Supplementary Note 1.

3.2.3. Advanced Computational and Experimental Strategies for Off-Target Detection and Validation

3.2.3.1 Computational Prediction

To choose the best CRISPR-Cas9 guide RNAs for genome editing, Gorodkin's group developed a computational tool that combines deep learning and binding energy models (1–6). While the binding energy model evaluates off-target risks by computing the binding affinities between the gRNA and putative off-target locations, the deep learning predictor uses a convolutional neural network to estimate on-target editing efficiency (2). CRISPR Roots, a program developed by Stefan Seemann of the University of Copenhagen, combines variant calling, off-target prediction, and RNA-seq differential expression analysis to evaluate the effectiveness of on-target editing and identify off-target impacts affecting alterations in gene expression (3). To determine possible off-target effects in genes with changed expression and evaluate the effectiveness of on-target editing, this method integrates off-target predictions, variant calling, and RNA-seq differential expression analysis. Researchers may use it to objectively assess somatic variations and differentially expressed genes linked to anticipated targets, confirming on-target alterations and prioritizing off-target locations for additional verification (3).

3.2.3.2 Experimental Off-Target Detection

University College London's Giandomenico Turchiano outlined thorough techniques for evaluating genome integrity in ex vivo gene-edited primary cells. Computing off-target prediction, chromosomal aberration screening, base editor off-target analysis, and in vitro and in-cell DNA double-strand break detection were among the technologies he discussed. He highlighted the necessity of a variety of methods by introducing MEGA, a multiplexed droplet digital PCR (ddPCR) method that concurrently finds different mutations from designer nucleases. In order to maximize the effectiveness of HDR, MEGA analyzes the amounts of episomal adeno-associated virus (AAV) DNA, provides information on DNA repair processes, and recommends RNP dosage thresholds. Along with highlighting connections between low-frequency indels and other genomic abnormalities, Turchiano shared CRISPR-Associated Site-specific Translocation sequencing (CAST-seq) results that showed complicated large deletions, massive deletions, and inversions at on-target sites, including events driven by homology and imbalance. The great sensitivity of CAST-seq, which can detect around one event per 10,000 cells, makes it more sensitive than traditional translocation assays, according to Toni Cathomen (Medical Center-University of Freiburg), who also talked about off-target analysis (7). With a linear detection range of 0.01% to 1%, CAST-seq can track changes in event frequency within this range. Cathomen's team worked with AstraZeneca to use CAST-seq on in vivo samples that had been modified using CRISPR-Cas9 that targeted *Pcsk9* using a "promiscuous" gRNA (8). Ninety of the 99 off-target sites identified by CAST-seq could

be tested using NGS, and 88 of them were verified as genuine off-targets by rhAmpSeq and CRISPECTOR validation (9).

3.2.3.3 Genomic-Instability Assessment and Translational Relevance

Comprehensive surveillance of genome integrity is a prerequisite for moving CRISPR-based therapies from bench to bedside.

Next-generation assays *CAST-seq, MEGA ddPCR, rhAmpSeq*, and *CRISPECTOR* offer orthogonal windows into on- and off-target events, capturing phenomena that span single-nucleotide indels to megabase-scale rearrangements and homology-driven translocations.

Applied together, these platforms provide near-single-cell sensitivity and quantitative insight into DNA-repair pathway bias, enabling predictive modelling of long-term genomic stability.

Initially validated in primary human T cells and hematopoietic progenitors, they are readily transferable to breast-cancer organoids and patient-derived xenografts, furnishing an advanced, clinically relevant toolkit for high-resolution safety profiling in translational CRISPR oncology **(Table S1)**.

Table S1. Key CRISPR off-target/genomic-instability assays with detection limits, model systems, and relevance to breast cancer editing

Assay	Measures	limit of detection	Validated Models	Strengths / Limits	Breast-cancer Relevance	References
CAST-seq	Translocations, large deletions/inversions at CRISPR on/off-targets	~1/10^4 cells; linear 0.01–1 %[1]	Human T cells, HEK293, HSPCs	Very high sensitivity for rare structural variants; needs deep sequencing & bioinformatics	Detects large rearrangements after CRISPR editing; adaptable to breast-cancer cell lines/PDX (patient-derived xenograft)	(10, 11)
MEGA ddPCR	Multiplex indels & HDR (homology-directed repair)/NHEJ (non-homologous end joining) balance	~0.05–0.1 %[2]	Human T cells, $CD34^+$ HSPCs	Quantifies low-freq edits; multiplex; requires custom probes	Adaptable for quantifying rare edits in breast cancer CRISPR models	
rhAmpSeq	Targeted deep-seq of predicted off-targets	~0.1–0.5 %[3]	Multiple human cell lines (e.g., HEK293)	Cost-effective for known loci; may miss unexpected sites	Validates candidate off-targets in engineered breast cancer lines	
CRISPECTOR	Genome-wide DSB (double-strand break)/indel mapping	~0.5–1 %[4]	HEK293, HSPCs (hematopoietic stem/progenitor cells)	Unbiased genome-wide detection; higher cost & depth	Enables genome-wide off-target discovery in breast cancer organoids or cell lines	

Supplementary Note 2.

4.3 Emerging CRISPR-Based Therapies

Since the initial set of CRISPR screening tests was published (12–14), because CRISPR technology allows for systematic gene function investigations in complex systems, it has completely changed the way that illness research is conducted. Through synthetic lethality (SL), in particular, CRISPR screening has accelerated the identification of novel therapeutic targets and expanded our understanding of cellular biology. This demonstrates how important CRISPR-Cas9 library screens are for identifying therapeutic targets and disease processes, which advances the development of safer, more efficient medicines, especially in cancer.

Building on these discoveries, the two main areas of CRISPR screening in drug target discovery are (1) discovering synthetic lethal genes and (2) investigating drug resistance mechanisms or finding effective combination treatments. Each application is examined in detail in the following sections.

Designing and creating sgRNA libraries, packaging lentiviral vectors to transfer sgRNAs into target cells, conducting positive or negative selection screens to associate gene edits with cell survival, analyzing the results using high-throughput sequencing and bioinformatics, and confirming candidate gene functions are all steps in the CRISPR-Cas9 library screening workflow (14,15). According to their objectives, CRISPR high-throughput screening is categorized as either positive or negative. Positive screens use selection pressure to find genes whose removal provides resistance, whereas negative screens use sgRNA depletion associated with decreased cell survival to discover important genes (16). By combining these procedures, a methodical approach to CRISPR screening is provided, enabling customization to certain research circumstances.

Immunotherapy and prognosis have a strong correlation with CD8+ T cell destiny (17–19). Without impairing their ability to operate in vivo, CRISPR-Cas9 may modify the differentiation pathways, metabolic pathways, and expression of inhibitory signaling molecules in CD8+ T cells (20). The behavior and variety of CD8+ T lymphocytes are influenced by metabolic pathways, and metabolic inhibitors function as immunomodulators in the therapy of cancer. However, the metabolic characteristics of immune cells and tumor cells are often different (21,22). Because methionine shortage causes CD8+ T cells to die and malfunction, competition for methionine between tumor cells and CD8+ T cells reduces the cytotoxicity of CD8+ T cells. Notably, tumor cells rely on *SLC43A2* for methionine absorption, while CD8+ T cells mainly use *SLC7A5*. By successfully restoring methionine availability, CRISPR-Cas9-mediated reduction of *SLC43A2* in tumor cells returns CD8+ T cell metabolism and function to normal (23,24). A major factor in determining the destiny of CD8+ T effector subsets is nutrient signaling. The amino acid transporter *SLC7A1* limits memory T cell development by modulating mTORC1 signaling, according to an in vivo CRISPR screen of metabolic regulators (24).

By targeting CD8+T cell metabolism and enhancing its general fitness, CRISPR-Cas9 can increase the effectiveness of immunological checkpoint blockades (ICBs) (12). By altering metabolic pathways, genome-wide CRISPR-Cas9 screening in mouse colorectal cancer (CRC) models revealed chondroitin sulfate synthase 1 (*CHSY1*) as a major cause of CD8+ T cell fatigue. In CD8+ T lymphocytes, *CHSY1* triggers the PI3K-AKT pathway and succinate metabolism. Liver metastases from colorectal cancer can be successfully decreased by combining anti-PD1 treatment with *CHSY1* suppression (25). By increasing metabolism, acetate supplementation improves T cell effector function and proliferation. In preclinical breast cancer models, CRISPR-Cas9 targeting of acetyl-CoA synthetase 2 (*ACSS2*) enhances effector T cell function, boosting antitumor immunity and enhancing chemotherapy results (26). Potential targets for CD8+ T cell immunotherapy have been found using CRISPR screening. By altering NF-κB signaling, the RNA helicase *Dhx37* was discovered to inhibit CD8+ T cell effector function, cytokine generation, and activation in triple-negative breast cancer. This makes *Dhx37* a viable target for immunotherapy (27). CRISPR screenings aid in determining that *PDIA3* can improve the CD8+T effector in glioblastoma (28). The ETS family transcription factor *Fli1* was discovered by CRISPR screening to be a crucial regulator restricting the function of effector CD8+ T cells. *Fli1* inhibits effector T cell activity by binding cis-regulatory elements of effector genes and reducing chromatin accessibility at ETS motifs (29). With less potential to effector cell development, CD8+T cells that lose *Fli1* are more effective against infections and cancer (30,31).

Cytotoxic CD8+ T cell depletion impairs immunological responses as cancer advances. The epigenetic regulators that drive important chromatin remodeling processes during the differentiation of fatigued CD8+ T lymphocytes may be found using CRISPR-Cas9 (32). The archetypal SWI/SNF complex, PBAF, pushes TCF+ progenitor T cells in the direction of exhaustion. When paired with immunotherapy, loss of PBAF increases the efficacy of PD-1 inhibition, improving tumor control (33). Exhausted CD8+ T lymphocytes had higher levels of the E3 ubiquitin ligase *Cblb*. CRISPR-Cas9 deletion of *Cblb* increases the generation of cytotoxic cytokines, improves tumor-killing capacity, and reduces CAR-T cell fatigue (34). Cytotoxic CD8+T cell activation and effector function are enhanced by CRISPR-Cas9-mediated deletion of the endogenous *TCRα* chain gene, which is more selective to pathologically targeted cells (35).

With the ability to precisely genetically modify T-cell treatments to improve their therapeutic activities, particularly in cancer immunotherapy, CRISPR technology holds revolutionary promise for T-cell therapies. Numerous therapeutic experiments are already producing edited T-cell products. However, further research is required to increase their cost-effectiveness, safety, and efficacy for wider clinical application.

While CRISPRa has been widely employed in preclinical research to produce gain-of-function (GOF) mutations, CRISPR technology has mostly been used to cause loss-of-function (LOF) mutations in T-Cell Receptor–Engineered T-cell (TCR-T) or CAR-T cells for treatment (36–38). Because CRISPR components must be present for an extended

period of time, safety issues are raised, which makes it less suitable for clinical settings. But base editing (39) and prime editing (40) GOF mutations may be introduced using CRISPRa without resulting in DSBs, offering a safer method of producing improved therapeutic T-cell products. Future studies should concentrate on finding targets that significantly improve T-cell activity in order to fully exploit this promise. Gaining greater knowledge about GOF mutations will help create T-cell treatments that are safer, more efficient, and have better clinical results. Recent studies have reported quantifiable efficiencies for *TP53* base editing in breast cancer. In T47D cells harboring the *TP53* L194F mutation, adenine base editors corrected the mutation with ~18–22% efficiency, partially restoring downstream *p53* pathway activity. Similarly, cytosine base editors achieved ~30% correction of the R175H *TP53* mutation in MDA-MB-231 cells, which was associated with increased apoptosis and reduced clonogenic survival. Although efficiencies remain moderate, these findings underscore the feasibility of base editing as a strategy to restore *TP53* function in TNBC (41).

Poor in vivo durability of genome-edited T cells is a significant obstacle for their use in cancer treatment. Reduced T cell survival and effectiveness may result from the patient's immune system becoming activated against CRISPR-Cas peptides, as these proteins are derived from bacterial infections, even if fatigue and the suppressive tumor microenvironment also play a part (42). According to reports, most healthy persons have both humoral and cellular immunity, and Cas9 is immunogenic (43). The efficacy of genome-edited T cells as a treatment may be diminished if this immune response results in their removal. Future genome editing techniques should concentrate on altering Cas9 to eliminate immunodominant epitopes while maintaining its gene-editing capabilities in order to solve this (43). In order to increase the longevity and therapeutic effectiveness of genome-edited T cells for cancer immunotherapy, methods to reduce the immune response against CRISPR-Cas systems should be developed.

Most contemporary T-cell therapies require ex vivo genetic editing, which is costly and time-consuming, and then the patient is transferred. To get around this, future research should concentrate on developing in vivo delivery strategies that use CRISPR components to specifically target certain T-cell populations. Because they can be designed to transport a variety of cargos and can be customized for organ- or cell-specific targeting, nanoparticles are a viable vehicle for this (44,45). A significant benefit over AAVs is the shorter time of active Cas protein expression offered by nanoparticles carrying CRISPR components for in vivo T-cell editing. Nanoparticles are a viable platform for targeted gene treatments because of their transitory activity, which enhances safety by lowering immune responses and off-target consequences. However, effectively delivering nanoparticles into T cells, which are typically nonphagocytic, remains a significant difficulty. For in vivo T-cell gene editing to advance, this obstacle must be removed (46). The full promise of personalized "living drugs," including commercially available T-cell products, can be realized when delivery methods develop, improving the accessibility and affordability of T-cell treatments.

Targeting cancer stem cells (CSCs) and therapy-resistant clones, which are major causes of tumor recurrence and treatment failure, is a promising application of emerging CRISPR-based therapeutics. CRISPR-Cas9 and similar techniques can decrease CSC survival and restore susceptibility to chemotherapy by precisely editing or silencing genes associated with drug resistance and stemness, such as *ABCB1*, *SOX2*, and *BMI1*. Because they allow for gene modification without DNA breakage, cutting-edge techniques like base editing and CRISPRi are particularly well-suited for delicate cancer cell populations. Lipid nanoparticles, tumor-targeted viral vectors, and exosomes are examples of novel delivery platforms that enhance the safety and specificity of CRISPR delivery to these difficult targets. CRISPR techniques may successfully eradicate little residual illness, stop recurrence, and improve long-term cancer patient outcomes when used in conjunction with immunotherapies or conventional treatments.

Table S2: Key oncogenic and tumor suppressor genes targeted by CRISPR-Cas9 in breast cancer, detailing subtype specificity, outcomes, experimental evidence, and clinical trial status.

Target Gene	Advantages	Disadvantages	Breast Cancer Subtype	Preclinical/Clinical Outcomes	Clinical Outcomes (trial ID & primary endpoint)	Experimental Evidence	Clinical Trial Status	Evidence Tier	Clinical Outcomes (trial ID & primary endpoint)	References
HER2	Disrupts oncogenic signaling; high specificity	Partial mutations may not fully suppress protein expression	HER2+	SKBR-3 and BT-474 cells and mouse xenografts; ↓ tumor volume and cell proliferation	None to date	*SKBR-3 and BT-474 cell growth was decreased by HER2 inactivation*	HER2 targeting shows efficacy in breast cancer cell line and xenograft models; preclinical only, no clinical trials identified	Cell line, animal (preclinical only)	CRISPR knockout of HER2 reduces growth and enhances response to antibody–drug conjugates (ADCs)	(47, 48)
TP53	Restores apoptotic pathways and targets therapy-resistant clones.	Complex mutations; low efficiency with prime editing	TNBC, HR+	T47D cells and xenografts; TP53 restoration induced apoptosis and ↓ tumor growth	Phase I trial NCT04502446 (solid tumors, incl. TP53-mutant malignancies, not breast-specific); primary endpoint: safety/feasibility	T47D cells' TP53 repair	NCT04502446 – Phase I; terminated; status last verified 2025 (TP53-linked malignancies, not breast cancer-specific)	Cell line, animal, human (Phase I – non–breast cancer specific, includes TP53-linked malignancies)	Prime-editing TP53 shows proof-of-concept for tumor-suppressor rescue despite low efficiency	(49)
ABCB1	Overcomes drug resistance and improves responses to chemotherapy and ADCs.	Off-target risks; variable expression	TNBC	TNBC cell lines; ↑ chemosensitivity and reversal of multidrug resistance; endpoint: cytotoxicity assays	None to date	ABCB1 deletion in TNBC cells	Preclinical only; no breast cancer clinical trials identified	Cell line, preclinical only	ABCB1 deletion overcomes multidrug resistance and potentiates chemotherapy efficacy	(50,51)
BRCA1	Restores DNA repair capacity and reduces	Complex mutations; HDR challenges	HR+, TNBC	BRCA1-mutant MCF-7 cells and mouse xenografts; HDR correction	Phase I trial NCT03919292 (BRCA-associated cancers); primary	*BRCA1 correction in MCF-7 cells*	NCT03919292 – Phase I; recruiting; status last verified Apr	Cell line, animal, human (Phase I – BRCA	HDR-mediated BRCA1 repair restores homologous-recombination capacity and	(52,53)

	tumor progression			restored DNA repair and ↓ tumor burden	endpoint: safety and preliminary efficacy		2025 (BRCA-associated cancers)	cancers, not breast-specific only)	suppresses tumor growth	
PTEN	Reduces tumor cell survival by inhibiting Akt signaling	Random mutations; off-target risks	HR+, TNBC	TNBC and HR^+ cell lines; PTEN re-expression ↓, AKT signaling, and ↓ tumor growth in xenografts	None to date	PTEN restoration in TNBC cells	Preclinical only; no breast cancer clinical trials identified	Cell line, preclinical only	CRISPR restoration of PTEN suppresses the PI3K/AKT pathway and limits tumor progression	(52,54)

Table S3: Improved synergistic uses of CRISPR-Cas9 in conjunction with CAR-T and ADC treatments for breast cancer, including information on subtype specificity, particular results, experimental support, clinical significance, trial status, and references. These tactics deal with resistance and enhance treatment results in TNBC and HER2-positive subtypes.

Therapy	Advantages	Disadvantages	Breast Cancer Subtype	Specific Outcomes	Experimental Evidence	Clinical Relevance	Clinical Trial Status	Evidence Tier	References
CRISPR + CAR-T (*PD-1/CBLB* Editing)	Enhances T-cell cytotoxicity and persistence through *PD-1/CBLB* knockout	T-cell exhaustion and Cas9 immunogenicity in solid tumors	HER2+, TNBC	60% tumor shrinkage and prolonged survival in HER2+ xenografts	In BT-474 models, CAR-T cells with *PD-1* deletion showed increased cytotoxicity.	Improves response rates in trastuzumab-resistant HER2+ breast cancer	NCT04650451 – Phase I/II HER2+ breast cancer trial (Discontinued, last verified 2025)	Animal (xenograft), human (Phase I/II)	(55–58)
CRISPR + ADCs (*ABCB1* Suppression)	Targets *ABCB1* to overcome drug resistance and enhance ADC efficacy	Off-target risks and limited tumor penetration	HER2+, TNBC	50% increased susceptibility of TNBC cells to trastuzumab emtansine	*ABCB1* deletion in MDA-MB-231 cells enhanced ADC response	Addresses ADC resistance in TNBC, improving progression-free survival	NCT04039230 – Phase I/II (Recruiting, last verified 2025)	Cell line, preclinical; human (Phase II ADC trials, indirect)	(59–62)
CRISPRi for Immune Modulation	Augments immune response by silencing immunosuppressive genes (e.g., TGFBR2)	Reduced efficiency due to transcriptional suppression	TNBC, HR+	40% tumor growth reduction with enhanced immune activation	CRISPRi downregulation of TGFBR2 increased T-cell infiltration in TNBC models	Enhances immunotherapy in immunosuppressive breast cancer settings	Preclinical only; no breast cancer clinical trials identified	Cell line, animal (preclinical only)	(63)
Dual CRISPR-CAR-T/ADC Strategy	Synergistically targets tumors by combining CAR-T/ADC with HER2 disruption	Delivery complexity and elevated off-target risks	HER2+, TNBC	70% tumor shrinkage in HER2+ animal models treated with both therapies	HER2 deletion combined with CAR-T/ADC therapy in SKBR-3 models	Maximizes therapeutic efficacy in TNBC and resistant HER2+ breast cancer	NCT04893109 – Phase II adjuvant (Recruiting, last verified 2025)	Cell line, animal (preclinical only)	(64–66)
Nanoparticle/Exosome Delivery	Improves delivery of CRISPR, CAR-T, and ADC	Low transfection efficiency and	HER2+, TNBC	A twofold increase in	Exosome-mediated HER2 targeting in	Reduces systemic toxicity by enabling	No CRISPR clinical trials yet	Animal, preclinical only	(67–69)

	components to tumors	scalability challenges		CRISPR delivery efficiency *in vivo*	HER2+ xenograft models	precise delivery in solid tumors			
Viral Vector Delivery	Provides effective transfection of CRISPR and CAR-T components	Immunogenicity and risk of insertional mutagenesis	HER2+, TNBC	Up to 80% editing efficiency achieved in TNBC cell lines	AAV-mediated CRISPR delivery in MDA-MB-468 cells	Facilitates scalable delivery for clinical translation	NCT04426669 – Phase I GI cancers (Completed, last verified 2025)	Cell line, human (Phase I)	(48,70)

Note: Evidence tier indicates whether findings derive from in vitro cell lines, in vivo animal models, or human clinical studies. Clinical trial statuses were verified on ClinicalTrials.gov (last check: Aug–Sept 2025).

Supplementary Note 3.

5.2 Genomic Integrity and Safety Concerns

The ability of CRISPR-Cas editing to precisely target certain gene loci, as opposed to depending on random integration, is a significant advantage over viral genome insertion. However, because the genome is so large, sequences that are identical to the target may be found elsewhere. These highly homologous off-target sites can occasionally be cleaved by Cas9 and other nucleases, according to studies; the cutting effectiveness depends on the location of the mismatch and the surrounding genomic context (71–74). To evaluate and mitigate the risk of unexpected genetic changes, off-target locations must be identified. To find highly similar regions in the genome and anticipate possible CRISPR-Cas9 off-target cleavage sites, a variety of computational techniques have been created (75–84). The prediction ability of these bioinformatic tools has been further enhanced by applying machine learning to big experimental datasets (5,85,86). However, biases associated with the reference genomes and input gRNAs that these algorithms utilize to make predictions restrict their potential. To verify real CRISPR activity at these locations, experimental screening in genome-edited cells is required once possible off-target areas have been discovered. Targeted sequencing of candidate loci is usually used for this validation; conventional sequencing panels can identify variations at a frequency as low as 5% or lower (87). However, depending on the sequencing depth, the detection limit for indels may be lower (0.2-1%) (88).

As an alternative, a number of experimental methods have been created to identify off-target locations that bioinformatic predictions that rely only on gRNA homology may overlook (89). The methodology and initial materials used in these techniques vary greatly; some map possible cleavage sites more thoroughly by employing cell-free genomic DNA in vitro (90–94), in intact live cells *ex vivo* (95–101), and *in vivo* animal models (8,99,102). The greatest number of off-targets are often reported by methods that employ DNA that is devoid of cells and nucleosomes, many of which cannot be confirmed in a cellular setting (103).

Obstacles to personalized off-target analysis include limited patient samples, logistical obstacles, and high prices. To thoroughly identify possible off-target locations, however, researchers can use a variety of techniques, including live cell and animal model studies, in silico predictions, and cell-free DNA tests. When these techniques are carefully included in therapeutic genome editing plans, unwanted alterations may be found and minimized, increasing safety and effectiveness (104).

Indeed, CRISPR-Cas9 can occasionally cause more extensive genomic changes, such as massive deletions, duplications, or translocations, in addition to localized off-target cuts. Mis-repaired DNA DSBs can result in these events (105), which may link non-adjacent chromosomal segments or alter the structure of the genome. Safe therapeutic genome editing depends on the discovery and mitigation of such rearrangements since they might have serious repercussions, such as malignant changes or genomic instability (106–108), chromothripsis (109), and aneuploidy (110,111). The most frequent causes of translocation events are: 1) Recombination with a homologous area of the genome and on-target cleavage (7); 2) cleavage of an off-target and on-target sequence at the same time (112); or 3) tracking many instances of on-target cleavage in processes that use multiplexed editing (106–108,113,114). Moreover, copy-neutral loss of heterozygosity (CN-LOH) and large deletions at either the distal end of the chromosome or near the cut site may occur (115,116).

It is still technically challenging to discover other genetic anomalies, particularly rare ones, except for big deletions. A number of specialized tests have been created to detect translocations between the on-target site and other genomic regions. By mapping unwanted genomic rearrangements more sensitively and precisely, these techniques hope to provide a more thorough evaluation of the effects of CRISPR editing (7,114,117–120).

The goal of in vivo CRISPR-based gene editing is to precisely alter certain DNA locations in targeted tissues. However, after delivery, a number of unexpected consequences might occur. These consist of: (1) altering unwanted DNA locations in the appropriate tissue (off-target, on-tissue); (2) altering the proper gene but in the incorrect tissue (on-target, off-tissue); and (3) altering unwanted DNA locations in tissues that are not the goal (off-target, off-tissue). Unintended alterations are especially concerning since they have the ability to affect germline DNA, which presents significant ethical and therapeutic problems because these alterations might be passed on to subsequent generations (121). Notwithstanding these reservations, techniques for identifying off-target Cas9 activity in vivo in animal models (8,99,108). The prevalence of unexpected genomic changes after in vivo delivery of genome editing tools in human patients has not yet been thoroughly investigated in a clinical investigation. For instance, off-target effects were only evaluated ex vivo using GUIDE-Seq in isolated hepatocytes in the seminal work utilizing *TTR*-targeting Cas9 administered by LNPs. This left a substantial knowledge vacuum about actual off-target hazards in the clinical situation (122). Despite the fact that this method helps identify potential off-target locations in the patient's genome, in vivo

validation of these results is lacking. To measure the frequency of on-target and off-target edits within the liver tissue itself, routine clinical liver biopsies could have been performed both before and after treatment, possibly from locations near and far from the hepatic artery (123), which is where the LNPs enter the liver. Although there is evidence that the identical LNPs caused detectable gene modifications in the ovaries, spleen, and adrenal glands, this approach offers little insight into CRISPR activity in other organs (124). While liver biopsies are widely available and often performed, many other organs, particularly delicate ones like the ovaries, do not typically undergo comparable sampling. This restriction makes it extremely difficult to thoroughly evaluate and guarantee the safety of CRISPR-based treatments given *in vivo* (125).

Table S4. Bridging Biological Complexity and Computational Precision: A Validation Blueprint for Predictive AI–Organoid–CRISPR Oncology

Focus Area	Main Limitation	Impact	Critical Examination	Proposed Innovation / Validation Step	Outcome	References
Tumor Microenvironment	PDOs lack immune, vascular, and stromal elements	Overestimates drug response	PDOs lack stromal–immune interaction and natural perfusion.	Ecological triangulation using co-cultures, organ-on-chips, and PDXs	Restores real tumor context	(126–131)
Immune Interactions	No active T-cell or macrophage signaling	Missed immune resistance patterns	Lack of immune cells hinders modeling of immune responses.	Add CAF/TAM/T-cell co-cultures, cytokine mapping	Better immune-response prediction	
Tumor Evolution	Clonal drift and epigenetic decay with passages	Model aging, false positives	Repeated passages distort tumor identity and reduce reproducibility.	Temporal robustness testing over therapy cycles	Captures long-term adaptation	
AI Model Reliability	Domain shift, overfitting, poor calibration	Inflated accuracy	Static data miss's tumor dynamics, limiting temporal accuracy.	Pre-registered, blinded design; external validation sets	True generalization, reduced bias	
Interpretability & Safety	Black-box models, unseen off-target edits	Erroneous targets or genotoxic risk	Poor interpretability and safety checks hinder clinical translation.	AI transparency metrics + CAST-seq & deep-seq auditing	Reliable, safe translation	
Clinical Predictivity	Disconnected from patient evolution	Poor relapse forecasting	Lack of long-term data limits resistance modeling.	Digital-twin pilots integrating liquid biopsies	Real-time alignment with patient data	

Table S5. Translational Barriers to CRISPR Breast-Cancer Therapy and Practical Mitigations

Challenge (Counter-Evidence)	Representative Findings	Next-Generation Mitigation	References
Low editing efficiency in solid tumors	<10 % on-target editing in orthotopic TNBC xenografts using standard SpCas9 RNPs.	Programmable lipid nanoparticles (LNPs) with tumor-microenvironment triggers (hypoxia + acidosis); AI-guided sgRNA redesign using spatial transcriptomics to target dominant clones.	(132)
Mosaicism and clonal heterogeneity	Single-cell multi-omics reveals mixed edit profiles and sub-clone–specific resistance in TNBC organoids.	Iterative, real-time gRNA updating driven by longitudinal ctDNA and single-cell sequencing; multiplexed Cas12a arrays to edit multiple resistance nodes simultaneously.	(133)

Cas9 immunogenicity	Pre-existing anti-SpCas9 antibodies were detected in >50 % of healthy donor sera.	Epitope-silent Cas9 variants engineered by deep mutational scanning; transient self-degrading RNPs that clear within 24 h.	(134)
Off-target translocations & large deletions	CAST-seq detects rare translocations (~1 per 10^4 cells) and kilobase-scale deletions in primary human cells.	Patient-specific off-target maps combining GUIDE-seq and long-read sequencing; prime/base editors to avoid double-strand breaks altogether.	(11,135)
Triple-negative breast cancer (TNBC) heterogeneity	Spatial transcriptomics shows the coexistence of basal-like, mesenchymal, and luminal subpopulations within a single tumor.	Adaptive AI dosing algorithms to re-rank targets during therapy; logic-gated exosome mimetics that release cargo only when multi-factorial cues (e.g., hypoxia + EGFR↑ + acidic pH) are present.	(136)

Supplementary References:

The following references provide additional supporting information.

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