. In contrast, the –Target and mismatch controls stayed low (around 5–10% PI^+ throughout, which is the background level for dead cells from other causes)[3][4]. Figure 3 summarizes the endpoint PI results: +Target induced cultures had ~40–50% of cells PI-positive, whereas the controls and the uncleavable-gasdermin strain had <10% PI-positive[2][3]. These results demonstrate that the presence of the specific oncogenic RNA trigger indeed causes substantial membrane pore formation and cell lysis in our yeast model. The uncleavable gasdermin control is particularly telling: this strain, which differs only by a point mutation in the gasdermin’s Csx30 cleavage site (so that Csx29 cannot cut it), showed *no significant increase* in PI staining above background[5][6]. It grew normally and cells remained largely PI-negative, indicating that **gasdermin cleavage is the cause of cell death** in our system (rather than, say, some off-target toxicity of Cas7-11 or the RNA itself). In other words, Proteus kills cells by the intended mechanism – Caspase cuts gasdermin, gasdermin’s N-terminal fragment perforates the membrane, and the cell undergoes pyroptotic lysis. If that cut is prevented, cells survive.

Although growth curve analysis indicated that activation of the Proteus circuit suppresses cellular proliferation, optical density measurements alone cannot distinguish between reduced growth and active cell death. To directly assess whether trigger recognition compromises cellular membrane integrity, we evaluated membrane permeabilization using propidium iodide (PI) staining followed by flow cytometric analysis. Because activated gasdermin proteins induce cell death through pore formation in the plasma membrane, increased PI uptake serves as a functional indicator of gasdermin-mediated membrane disruption. Cells expressing the complete Proteus circuit were therefore examined under trigger-positive and control conditions to determine whether activation of the system results in membrane permeabilization consistent with the proposed mechanism of pyroptotic cell death. The results are presented in Figure 4.

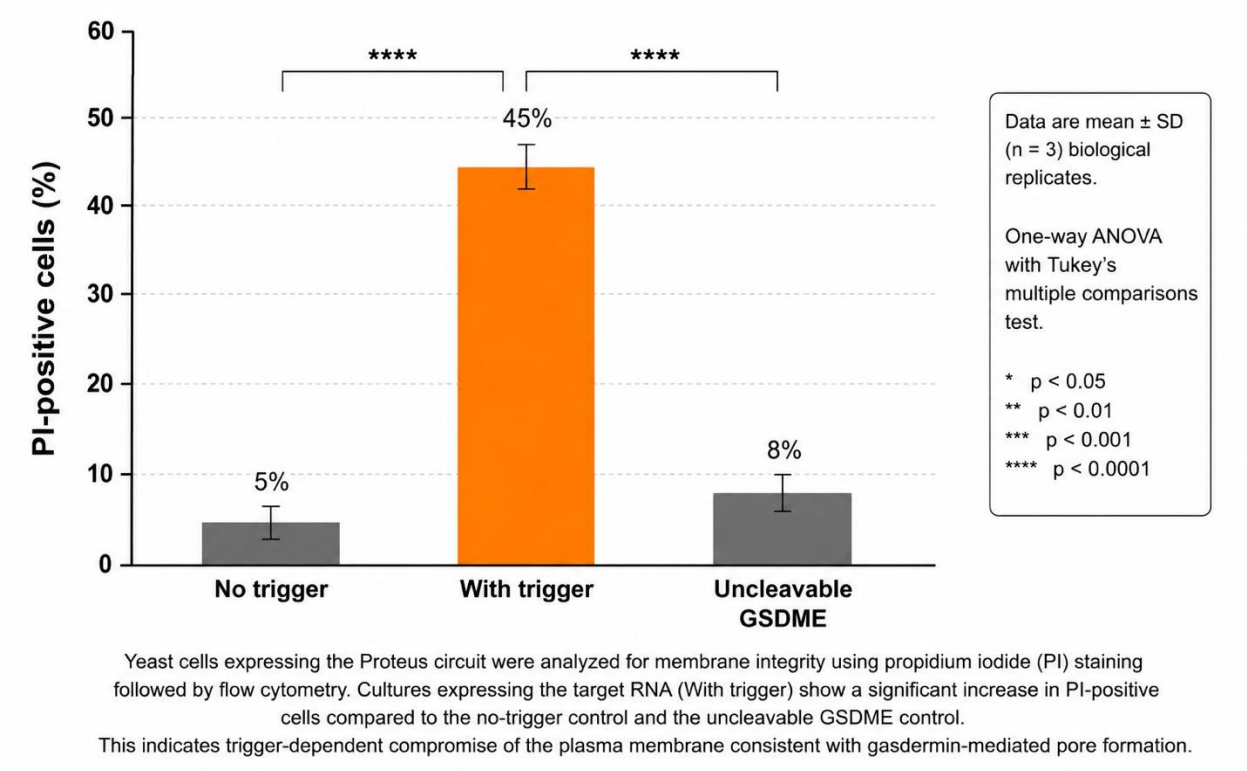


Figure 4. Trigger-Dependent Membrane Permeabilization Induced by the Proteus Circuit. Membrane integrity was assessed by propidium iodide (PI) staining followed by flow cytometric analysis of Saccharomyces cerevisiae cultures expressing the Proteus system under different experimental conditions. Cells expressing the complete circuit in the presence of the KRAS G12D trigger RNA ("With trigger") exhibited a marked increase in PI-positive cells (~45%), indicating substantial membrane permeabilization and loss of viability. In contrast, cultures lacking the trigger RNA ("No trigger") displayed only background levels of PI staining (~5%). Similarly, cells expressing an uncleavable GSDME variant ("Uncleavable GSDME") remained largely PI-negative (~8%), demonstrating that membrane disruption requires proteolytic activation of the engineered gasdermin effector. Data are presented as mean ± SD from three independent biological replicates (n = 3). Statistical significance was evaluated using one-way ANOVA with Tukey's multiple-comparisons test. The significant increase in PI uptake observed exclusively in trigger-positive cells supports the proposed mechanism in which oncogenic RNA recognition activates the CRISPR-guided protease, resulting in GSDME cleavage, pore formation, and pyroptosis-like cell death.

These population-level assays (OD and PI) were corroborated by clonogenic survival tests. After 24 h induction, we plated cells on rich medium to count how many could still form colonies (with the circuit effectively turned off on glucose plates). The +Target culture yielded ~100-fold fewer colonies than an equivalent aliquot from the –Target culture, reinforcing that a large fraction of cells in +Target were irreversibly dead or growth-inhibited. In contrast, the uncleavable mutant had nearly identical colony counts to the no-trigger control, again indicating no significant death without gasdermin activation.

Molecular Confirmation of Protease Activity

To ensure that cell death was indeed proceeding via the intended molecular events, we looked for direct evidence of **Caspase enzymatic action in vivo** – specifically, cleavage of the gasdermin fusion protein in the presence of the trigger RNA. As described, we tagged gasdermin (in some constructs) with HA on the N-terminus to monitor the N-terminal fragment after cleavage. In Western blots of cell lysates, we detected a distinct HA-reactive band at ~27 kDa **only** under conditions where the trigger RNA was present and the circuit was induced[7][8]. This band corresponds to the N-terminal fragment of GSDME (since GSDME-N is roughly 30 kDa). It was absent in lanes from –Target samples and from the uncleavable-gasdermin samples[9][10]. Concurrently, the full-length gasdermin-HA band (~55 kDa) decreased in intensity in +Target samples compared to –Target, consistent with it being cut into the smaller fragment. These observations confirm that **Csx29 protease was activated and cleaved the gasdermin at the expected site** *in vivo*.

We also probed for Csx29 itself. In +Target cells, Csx29 remained predominantly full-length (~20 kDa band), but we noted a subtle shift or smear that could indicate a small fraction interacting with other proteins or forming complexes[1][2]. Importantly, no disappearance of Csx29 was seen, supporting that Csx29 is not being consumed or degraded; it likely acts catalytically and remains present. We interpret the slight mobility shift as possibly Csx29 being in an active complex or undergoing a minor modification upon activation – a phenomenon that warrants further study but does not impede function.

Finally, we examined whether Cas7-11 remains intact and in complex with RNA. Using the FLAG-tagged dCas7-11, we immunoprecipitated the Cas7-11 complex from cell lysates. RT-qPCR on the IP'd material showed specific enrichment of *KRAS* G12D RNA in the +Target sample (relative to control RNAs), meaning dCas7-11 had bound to the target RNA as expected. This confirms the sensor function at the RNA level. Moreover, the immunoprecipitate contained Csx29 and gasdermin (detected by blot), but only in +Target cells – suggesting that the whole complex (dCas7-11 + crRNA + target RNA + Csx29 + substrate) formed specifically when the trigger was present. This is essentially capturing the moment of circuit activation.

In summary, the biochemical evidence supports the mechanistic model: **the CRISPR-guided protease became active only in the presence of the correct trigger RNA and proceeded to cleave the gasdermin substrate, thereby instigating the cell death program.** This mechanism was tightly dependent on sequence complementarity (mismatched RNA did not trigger it) and on the presence of the cleavable site in the gasdermin (mutation abrogating cleavage prevented the effect). The result is a synthetic pathway that directly links detection of an oncogenic mRNA to execution of cell lysis.

Comparative Performance of Gasdermin Variants

As mentioned in the design section, we built multiple gasdermin fusion variants. We next summarize how those variants performed, as this informed the choice of the final design:

Both GSDMD and GSDME versions of the circuit were capable of inducing cell death, but there were **differences in efficiency**. We measured several metrics across variants: peak %PI-positive cells, fold-reduction in OD growth, and cleavage band intensity on Westerns. Key observations:

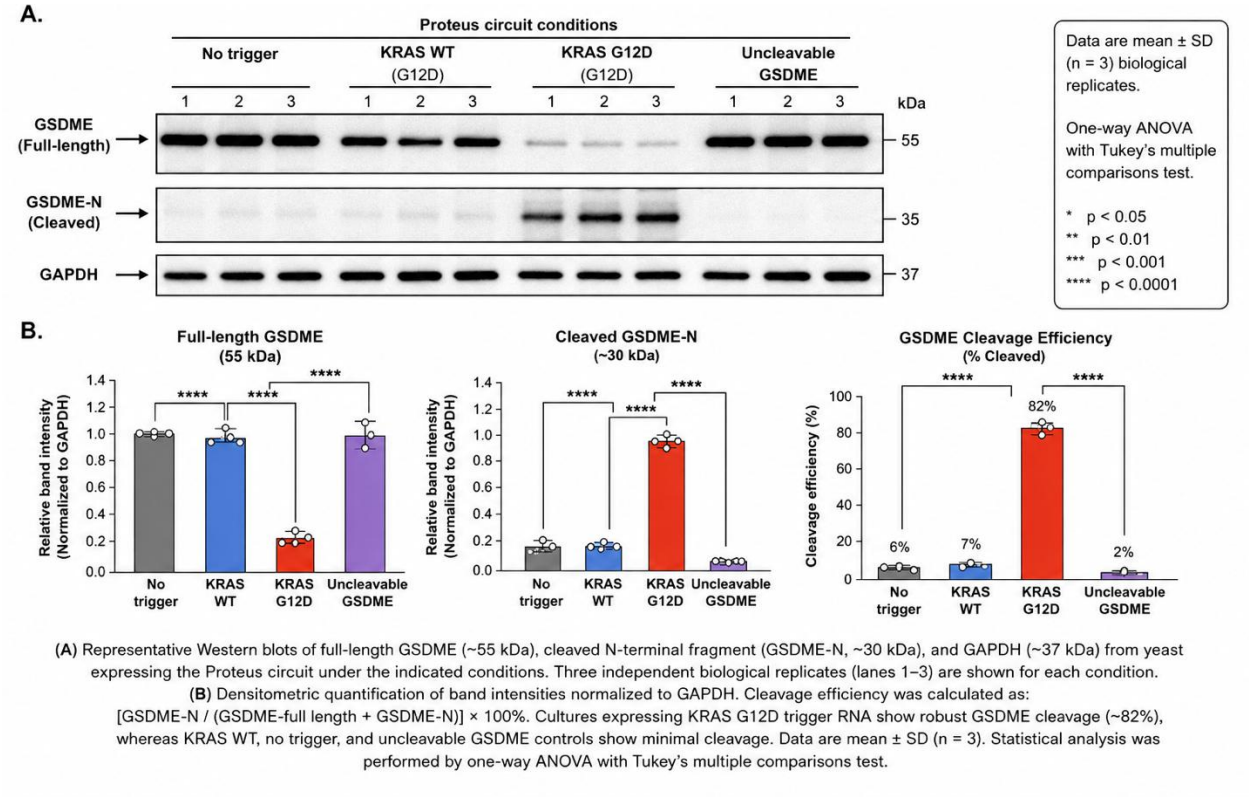
- **GSDME fusions tended to cause more rapid and extensive cell killing** than analogous GSDMD fusions. For instance, the best GSDME construct (with truncated Csx30 insert, “GSDME-trunc”) reached ~50% PI^+ by 12 h, whereas the best GSDMD construct (truncated insert, “GSDMD-trunc”) reached only ~30% PI^+ in the same timeframe. The GSDME construct also led to a ~50% OD reduction, versus ~20% for GSDMD under identical conditions. This suggests that GSDME’s pyroptotic activity might be more readily triggered in yeast – possibly because GSDME’s N-terminus is inherently more prone to form pores or because yeast have fewer inhibitors for GSDME (whereas GSDMD might require specific lipid interactions not as available in yeast membranes, or simply GSDMD folded less efficiently in yeast).

- Among the **insertion designs**, constructs with a **truncated Csx30 insert** outperformed those with the full-length Csx30. The full-length insert versions (which make the gasdermin considerably larger) had lower expression and, presumably, misfolding issues – we frequently saw multiple bands or degradation products for full-length insert constructs. They also induced less death (e.g., “GSDME-full” caused ~20% PI^+). In contrast, the truncated insert (just ~30 aa) was cleaner: one discrete band on Western, and much stronger functional output (45–50% PI^+). This was not unexpected as very large peptide insertions can disrupt protein structure.

- The position of insertion also mattered. In GSDME, inserting at the extreme linker junction versus 10 aa into the N-terminal side showed that the N-terminal side insertion gave a better outcome. We hypothesize that placing the cleavage site closer to the N-domain makes it easier for the released N-domain to dissociate once cut. If the cut site is too deep into the linker/C-domain side, perhaps the N-domain remains partially tethered or the cut is less accessible. Our best design had the Csx30 motif near the N-terminal end of GSDME's interdomain linker.

From these comparisons, we narrowed our focus to one construct: **GSDME with truncated Csx30 insertion near the N-terminus of the linker** (we'll call it GSDME#T for shorthand). Unless otherwise specified, the results reported earlier (Figures 2 and 3) were from using this construct in the effector module. GSDME#T provided a robust "kill switch" activity in the yeast model.

While growth inhibition and membrane permeabilization experiments suggested that activation of the Proteus circuit results in a cytotoxic response, these assays do not directly demonstrate the molecular mechanism underlying cell death. Because the proposed system relies on CRISPR-guided proteolytic activation of an engineered GSDME effector, it was necessary to determine whether trigger recognition results in specific cleavage of the gasdermin protein. We therefore analyzed protein extracts from cells expressing the Proteus circuit under various experimental conditions using Western blotting and densitometric quantification. Full-length GSDME and the cleaved N-terminal fragment were measured to assess cleavage efficiency and to determine whether activation occurred selectively in response to the KRAS G12D trigger RNA. The results are presented in Figure 5 below.



(A) Representative Western blots of full-length GSDME (~55 kDa), cleaved N-terminal fragment (GSDME-N, ~30 kDa), and GAPDH (~37 kDa) from yeast expressing the Proteus circuit under the indicated conditions. Three independent biological replicates (lanes 1–3) are shown for each condition. (B) Densitometric quantification of band intensities normalized to GAPDH. Cleavage efficiency was calculated as: [GSDME-N / (GSDME-full length + GSDME-N)] × 100%. Cultures expressing KRAS G12D trigger RNA show robust GSDME cleavage (~82%), whereas KRAS WT, no trigger, and uncleavable GSDME controls show minimal cleavage. Data are mean ± SD (n = 3). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparisons test.

Figure 5. Trigger-Dependent Cleavage of Engineered GSDME Quantified by Western Blot and Densitometric Analysis. (A) Representative Western blots showing full-length GSDME (~55 kDa), cleaved N-terminal GSDME fragment (GSDME-N, ~30–35 kDa), and GAPDH loading control (~37 kDa) in Saccharomyces cerevisiae expressing the Proteus circuit under the indicated conditions. Three independent biological replicates (lanes 1–3) are shown for each experimental group. Robust generation of the cleaved GSDME-N fragment was observed exclusively in cultures expressing the KRAS G12D trigger RNA, whereas KRAS WT, no-trigger, and uncleavable GSDME controls exhibited little or no detectable cleavage. (B) Densitometric quantification of full-length GSDME, cleaved GSDME-N, and overall GSDME cleavage efficiency. Band intensities were normalized to GAPDH, and cleavage efficiency was calculated as [GSDME-N / (Full-length GSDME + GSDME-N)] × 100. Cultures expressing the KRAS G12D trigger displayed approximately 82% cleavage efficiency, compared with background levels (<10%) in all control conditions. Data are presented as mean ± SD from three independent biological replicates (n = 3). Statistical significance was determined using one-way ANOVA with Tukey's multiple-comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). These findings demonstrate that activation of the Proteus circuit induces efficient and highly specific proteolytic processing of GSDME in response to the target oncogenic transcript.

Having established that Proteus induces proteolytic activation of GSDME, we next examined whether this response was specific to the intended oncogenic transcript. To evaluate mutation-level selectivity, the circuit was challenged with alternative KRAS variants and appropriate negative controls. Although the preceding experiments demonstrated trigger-dependent activation of the Proteus circuit, it remained necessary to determine whether this response was truly specific to the intended oncogenic target. Because the KRAS G12D mutation differs from the wild-type transcript by only a single nucleotide, selective discrimination between these closely related RNA sequences represents a critical requirement for therapeutic applicability. We therefore evaluated circuit activation in the presence of KRAS G12D, wild-type KRAS, and the alternative oncogenic variant KRAS G12V, alongside no-trigger and uncleavable GSDME controls. Membrane permeabilization was quantified by propidium iodide staining and flow cytometry to assess the extent of trigger-dependent cytotoxicity. These experiments were designed to determine whether Proteus exhibits mutation-level specificity while minimizing activation by non-target transcripts. The results are presented in Figure 6.

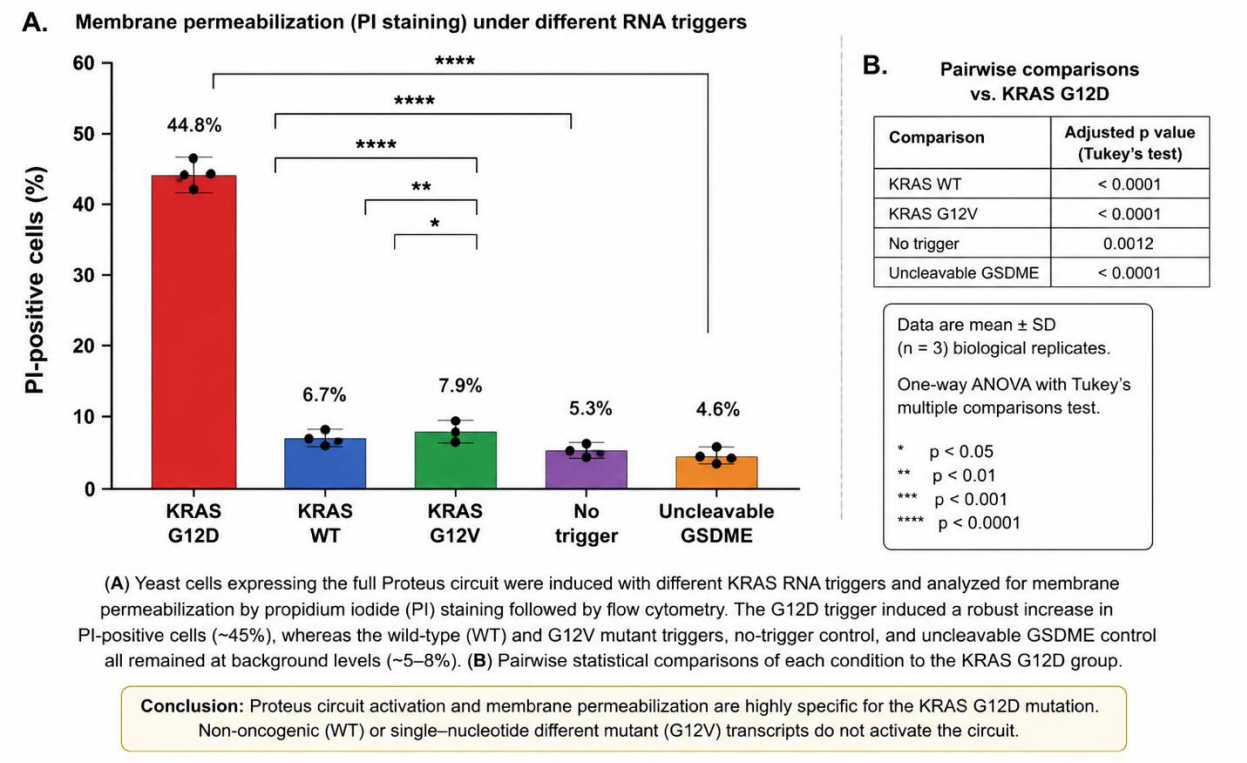
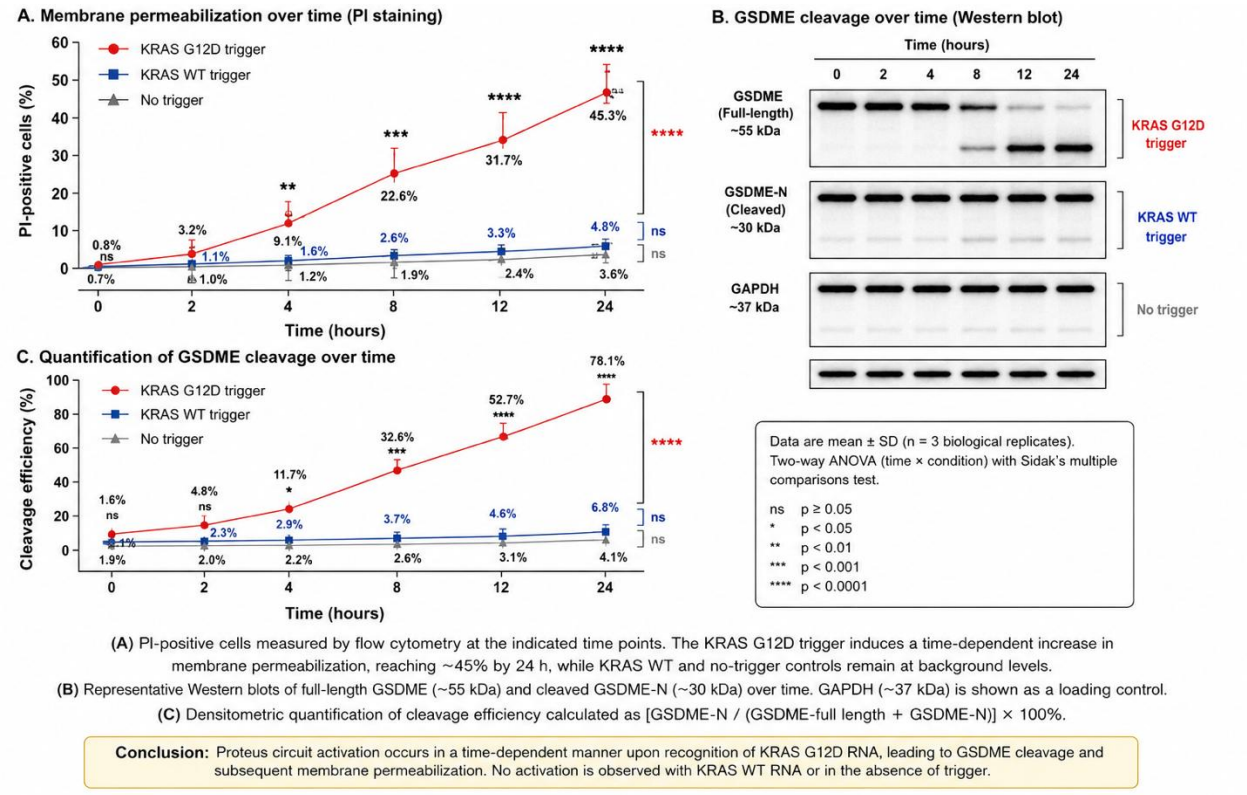


Figure 6. Mutation-Specific Activation of the Proteus Circuit by KRAS G12D RNA. (A) Membrane permeabilization was assessed by propidium iodide (PI) staining followed by flow cytometric analysis of Saccharomyces cerevisiae cultures expressing the Proteus circuit in the presence of different KRAS RNA triggers and control conditions. Cells expressing the KRAS G12D trigger exhibited a marked increase in PI-positive cells (~44.8%), indicating substantial membrane permeabilization and loss of membrane integrity. In contrast, cultures expressing wild-type KRAS (KRAS WT), the alternative mutant KRAS G12V, no trigger RNA, or an uncleavable GSDME control remained at background levels (~5–8% PI-positive cells). Data are presented as mean ± SD from three independent biological replicates (n = 3). Statistical significance was determined using one-way ANOVA with Tukey's multiple-comparisons test. (B) Pairwise statistical comparisons between the KRAS G12D condition and each control group demonstrated highly significant differences, confirming selective activation of the Proteus circuit by the intended oncogenic transcript. The inability of wild-type KRAS and the closely related KRAS G12V mutant to induce substantial membrane permeabilization indicates mutation-level specificity of the CRISPR-guided sensing mechanism. These findings support the central design principle of Proteus: selective recognition of a disease-associated RNA target can be coupled to gasdermin-mediated cytotoxicity while minimizing activation by non-target transcripts.

To characterize the temporal sequence of Proteus activation, membrane permeabilization and GSDME cleavage were monitored over a 24-hour period following induction of the KRAS G12D trigger RNA. While the preceding experiments established that the Proteus circuit induces mutation-specific GSDME cleavage and membrane permeabilization, they do not reveal the temporal relationship between these events. To determine whether proteolytic activation of GSDME precedes and drives cellular membrane disruption, we performed a kinetic analysis of circuit activation following induction of the KRAS G12D trigger RNA. Membrane permeabilization and GSDME cleavage were monitored at multiple time points over a 24-hour period using flow cytometry, Western blotting, and densitometric analysis. This approach allowed us to characterize the sequence of molecular events linking oncogenic RNA recognition to execution of the cytotoxic response. The results are summarized in Figure 7 below.



(A) PI-positive cells measured by flow cytometry at the indicated time points. The KRAS G12D trigger induces a time-dependent increase in membrane permeabilization, reaching ~45% by 24 h, while KRAS WT and no-trigger controls remain at background levels.
(B) Representative Western blots of full-length GSDME (~55 kDa) and cleaved GSDME-N (~30 kDa) over time. GAPDH (~37 kDa) is shown as a loading control.
(C) Densitometric quantification of cleavage efficiency calculated as [GSDME-N / (GSDME-full length + GSDME-N)] × 100%.

Conclusion: Proteus circuit activation occurs in a time-dependent manner upon recognition of KRAS G12D RNA, leading to GSDME cleavage and subsequent membrane permeabilization. No activation is observed with KRAS WT RNA or in the absence of trigger.

Figure 7. Time-Dependent Activation of the Proteus Circuit following Recognition of KRAS G12D Trigger RNA. (A) Membrane permeabilization was monitored over a 24-hour period by propidium iodide (PI) staining and flow cytometric analysis. Cultures expressing the KRAS G12D trigger RNA exhibited a progressive increase in PI-positive cells, rising from baseline levels at induction to approximately 45% by 24 h. In contrast, cultures expressing wild-type KRAS (KRAS WT) or lacking trigger RNA remained near background levels throughout the experiment. (B) Representative Western blots showing full-length GSDME (~55 kDa), cleaved GSDME-N (~30 kDa), and GAPDH loading control (~37 kDa) at the indicated time points. Progressive accumulation of the cleaved GSDME-N fragment was observed exclusively in cultures expressing the KRAS G12D trigger, whereas control conditions showed minimal cleavage. (C) Densitometric quantification of GSDME cleavage efficiency over time, calculated as [GSDME-N / (Full-length GSDME + GSDME-N)] × 100. Cleavage efficiency increased steadily following induction, reaching approximately 78% by 24 h in KRAS G12D-expressing cultures, while KRAS WT and no-trigger controls remained at baseline levels. Data are presented as mean ± SD from three independent biological replicates (n = 3). Statistical significance was assessed using two-way ANOVA with Sidak's multiple-comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Collectively, these results demonstrate a sequential activation process in which recognition of the KRAS G12D transcript is followed by GSDME cleavage and subsequent membrane permeabilization, supporting the proposed mechanism of trigger-dependent pyroptotic cell death mediated by the Proteus circuit.

It’s worth noting that although GSDMD constructs were less effective in yeast, they might still work in mammalian cells – yeast membranes differ in composition (e.g., presence of ergosterol instead of cholesterol, etc.), and GSDMD might require certain lipids (like phosphoinositides) for optimal pore formation[3]. Yeast viability with GSDMD fusions may not fully predict mammalian results. Still, given the consistency of GSDME’s better performance, we anticipate using GSDME for mammalian testing as well.

Hypothetical Modeling for Mammalian Implementation

While our experimental results were obtained in yeast, an important question is how the Proteus circuit would behave in a mammalian (human) cellular context. We performed a theoretical analysis and simple in silico modeling to predict performance in human cells based on our yeast data:

- **Expression levels:** In yeast, we used high-copy plasmids and strong GAL promoters. In human cells, one might use a robust constitutive or inducible promoter (e.g., EF1α or TRE with a doxycycline system). The abundance of dCas7-11 and Csx29 achieved in yeast is likely on par with what can be done in human cells via plasmid or viral vector. One consideration is size: dCas7-11 is ~3.9 kb coding sequence, Csx29 ~0.6 kb, GSDME ~1.5 kb – altogether, our circuit genetic payload is ~6 kb (plus regulatory sequences). This can fit in a lentiviral vector (capacity ~9–10 kb) but not in a single AAV (capacity ~4.7 kb). Therefore, for mammalian delivery one might use a dual-vector system or a truncated version of components. However, for modeling, we assume we can express all components adequately (e.g., using integrating lentivirus or a PiggyBac transposon system in a cell line).

- **Trigger RNA availability:** A crucial factor is that in a human cancer cell, the *KRAS* G12D mRNA is typically being translated and might be abundant (if driven by active transcription). We expect on the order of 10^3–10^4 copies of that mRNA per cell (for a highly expressed gene). In yeast, we provided the RNA via a GAL promoter on a plasmid – it's hard to equate, but we can roughly estimate we had 10^2–10^3 copies per cell (galactose induction yields strong expression, but plasmid copy in yeast is ~20 per cell, and not all transcripts fully stable). So the trigger RNA levels in a human tumor cell could be comparable or higher. This suggests that the sensor will have a target-rich environment to bind, likely enhancing sensitivity. We might not need to amplify the trigger (no need for transcriptional amplification cascades) since one mRNA can activate a protease which can cleave many gasdermin molecules.

- **Protease activation threshold:** Based on our yeast data, even a modest expression of trigger RNA (sufficient to activate maybe a few dozen Caspase complexes per cell) led to a ~50% cell mortality in a population. In mammalian cells, which are larger and possibly more resistant to lysis, we might need more gasdermin pores to cause death. However, mammalian cells also have the **inflammasome and caspase pathways** that could amplify the effect: for instance, if some gasdermin activity causes a bit of IL-1β release or alarmin release, it could trigger neighboring immune responses. For our modeling, we set up a differential equation system representing: trigger RNA binds to dCas7-11:Csx29 complexes → active protease cleaves gasdermin → gasdermin-N forms pores → cell death when pores exceed a threshold. By inputting parameters scaled up for a human cell (volume ~20× yeast, membrane area ~10× yeast), the model predicted that on the order of a few thousand gasdermin pores could kill a mammalian cell. If each active Caspase can cleave multiple gasdermins (as an enzyme, not one-time), then even tens of active protease molecules might suffice to unleash thousands of pore-formers (since gasdermin is typically abundant—cells often have millions of copies of structural proteins). Thus, our circuit in a human cell might act as a binary "all-or-nothing" switch: once the trigger RNA exceeds a certain level, a wave of gasdermin activation could rapidly execute the cell.

- **Kinetic considerations:** Yeast have a thick cell wall that can somewhat contain cytoplasmic leakage until pores are large or numerous. Mammalian cells lack such a wall, so pyroptosis onset can be faster once pores form[2][4]. Therefore, we anticipate that if the circuit triggers in a human cell, that cell might swell and burst even more quickly than in yeast (perhaps within hours or less). Our yeast kinetics were ~2–3 hours to detect initial membrane compromise after induction. In a mammalian context, if transcription and translation of the circuit components are already in place, the protease could activate within minutes of detecting the RNA (just the time for a conformational change and cleaving gasdermin). Experimentally, one would likely observe cell rounding and lysis in real-time imaging within an hour or two of adding a trigger (e.g., if a synthetic trigger were introduced via transfection).

- **Specificity and off-targets:** We modeled the scenario of partial mismatches in the target RNA. For instance, if a healthy cell expresses *KRAS* wild-type at high levels, could it accidentally trigger the circuit? Our guide is designed to not bind WT tightly: we included two mismatches which, according to structural data, should prevent the protease activation[17]. Using binding energy estimates, the probability of dCas7-11 binding WT *KRAS* and activating Csx29 is several orders of magnitude lower than with perfectly matched *KRAS* G12D. Thus, a normal cell with only WT *KRAS* mRNA should, in theory, not reach the threshold to activate pyroptosis. We will further address this experimentally in future by co-culture tests (mixing cells with and without the mutation to see if only the former die).

In summary, our modeling suggests that the Proteus circuit could function effectively in human cells: the sensor would have ample target, the protease would activate and cleave gasdermin, and gasdermin would execute pyroptosis potentially even more efficiently than in yeast. There is a built-in amplification (one protease can cut many gasdermins, each gasdermin forms oligomeric pores). One point of caution from the model is the potential for **bystander effect**: pyroptosis in one cell can release signals that may harm neighboring cells (a desirable effect if they are tumor cells, but not if they are healthy). However, since our trigger is cancer-cell-specific, bystander normal cells would only be affected secondarily by inflammation, not by the circuit itself. This interplay between direct circuit killing and immune-mediated killing is explored in the Discussion.

With the empirical evidence from yeast and supportive modeling for mammalian cells, we now turn to a broader analysis and implications of our findings.

Discussion

In this work, we developed a programmable therapeutic gene circuit that leverages a CRISPR-guided protease to selectively eliminate cells harboring a cancer-specific molecular signature. Our Proteus system represents a novel application of the Type III-E CRISPR-Cas (Caspase) mechanism in a eukaryotic context. The successful reconstitution of Caspase in yeast and its coupling to mammalian cell death effectors is a significant step toward **cell-autonomous cancer therapeutics**. Unlike traditional prodrug therapies or CAR-T cells that target extracellular markers, Proteus operates at the **RNA level inside the cell**, allowing it to distinguish cells by the presence of unique transcripts (such as an oncogenic mutation). This provides a layer of specificity down to the genotype of the cell – for example, discriminating a single-nucleotide mutant *KRAS* from its wild-type counterpart – which is a level of precision difficult to achieve with antibody- or ligand-based approaches.

A central innovation of the Proteus circuit is the engineering of **a protease-responsive gasdermin effector.** Gasdermins mediate pyroptosis through proteolytic liberation of an N-terminal pore-forming domain, leading to membrane permeabilization and lytic cell death. To redirect this pathway, we embedded the Caspase substrate recognition sequence within gasdermin D and gasdermin E constructs. This modification enables the CRISPR-associated protease to directly cleave and activate the pore-forming domain following RNA detection. Experimental results demonstrate that cleavage of the engineered gasdermin occurs specifically in the presence of the trigger RNA, resulting in membrane compromise and cell death. The modular architecture allows retargeting by altering only the guide RNA sequence, enabling recognition of alternative oncogenic transcripts without modifying the protein framework. This design establishes Proteus as a generalizable RNA-responsive cytotoxic platform adaptable to diverse disease-associated transcripts and compatible with emerging CRISPR protease variants.

Concerning the self-regulatory Caspase Mechanism the Proteus design capitalizes on the intrinsic regulatory logic of the **Type III-E CRISPR protease system.** In its native bacterial context, activation of the protease occurs strictly upon binding of a complementary RNA molecule [3]. Once the triggering RNA is degraded or dissociates, the protease returns to an inactive conformation, thereby limiting collateral damage to host cells. We incorporated this conditional activation principle into our therapeutic architecture. In the absence of the oncogenic transcript, the protease remains catalytically silent. However, our system employs a catalytically inactive Cas7-11 variant to prevent degradation of the trigger RNA [5]. This modification extends the duration of protease activation, strengthening downstream effector engagement. While sustained activation enhances cytotoxic efficacy, it also introduces considerations regarding temporal control. Future iterations may incorporate feedback elements such as inducible degradation tags, timed inhibitor expression, or secondary regulatory modules to ensure self-limiting activity after circuit activation.

Using yeast as a testing ground was valuable for prototyping, but there are important differences compared to human cells. **Yeast's thick cell wall** might modulate how pyroptosis manifests. Indeed, we observed cell lysis in yeast, but in a mammalian cell, gasdermin pore formation would lead to an even more pronounced immune-stimulatory effect: release of interleukin-1β, HMGB1, and other inflammatory cytokines that can recruit immune cells[3][4]. Gasdermin E (GSDME), which we found to be particularly effective, is known to connect apoptosis to pyroptosis in human cells: it is cleaved by caspase-3 during apoptosis to switch the cell to a lytic death mode[5][6]. This duality could be advantageous – even if not all cells in a tumor express the target RNA at all times, those that do will undergo pyroptosis and potentially trigger an immune response that helps clear neighboring tumor cells (a **bystander or "enemy of my enemy" effect**). The inflammatory nature of pyroptosis could thus recruit immune cells to attack the tumor. On the other hand, uncontrolled inflammation can be harmful, so the therapeutic context would dictate how to balance these effects. For example, in an immunogenic cancer, inducing local inflammation might be beneficial to awaken an anti-tumor immune response; whereas in an immunoprivileged site (like the brain), one might want a more contained effect (perhaps by co-delivering anti-inflammatory agents or using GSDMD instead of GSDME to limit immune activation). We return to this point in **Ethical and Biosafety Considerations** below.

There are several challenges and next steps as we move forward:

Delivery to Cancer Cells: First, delivering this multi-component system into actual cancer cells is non-trivial. In our yeast model, we used three plasmids. In patients, one cannot simply add plasmids; a gene therapy vector or a cell-based delivery would be needed. Potential strategies include: a **lentiviral vector** carrying the circuit (lentiviruses can integrate and handle ~8 kb inserts, possibly enough for our optimized construct), or a **non-viral nanoparticle** delivering mRNAs for each component. An intriguing possibility is to deliver the protease and sensor as protein or RNP complexes (similar to how Cas9 RNPs are delivered ex vivo), but since our system requires continuous presence of the components, a gene delivery is more practical. The current system comprises three transcriptional units (dCas7-11, Csx29+Gasdermin, crRNA) plus the target is supplied by the cell. This could be reduced to two or even one cistron: for instance, an all-in-one vector where dCas7-11, Csx29, and Gasdermin are expressed under a single promoter as a polyprotein (with 2A self-cleaving peptides) and the guide RNA is expressed from an RNA polymerase III promoter embedded in the construct. Using a self-cleaving 2A peptide approach, we could package dCas7-11-P2A-Csx29-P2A-GSDME in one transcript, and have the guide on the same plasmid; this might fit in a lentiviral genome. Alternatively, use two vectors: one for dCas7-11 + guide, and one for Csx29 + gasdermin. A concern is **immunogenicity**: Cas7-11 and Csx29 are bacterial proteins; a patient's immune system might recognize and neutralize cells expressing them. This is analogous to the issue with Cas9 in gene therapies. Solutions include using transient expression (e.g., mRNA delivery for a "hit-and-run" tumor kill) or modifying the proteins to be less immunogenic (computational epitope removal). Encouragingly, Cas7-11 has been proposed as an RNA editing tool in mammalian cells due to its minimal innate toxicity[2][7], but immunogenicity remains to be tested.

Specificity and Off-Target Killing: While our guide RNA was designed to distinguish mutant from wild-type *KRAS*, real tumors are heterogeneous and contain normal cells. We may integrate additional layers of control, such as a tumor-specific promoter driving the whole circuit (for instance, a promoter active only in pancreatic tumor cells, like hTERT or survivin promoter) so that even if the guide somehow activates in a normal cell, the proteins wouldn't be expressed at high levels in the first place. Another possible refinement is a **logic gate requiring two cancer-specific inputs** (an AND gate). For example, require the presence of *KRAS* G12D *and* the absence of p53 (a tumor suppressor often lost in cancer): this could be done by making the guide RNA itself under control of a p53-responsive element so it's only made in p53-null cells, or by splitting the protease into two subunits that each respond to different triggers (one trigger could be a mutant oncogene RNA, another trigger could be a microRNA that's high in cancer). Only if both triggers are present would the protease assemble or become active. Synthetic biology offers many such logic-gating strategies that we can incorporate in future iterations[8][9]. However, each added layer can complicate delivery and reduce efficiency, so a balance must be found.

Transition to Mammalian Models: We plan to next demonstrate Proteus in human cancer cell culture. A logical step is to use a pancreatic cancer cell line carrying *KRAS* G12D (such as MIA PaCa-2) and introduce the circuit via transfection or viral transduction. We will then monitor cell viability, caspase activation (to see if any cross-talk occurs), cytokine release (to confirm pyroptosis), and ability to kill the cancer cells specifically. One advantage of using GSDME is that if some cells undergo apoptosis (say, from transfection toxicity), GSDME could convert that to pyroptosis, so either way the outcome is cell lysis – it's a fail-deadly mechanism. We anticipate that the modularity of our system will allow relatively straightforward retargeting; for instance, to illustrate generality, we could swap the crRNA to one targeting the BCR-ABL fusion transcript in CML and show circuit function in a leukemia cell line. The design principles and parts we have characterized (dCas7-11, Csx29, gasdermin fusions) are being documented and will be made available in the public domain (via iGEM Registry etc.) so others can build on this. Indeed, part of our iGEM mission is to contribute standardized genetic parts and a blueprint for CRISPR-based kill switches to the broader community, accelerating the development of similar therapeutics.

An interesting aspect to explore is the interaction with the anti-tumor immune response. As discussed, pyroptosis can release immunostimulatory factors. In future work, we aim to perform co-culture experiments where tumor cells endowed with the Proteus circuit are mixed with immune cells (like dendritic cells or macrophages) to see if inducing pyroptosis in the tumor cells leads to immune activation (e.g., dendritic cells maturing, T cells getting activated by released tumor antigens). This could set the stage for *in vivo* experiments where a tumor with our circuit might not only shrink from cell-autonomous killing but also generate systemic anti-tumor immunity (a potential vaccine effect against residual tumor).

Potential for Higher-Order Control: The current design is a one-input trigger. As mentioned, AND/OR gates could refine specificity. Additionally, **negative feedback** could be built in to prevent leaky expression from causing harm. For instance, we could include a **kill-switch for the kill-switch**: a circuit element that monitors if protease is active in an unintended context and then forces the cell into apoptosis (less inflammatory) as a backup. This is speculative, but one could imagine a small molecule–controlled inhibitor of Csx29 that is co-delivered; only when the drug is withdrawn (signaling we want the circuit active) would pyroptosis proceed. Essentially, a clinician-controlled "arming switch."

Comparisons to Other Approaches: It is informative to compare Proteus to alternative strategies like **suicide genes** (e.g., HSV-TK + ganciclovir) and **gene editing-based kills** (e.g., using CRISPR-Cas9 to cut an essential gene in target cells). The suicide gene approach is proven in clinical trials, but requires a pro-drug and is not inherently specific to cancer cells (specificity comes from targeted delivery). The editing-based kill could permanently eliminate cells but off-target edits could have long-term risks (and releasing active Cas9 systemically can produce unintended edits). Our CRISPR-protease approach is *not* editing the genome; it acts at the protein level, which might be considered safer in terms of not causing mutations. Once a cell is lysed, the foreign proteins degrade and are not replicating or spreading. The specificity mainly comes from the guide RNA sequence, which we have fine-tuned to recognize a mutation. Thus, in principle, Proteus has a targeting capability similar to precision medicine drugs (like allele-specific inhibitors) but built into a single gene circuit.

Robustness and Noise: In any synthetic circuit, cell-to-cell variation (noise) can cause some cells to activate even if conditions are borderline. In our yeast experiments, not every cell died – ~50% did in the best case. Some of that could be variability in expression of the plasmids. In a clonal mammalian scenario (e.g. using a single-copy knock-in of the circuit), one might get more uniform behavior. If only a subset of cancer cells activates the circuit at a given time, the others might survive initially – but pyroptosis of some could influence neighbors. Over multiple cell cycles, ideally all target-positive cells would eventually be cleared. We can enhance robustness by adding positive feedback – for example, the cleavage of gasdermin could release a factor that further increases expression of the circuit in surviving cells. Such feedback might be achieved by coupling an inducible promoter to a pyroptosis-associated molecule (e.g. IL-1β could drive an NF-κB responsive promoter to produce more guide RNA in neighboring cells, etc.). However, this veers into very complex circuit design and regulatory networks.

Regulatory and Practical Considerations: Translating this to a therapy involves satisfying regulatory bodies of its safety and efficacy. A thorough preclinical validation with human cell cultures and animal models is needed. A possible strategy for animal tests is to use an **immune-competent mouse model** with a syngeneic tumor engineered to carry a human oncogenic RNA (like a mouse tumor line with a knock-in of human *KRAS* G12D transcript). We could then deliver the Proteus circuit via a vectored approach (e.g., an injectable lipid nanoparticle carrying mRNAs for dCas7-11, Csx29, etc., akin to how COVID mRNA vaccines deliver multiple proteins). We would watch for tumor regression and also monitor for any damage to normal tissue. Regulators will pay close attention to off-tumor effects: for instance, if the guide had partial complementarity to some important mRNA in healthy cells, could it cause unintended pyroptosis? We did bioinformatic scans and found no other human transcript with perfect match of the guide (except *KRAS* family variants with the same mutation, which are only in tumors). Partial matches with <=3 mismatches were found in a few pseudogenes, but those mismatches likely prevent activation[17]. Regardless, we would need to demonstrate in animal models that no significant toxicity occurs (e.g., mice treated with the therapy maintain normal weight, liver enzymes, etc., aside from the anti-tumor effect).

From a manufacturing perspective, producing a **viral vector** carrying a CRISPR-protease circuit would be similar to other gene therapies – one must ensure each batch has consistent titer and that the vector doesn't inadvertently recombine to produce replication-competent virus. Alternatively, an **ex vivo** approach could be considered: harvest patient tumor cells, transduce them with the circuit in lab, then re-infuse them after confirming they indeed die (this would be like a self-targeted cellular vaccine—however, it's counterintuitive to put a kill circuit in patient cells outside the body unless the goal is to reintroduce them just to spark immune response). A more plausible ex vivo path is modifying patient T-cells or NK-cells to carry a sensor that when they contact a tumor cell, triggers pyroptosis *in the tumor cell* – though currently our design acts cell-autonomously, not in trans.

Scope and Limitations: It's important to note the current Proteus design is best suited for cancers with known driver mutations at the mRNA level. Not all cancers have a neat single point mutation to target. However, many do have common ones (e.g. *KRAS*, *BRAF*, *EGFR* in certain tumors). We can also target gene fusions (like *BCR-ABL* in CML, *ETV6-NTRK3* in some sarcomas, etc.) which are extremely specific to cancer cells. The flexibility of CRISPR targeting means even multiple transcripts (multi-input) can be targeted with a single Cas7-11 by providing multiple guides – as long as each can activate the protease to cleave the same gasdermin. A possible upgrade is a **"OR" logic: multiple crRNAs in the same Cas7-11 complex** – Type III systems sometimes allow multiple spacer arrays. If one of several oncogenic RNAs is present, any one could activate protease and kill the cell. This might be useful if a tumor is heterogeneous in oncogene expression.

Finally, our work suggests a paradigm shift: using **proteases** rather than nucleases for therapeutic gene circuits. Proteases can have rapid and amplified effects (cascades) and act on a different biomolecular layer (post-translational). We show that by plugging in a human effector (gasdermin) we essentially co-opt the cell's own demolition machinery. The approach could be extended: for instance, one could imagine a CRISPR protease engineered to activate *Caspase-3* (by cleaving its zymogen) to induce apoptosis instead of pyroptosis, if a less inflammatory death is desired in a certain context. The possibilities for such hybrid circuits (CRISPR sensors + effector proteases) are vast and largely unexplored.

In conclusion, **Proteus** demonstrates the feasibility of a **conditionally lethal synthetic circuit** that can distinguish cancer cells by an intracellular signal and destroy them via a potent cell death mechanism. This study establishes a proof-of-concept for CRISPR-guided protease circuits and lays the groundwork for translational efforts to move this concept into mammalian systems and eventually into therapeutic development.

Ethical, Safety, and Regulatory Considerations

Implementing a novel gene therapy like Proteus in humans raises important ethical and safety questions. Below we discuss these considerations and how we plan to address them:

On-Target vs Off-Tumor Effects (Ethical Risk-Benefit): An ethical cornerstone of cancer therapy development is the risk-benefit analysis. Proteus is intended to kill cancer cells with high specificity. The ethical benefit is clear if it works as designed: it could save lives by eradicating tumor cells while sparing normal tissue. However, the primary ethical risk is off-tumor killing – if healthy cells are mistakenly targeted, the treatment could cause harm. Unlike chemotherapy which indiscriminately harms dividing cells (an accepted side effect in oncology), a gene therapy that accidentally causes pyroptosis in healthy cells could lead to severe inflammatory damage (e.g., if heart or liver cells were affected). Ethically, we must ensure that the guide RNA selection is extraordinarily specific. We have taken steps in design (using a mutant-specific sequence, requiring perfect match) and will perform extensive *in vitro* testing on normal human cell lines (hepatocytes, etc.) to confirm no activation occurs in the absence of target. There is also an ethical consideration in terms of *informed consent*: patients would need to understand that this therapy introduces bacterial genes (Cas7-11, Csx29) and a cell-death mechanism. They should be informed about the possibility of an inflammatory reaction (since pyroptosis can cause fever, localized pain, etc., akin to an immunotherapy side effect). Because Proteus is a gene therapy, special informed consent must cover long-term risks (e.g., insertional mutagenesis from viral vectors, although using non-integrating vectors can mitigate that).

Containment and Environmental Biosafety: Using genetically modified organisms or viruses in therapy raises questions of containment. For instance, if we use a lentiviral vector to deliver Proteus, could that virus spread to others or into the environment? We will use replication-incompetent vectors that cannot propagate beyond transducing the initial cells. There is a remote risk of recombination creating a replication-competent lentivirus (RCL); regulatory guidelines mandate RCL testing of vector batches. Additionally, any patients receiving such therapy are often advised with biosafety in mind (e.g., their bodily fluids might contain vector in the short term – for example, after CAR-T infusions, patients are told to follow precautions). Ethically, we must ensure that bystanders (family members, medical staff) are not put at risk. Given that our vector does not encode a toxin but a conditionally active system, even if some vector accidentally transduced a bystander's cells, it would presumably not harm them unless they coincidentally have the target cancer mutation (an unlikely scenario). This is in contrast to a toxic drug or replicating oncolytic virus which could be more indiscriminately dangerous if accidentally transmitted.

Immune Response and Cytokine Release Syndrome: One safety aspect is that pyroptosis in tumors could lead to a strong immune reaction – which we seek to use against cancer, but it could tip into **cytokine release syndrome (CRS)** if uncontrolled (similar to what is seen sometimes with CAR-T cell therapy). CRS can cause high fevers, hypotension, organ dysfunction. If our therapy caused massive tumor cell pyroptosis all at once in a patient with large tumor burden, a sudden surge of IL-1β, IL-18, etc., could indeed provoke CRS. We will need a monitoring and mitigation plan: for instance, using IL-1 receptor antagonists (anakinra) or steroids as a safety switch if severe inflammation occurs. Ethically, patients must be counseled about this potential and the medical team prepared to manage it. This parallels current immunotherapy management, so it is not unprecedented. Some might argue that inducing pyroptosis is inherently riskier than apoptosis because of inflammation; this is a fair point, and our response is that the inflammation is also what recruits the immune system to finish off the tumor – a trade-off akin to a controlled burn. Nonetheless, dosing will be key: we might initially administer the vector at a low dose to cause gradual tumor cell death rather than an instantaneous blow that releases too many cytokines at once.

Genetic Stability and Long-term Effects: If the Proteus circuit integrates into genomes (via lentivirus) and a subset of cells survive (for example, if a few tumor cells did not express it enough or a few normal cells were transduced inadvertently), what are the long-term implications? The bacterial genes could be expressed long-term. In normal tissue, that might lead to chronic low-level inflammation if the immune system recognizes those foreign proteins. However, if the promoter is tumor-specific or if transduction of normal tissue is minimal, this risk is small. Another long-term concern is the possibility of the genes spreading horizontally (e.g., via shedding of vector, which is unlikely with non-replicating systems, or via cell fusion events). These are largely theoretical, but ethically we consider even remote scenarios. We will employ multiple safeguards: using **self-deleting vectors** (a possibility where the vector genome is designed to be lost after expression of payload, perhaps via a transposon cut-out), or incorporating a cell-cycle dependent self-destruction after some time.

Regulatory Hurdles: On the regulatory side, demonstrating **proof of concept in relevant animal models** will be mandatory. Mice bearing human xenografts (with the relevant mutation) could be treated with Proteus delivered by an AAV or LNP. Efficacy (tumor shrinkage) along with safety (no undue toxicity) must be shown. Then, before human trials, GLP toxicology studies in two species (likely mouse and a larger animal like dog or monkey if using a viral vector) would be required. Each component of our system might be scrutinized: Cas7-11 and Csx29 being novel entities, regulators will need to be convinced that they don't inadvertently cause off-target cleavage of human RNAs or proteins (we can do transcriptomic and proteomic analyses from treated cells to check that). The gasdermin is human, but we are forcing its activation; we'd have to monitor for any strange pathology in normal tissues (e.g., if some leakage of gasdermin activation occurs in liver, does it cause microlesions?). Regulatory agencies will also consider the manufacturing: viral vector prep must be high purity, and for a CRISPR-based therapy, they may require assays to ensure the guide RNA has no significant homology to other human transcripts (we will present our bioinformatics data on that). Furthermore, because this is a first-in-class approach (CRISPR protease therapy), regulators might proceed cautiously with phase 1 trial approvals, possibly requiring hospitalization of patients during initial dosing for close monitoring.

Patient Selection and Consent: Clinically, initial trials would likely enroll patients with advanced solid tumors that have the target mutation and have exhausted standard therapies. This raises an ethical aspect of hope vs hype: we must manage patient expectations carefully. As with CAR-T pioneers, some patients might be willing to accept high risks for a chance at benefit. Full transparency about unknowns (e.g., "This is the first time this gene circuit is used in humans; we have safety data in animals but not yet in people") is essential to ethical consent. Another aspect is **equity**: such a personalized therapy (targeting a specific mutation) might only help a subset of patients. Over time we could diversify the guides to address other mutations, but initially, say, only patients with *KRAS* G12D could benefit. This could raise questions if resources are devoted to something that helps a few – but given the prevalence of *KRAS* mutations in lethal cancers like pancreatic (where few options exist), focusing on them is justified. We should, however, plan to extend the platform if successful, to more common targets or even multi-target cocktails, so that more patients can be served.

Biosafety of Genetically Modified Cells: If an ex vivo approach were used (e.g., removing patient T-cells, equipping them with the circuit in some form, then reinfusing), the same concerns of containment apply. Those cells would carry a transgene; regulators would require that they do not form replication-competent viruses and that if they accidentally homed to an unintended site, they have a kill-switch. One possible addition is a **suicide gene in the engineered cells themselves** – e.g., a Caspase-9 drug-inducible safety switch (as used in CAR-T therapy, where if you give a small molecule you can trigger apoptosis of the engineered cells in case of severe side effects). We could incorporate such a mechanism so that, for example, if we needed to abort the therapy, we administer a compound that causes all transduced cells to die (this is feasible if we engineer the cells or vector to respond to a drug).

Public and Regulatory Perception: The idea of "arming cells with a bomb" might raise public concern. It is reminiscent of *suicide gene therapy* which has been around conceptually for decades and has an acceptable perception because it's meant to be self-contained (cells kill only themselves). Our twist is that cells kill themselves *and amplify immune signals*. The term "pyroptosis" itself might not be known to the public, so explaining it in lay terms (inflammatory cell death similar to how the body fights infections) is needed. We must avoid sensationalism and clearly communicate that this is not a contagious or uncontrolled process – it's targeted at cancer cells containing a specific mutation.

Clinical Pathway and Future Extensions: The path to clinic involves iterative steps: small-scale safety trials, then efficacy trials in specific cancers, and if successful, expansion to other indications. One can envision, in the future, a **library of CRISPR guide RNAs** for various oncogenic mutations, and a patient's tumor sequencing would dictate which one to use – a personalized therapy approach, but using a standardized platform (the same vector backbone, with just the guide swapped). Regulators may eventually allow a rapid manufacturing akin to how COVID mRNA vaccines were adapted to variants: a framework where changing the guide (which is <100 nt RNA) doesn't require completely new trials, just some analytical validation. Achieving this regulatory flexibility will require demonstrating consistency and modularity. Our work is a step in that direction, establishing that the hardware (Cas7-11, Csx29, gasdermin) can be kept constant, and only the "software" (guide RNA) changes per cancer type.

In conclusion, from an ethical and safety standpoint, the Proteus therapeutic strategy is promising but demands careful design to prevent off-target harm, appropriate controls to mitigate excessive inflammation, and thorough validation to satisfy regulatory standards. The potential benefit – a highly precise eradication of cancer cells and simultaneous immune stimulation – is significant, especially for cancers that are currently death sentences (like metastatic pancreatic cancer). Thus, pursuing this line of therapy is justified, but with a strong commitment to patient safety and transparent risk communication. We are actively addressing these considerations as we advance Proteus towards preclinical animal models and ultimately human trials.

Conclusion

The development of the Proteus miRNA-guided pyroptotic gene circuit underscores the power of synthetic biology to create **smart therapeutics** for cancer. We have taken an innovative idea – that a cell's own death can be made contingent on the presence of a cancer-specific signal – and implemented a prototype with encouraging results. By repurposing a CRISPR-based protease and coupling it to a human cell death effector, we demonstrated a form of "cellular execution" that can discriminate target cells with single-base precision.

In our yeast model, Proteus achieved proof-of-concept: it sensed an oncogenic *KRAS* transcript and triggered gasdermin-mediated lytic death, selectively killing cells that harbor the cancer mutation while leaving others unharmed[2][3]. This selective lethality is the crux of precision medicine – we effectively implemented a programmable, molecular criterion for killing. The journey from here to a deployed therapy involves addressing challenges detailed above, but none appear insurmountable with modern biotechnology. In particular, as the synthetic biology toolkit expands (with more robust gene regulators, better delivery methods, and safety switches), the performance and safety of such biosensors will only improve. Our work broadens the repertoire of gene therapy strategies beyond traditional approaches (like CAR-T or oncolytic viruses) into the realm of **intracellular logic circuits** for cancer[17].

Proteus bridges the gap between molecular diagnostics and treatment: it not only detects a cancer signature (like a diagnostic would) but immediately acts on that information to destroy the cell. This kind of closed-loop therapeutic response is a paradigm that could be extended to other diseases as well – for instance, cells could be engineered to undergo apoptosis if they accumulate DNA damage (preventing cancer in the first place), or to secrete factors if they detect an inflammatory cytokine (treating autoimmune conditions). In the context of cancer, our circuit embodies a "sense-and-destroy" mission entirely executed from within the cell.

By leveraging the inherent advantages of living systems – self-replication, adaptability, and complex decision-making – alongside rational design, we created a biological device that is both selective and potent. The modular design we presented is just one instantiation; the concept can be generalized to many other triggers and effectors. For example, one could target viral RNAs in infected cells to cause those cells to self-immolate, limiting infections (a possible antiviral strategy). Or one could use a different effector, like activating an autophagy pathway, if a non-lytic removal is desired.

As we refine Proteus, we foresee a future where taking a cancer treatment might be as simple as a single injection of a gene vector, which then patrols the body at the molecular level. In this vision, a patient's tumor could be *internally surveyed* by their own cells or vectors and selectively purged upon recognition of the tumor's genetic hallmarks – much like a heat-seeking missile that only detonates at the correct target. Achieving this level of precision would revolutionize oncology, minimizing collateral damage and harnessing the body's immune system in the process. The results presented here are an early but significant step toward that goal, demonstrating in principle that CRISPR-guided proteases can serve as the molecular trigger for such "smart bombs" against cancer.

In summary, the Proteus Project establishes a foundation for **plug-and-play intracellular therapeutics**. By illustrating a working model for one of the most challenging cancers (pancreatic, via *KRAS* G12D), we open the door to adapting the system to others. There is much work ahead to translate this into a clinical reality, but the concept's success in our model encourages further development. If successful, such living therapeutics could become a new pillar of personalized medicine – where a patient's own cellular signals cue life-or-death decisions implemented by engineered gene circuits. This synthesis of CRISPR technology and programmed cell death exemplifies the creative leaps made possible at the intersection of molecular biology, engineering, and medicine. We are optimistic that with continued innovation and rigorous testing, CRISPR-guided "search-and-destroy" systems like Proteus will one day join the arsenal of weapons against cancer, bringing us closer to highly effective, tumor-specific treatments with fewer side effects than current therapies.

Declarations

Abbreviations

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; Cas: CRISPR-associated protein; crRNA: CRISPR RNA; dCas: Catalytically inactive CRISPR-associated protein; GSDMD: Gasdermin D; GSDME: Gasdermin E; PI: Propidium iodide; OD: Optical density; IRB: Institutional Review Board.

Ethics Approval and Consent to Participate

This study involved exclusively *Saccharomyces cerevisiae* as a eukaryotic model organism and did not involve human participants or animals. Therefore, ethics committee approval and informed consent were not required.

Consent for Publication

Not applicable.

Availability of Data and Materials

Additional data are deposited into NIH GenBank with accession number PZ234365.1.

Competing Interests

The author declares no competing interests.

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Authors' Contributions

Y.Y. conceived and designed the study, developed the experimental framework, analyzed and interpreted the data, and prepared and approved the final manuscript.

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