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The NTR/Prodrug Revolution: Tools for Controlling Cell Loss and Regeneration

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eLife Assessment

This Review Article nicely synthesizes the development, applications, and recent technical advances of the nitroreductase/prodrug system, highlighting how it enables precise spatiotemporal cell ablation and experimental platforms for studying regenerative mechanisms and screening for pro-regenerative or protective compounds. Together, the article provides a conceptual and practical overview that will help researchers adopt and further develop this versatile approach in regenerative biology. It will be of interest to researchers studying regeneration, disease modelling, and targeted cell ablation, particularly those working with zebrafish and other genetic model systems.

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Abstract

Here, we review the history, advancements, and broad utility of the NTR/prodrug system, and suggest future strategies for developing versatile ablation models. As a chemogenetic tool, the nitroreductase (NTR)/prodrug system enables precise spatiotemporal control over cell ablation. The technology leverages bacterial nitroreductase enzymes (e.g., *nfsB*) to convert inert prodrugs into cytotoxic agents, thereby allowing researchers to induce targeted cell death. Although the NTR/prodrug approach was first implemented in transgenic mice, it was subsequently adapted to zebrafish, where it has been extensively optimized and applied. Consequently, zebrafish remain the primary focus of this review. Nevertheless, the utility of the NTR/prodrug system has expanded to other important model organisms, including *Drosophila*, *Nematostella*, *Xenopus*, medaka, and rats, enabling detailed studies of tissue damage and regeneration. This review highlights how the NTR system has been deployed to model a spectrum of human diseases, including Parkinson's disease, retinal degeneration, demyelinating disorders, and kidney disease. These models provide valuable platforms to study pathogenesis *in vivo*. Furthermore, the precise and controllable nature of NTR ablation makes it an ideal tool for high-throughput chemical and genetic screens aimed at discovering pro-regenerative and protective compounds. The development of NTR2.0, an enzyme variant with over 100-fold greater activity, along with more potent prodrugs such as ronidazole (RNZ), has dramatically broadened experimental possibilities. These improvements permit chronic ablation and long-term disease modeling at well-tolerated drug concentrations. Here we present some key considerations including transgenic design for optimal cell-type specificity, calibrating expression levels for desired ablation kinetics, and suitable controls to allow interpretation. These best practices will allow the researcher to develop a precise, reproducible, and versatile platform for either modeling human disease or dissecting regenerative mechanisms.

Introduction

Why Regeneration?

We often regard ourselves as having limited regenerative capacity, yet numerous human tissues exhibit substantial renewal: the liver restores lost mass, the skin undergoes continuous stem cell-driven turnover, skeletal muscle repairs through satellite cells, bone heals via remodeling, blood is replenished by hematopoietic stem cells, the intestinal epithelium renews within days, and the endometrium regenerates cyclically. However, the regenerative capacity of human tissues is modest when contrasted with that of many other species, in which entire organs or appendages can be restored following injury [1,2]. This capacity is profound in many invertebrates; for instance, hydra and planarians can regenerate complete organisms from minute fragments [3].

Understanding these enviable examples provides a framework for uncovering the cellular and molecular mechanisms that organisms employ to repair or replace damaged tissues, with the ultimate goal of developing new strategies to combat human injuries, degenerative diseases, and age-related decline [4,5]. An essential requirement for this research is the ability to induce controlled, reproducible injury to study the subsequent repair processes.

The laboratory mouse *(Mus musculus)* is a critical vertebrate model for human biology and has been essential for developing inducible, cell-type-specific genetic tools in mammals. Like adult humans, adult mice exhibit regenerative and reparative abilities across several tissues; however, their capacity for epimorphic regeneration (regrowth of complex structures such as heart tissue or appendages) is largely confined to early postnatal stages. For developing precise ablation tools, a genetically tractable and cost-effective vertebrate with robust adult regeneration is desirable. The zebrafish (*Danio rerio)* is uniquely suited for this role. Its external development, transparency, and high fecundity facilitate direct *in vivo* visualization and high-throughput screening [6]. Critically, zebrafish exhibit widespread regenerative abilities in adulthood, fully restoring complex organs including the heart [7,8], retina [9,10], spinal cord [11,12], and fins [13,14]. These attributes make zebrafish a practical system for dissecting the mechanisms of vertebrate regeneration [15].

Methods of Ablation in Zebrafish

Regeneration studies rely on experimental injuries, and the nature of the injury profoundly shapes the ensuing repair response. In zebrafish, the major injury paradigms can be broadly grouped into the following categories:

1. Physical Injury: Direct tissue damage via surgical intervention that have been used successfully to study adult regeneration, including fin amputation [16], ventricular resection [7], brain lesioning [17], scale removal [18,19] and transection of both tendons [20] and ligaments [21]. Severing of the spinal cord predictably leads to paralysis in zebrafish, but as testament to their regenerative capacity, full mobility is returned by 8 weeks [11,22,23]. Several non-surgical physical methods can be used to induce tissue damage in animal models, with their suitability largely determined by the subject's size and developmental stage. In adult models, approaches include cryoinjury to simulate myocardial infarction [24,25], intense light exposure to trigger photoreceptor degeneration in retinal regeneration studies [26], and acoustic trauma to model inner-ear damage and hearing loss recovery [27,28]. The acoustic method has also been applied to larval zebrafish to target lateral line hair cells [29]. Larval and embryonic zebrafish, owing to their small size and optical transparency, permit highly precise laser ablation of single neurons [30–32] or even individual cardiomyocytes [33]. Additionally, thermal injury has been employed in larvae to model burns and skin regeneration, revealing rapid inflammatory cell recruitment [34] and keratinocyte migration, but impaired sensory axon regeneration compared to mechanical injury [35].

Regardless of the method, each of these physical injury approaches perturbs tissue integrity in characteristic ways, disrupting multiple components of tissue architecture, including extracellular matrix, mesenchymal organization, and local vasculature. This disruption drives

rapid physiological and robust inflammatory responses involving neutrophils and macrophages, all of which critically influence regenerative outcomes [34–36].

2. Cell-Specific Toxins: While physical injury has provided key insights into regenerative biology, these approaches are often constrained by limited precision and cell-type specificity. Pharmacological methods offer a complementary strategy, using small molecules to induce targeted, dose-controlled damage. Table 1 summarizes representative compounds used for cell-specific ablation in zebrafish. However, many of these agents introduce off-target effects, ranging from dose-dependent systemic toxicity to unintended tissue injury, which can make downstream regenerative responses harder to interpret. For example, MPTP and MPP^+ mainly target dopaminergic neurons but can also impact noradrenergic and serotonergic systems via uptake through their neurotransmitter transporters [37,38]. Similarly, aminoglycoside antibiotics, while commonly used to ablate lateral line hair cells, can also damage support cells and afferent neurons at higher concentrations, and exhibit compound-specific differences in ototoxicity [39–42]. Streptozotocin, widely used to ablate pancreatic β-cells, is known to cause broader cytotoxicity beyond the target population [43]. To achieve higher precision and reduce off-target effects as well as expand the kinds of cell that can be ablated, the field has increasingly turned to genetic strategies. These methods allow damage to be targeted with exquisite specificity to predefined cell types.

3. Optogenetic cell ablation: Due to the transparent nature of young zebrafish, this model is highly amenable to optogenetic techniques that enable precise, non-invasive control of cellular signaling, neuronal circuit activity, and targeted cell ablation (see reviews [52,53]). KillerRed is a genetically encoded photosensitizer that produces reactive oxygen species (ROS) upon green or yellow light illumination, killing nearby cells [54,55]. When expressed under cell-specific promoters, this technique allows researchers to eliminate defined cell populations in living embryos or larvae with precise spatiotemporal control [54–56]. A key advantage of this method is its speed, as KillerRed-expressing cells can be ablated within hours of light exposure. This approach is particularly useful for modeling diseases in which ROS-mediated cell death is central, such as neurodegeneration [57] and cardiomyopathies [58], but it has not yet been applied in conventional regeneration studies. Although speculative, this method appears best suited for discrete, optically accessible cells [54] and is likely to be impractical for ablation of large populations, whole tissues, or cells deep within the adult body, where localized illumination is more difficult. Because this method relies on controlled optical delivery via microscopy, it also limits throughput making high-volume studies challenging.

4. Chemogenetic, cell-specific ablation: Chemogenetic ablation relies on transgene-driven, cell-specific expression of an exogenous protein that converts an otherwise innocuous chemical into a cytotoxic agent. By eliminating only the intended population and leaving neighboring tissues intact, these methods generate narrowly focused injuries whose regenerative responses differ substantially from those induced by broader, multi-tissue physical damage. Four such chemogenetic systems established in zebrafish include:

i. Human Diphtheria Toxin Receptor and Diphtheria toxin: The human pathogen *Corynebacterium diphtheriae* produces diphtheria toxin (DT), which enters cells by binding the human diphtheria toxin receptor (hDTR) [59,60]. Because endogenous receptors in non-primates do not bind DT efficiently, these animals are naturally resistant to the toxin. This strategy allows researchers to engineer specific cell types to express hDTR, making them uniquely susceptible to DT-induced ablation [61]. In mouse research, the DTR/DT system has become one of the most commonly employed strategies for conditional cell ablation, owing to its rapid and reliable elimination of targeted cell populations across many tissues. However, the method has caveats, like the fact that DT alone can cause kidney damage [62] and that repeated DT exposure induces production of neutralizing antibodies, preventing long-term or chronic ablation.

Although DTR/DT approaches are widely used in mouse studies, their application in zebrafish has been more limited [63,64]. A noteworthy example is the transgenic line generated by Jimenez et al., in which hDTR driven by the hair-cell-specific myo6b

promoter enabled selective depletion of sensory hair cells following DT administration, with full regeneration occurring within days [63]. Some studies in zebrafish have instead expressed diphtheria toxin subunit A (DTA) directly in target cells [65,66], although constitutive DTA expression lacks temporal control and is not suitable for regeneration studies. Temporal control can be introduced through Cre/lox-inducible DTA systems [67] but issues such as recombination efficiency, mosaicism, and promoter leakiness may limit precision [68].

ii. Inducible caspase systems: Caspase cascade in apoptosis begins with upstream initiator caspases (e.g., caspase 8) activated by dimerization, which then activates effector caspases (e.g., caspase 3) to execute cellular dismantling [69–71]. In zebrafish, researchers have exploited induction of caspase-8 dimerization to achieve temporal and spatial control of cell-specific ablation in two main ways: 1) activated by the FK1012 chemical inducer of dimerization [64,72]; and, 2) expression of a fusion between caspase and the modified estrogen receptor ligand-binding domain (ERT2), whose activity is induced by binding of tamoxifen [73–75]. Compared to FK1012, tamoxifen pharmacokinetics are better characterized in zebrafish, and a convincing study showing lifelong regeneration of cerebellar Purkinje cells in zebrafish strongly supports the use of the ER-T2/tamoxifen inducible ablation approach for probing cell loss and recovery [73].

iii. HSV-TK: The herpes simplex virus thymidine kinase (HSV-tk) has been applied in zebrafish for conditional cell ablation. Transgenic expression of this 'suicide gene' (here defined as a gene encoding an enzyme that converts a nontoxic prodrug into a cytotoxin [76]) in a defined cell population converts the antiviral prodrug ganciclovir into toxic nucleotides that are lethal to proliferating cells, but this dependence on cell division has limited the utility of the approach [77].

iv. Nitroreductase (NTR): Bacterial nitroreductase genes (nfsB) are another class of suicide genes. These genes encode nitroreductases (NTR), enzymes that convert nitro-containing prodrugs (e.g., nitroimidazoles and nitrofurans) into cytotoxic metabolites. As a result, transgenic animal cells expressing NTR become vulnerable to treatment with prodrugs such as metronidazole (MTZ), ronidazole (RNZ), nifurpirinol (NFP) and CB1954. Unlike HSV-TK, the NTR/MTZ system operates as a 'cell-cycle-independent' method for targeted cell ablation and improved variants of NTR can efficiently ablate fully differentiated cell types [78,79].

Toxin	Target Cell Type	Tissue/Organ	Key Limitation	Ref.
Acetaminophen	Hepatocytes	Liver	Dose-dependent toxicity with systemic side effects.	[44]
Aminoglycosides (neomycin, gentamicin)	Sensory hair cells	Lateral line, inner ear	Differential effects by compound. Incomplete ablation at some doses.	[39–42]
Caerulein	Acinar cells	Pancreas	Induces pancreatitis and leads to destruction of adjacent tissue	[45,46]
Cisplatin	Sensory hair cells	Lateral line, inner ear	Damages support cells and delays regeneration. Nephrotoxicity and ototoxicity.	[47]
Copper sulfate (CuSO4)	Sensory hair cells, support cells	Lateral line	At higher doses also damages support cells and afferent neurons, impairing regeneration.	[48]
6-Hydroxydopamine (6-OHDA)	Dopaminergic neurons	Brain	Broad catecholaminergic toxicity. May require direct injection in some models.	[37]
Ouabain	Retinal neurons	Retina	Dose-dependent and can damage multiple retinal cell layers.	[49,50]
MoTP	**Melanocytes**	**Skin (pigment system)**	Ablation is developmentally restricted	[51]
MPTP / MPP^+	Dopaminergic neurons	Brain	Species-dependent metabolism. Strict handling required. Off-target effects.	[37]
Streptozotocin (STZ)	Pancreatic [β-cells	Pancreas	Off-target effects, e.g. hepatotoxicity	[43]

The NTR/prodrug system is now a staple of zebrafish research, although, somewhat surprisingly, the approach was first developed in the mouse. Since then, it has been adapted for use in a wide range of model organisms, including:

- **Drosophila melanogaster** — Teeters *et al.* used NTR and RNZ to ablate multiple, diverse cell types during development, demonstrating rapid, temperature-independent ablation using a simple drug-feeding protocol [80].
- **Nematostella** — Gavgani *et al.* used NTR and NFP to ablate neurons and reveal their requirement in body-axis regeneration [81].
- **Xenopus laevis** — Two NTR/MTZ models, one targeting oligodendrocytes and the other rod photoreceptors, achieved reliable, cell-specific ablation with subsequent regeneration. One targeting study noted temperature-dependent NTR activity, with reduced efficiency below 22 °C [82–85].
- **Medaka** — Willems *et al.* used NTR and MTZ to conditionally ablate osteoblasts and assess regeneration following drug withdrawal [86].
- **Rat** — Kwak *et al.* applied NTR and CB1954 to ablate neonatal cerebellar and ventricular progenitors, resulting in ataxia and reduced cerebellar volume [87].
- **Zebrafish** — *The most extensively used model for NTR/prodrug-mediated cell ablation, with applications across a wide range of tissues to study regeneration and to model human disease* (Figure 1 and Supplemental Table 1).
- **Mouse** — The first *in vivo* NTR/CB1954 transgenic models were generated in mouse, where NTR expression enabled selective ablation of T cells, and mammary luminal epithelial cells [88,89]. These initial studies were followed by ablation studies of adipocytes, neurons, and kidney podocytes, showing the versatility across diverse tissues of this ablation method [90–92]. However, most of these efforts were carried out in the context of evaluating NTR as a suicide-gene strategy for cancer therapy in humans, rather than a tool for studying cell regeneration. Subsequent work in mammals shifted toward the DTR system, which is the predominant method of chemogenetic, cell-specific ablation in mouse.

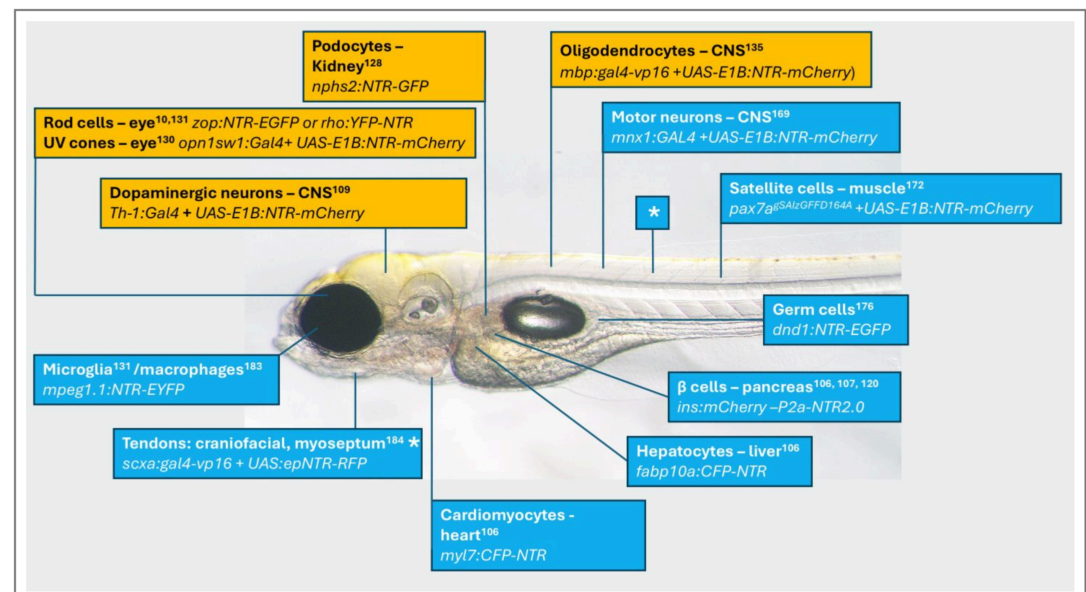

Figure 1. Commonly targeted cell types for ablation studies in zebrafish. Supplemental Table 1 for more complete list. Image of a 5 days post-fertilization (dpf) casper zebrafish larva[182] with approximate position of cell type ablated. Name of cell, and transgene provided along with reference. Orange highlights indicate transgenic models used to study human pathologies. * same line two different tissues.

Development of Nitroreductase as a Suicide Gene

Bacterial nitroreductases (NTRs) from *E. coli (nfsA* and *nfsB*) were first charaderized in the 1970s-80s for their role in reducing nitroaromatics [93–95], a function later harnessed in the 1990s for Directed Enzyme Prodrug Therapy (DEPT) [89,96,97]. DEPT strategies use viral or antibody-based systems to deliver a 'suicide gene' product to tumors (e.g. NTR) [98]. Once localized, the enzyme converts an administered prodrug into a cytotoxic agent, selectively killing the cancer cells.

The prodrug CB1954 is harmless to human cells but becomes a powerful, DNA-damaging toxin after activation by NTR [99,100] Even though the NTR/CB1954 approach has been tested in clinical trials [101,102], its effectiveness in mitigating cancers is limited due to low NTR activity and slow prodrug metabolism. Despite these therapeutic shortcomings, this work established NTR/CB1954 as a potent conditional cell-killing strategy. However, this system is subject to a "bystander effect," where activated metabolites diffuse into and kill neighboring cells. This attribute reduces the precision of targeted ablation and introduces ambiguity when assessing subsequent regeneration.

To overcome these limitations, researchers turned to alternative prodrugs. Metronidazole (MTZ), a nitroimidazole antibiotic, emerged as a particularly effective option as it is non-toxic to eukaryotic cells until reduced by bacterial NTR. Unlike CB1954, the activated metabolites of MTZ are short-lived and largely confined within the target cell, minimizing bystander effects [103–105]. This property made the NTR/MTZ system especially well-suited for regeneration and developmental studies, which benefit from precise, cell-specific ablation. In zebrafish, NTR/MTZ-mediated ablation consistently induces apoptosis across multiple tissues, as first shown in hepatocytes, cardiomyocytes, and pancreatic β-cells [106,107]. Across tissues, NTR/MTZ ablation elicits stress-linked apoptotic pathways, with hepatocytes showing elevated ROS and dopaminergic neurons exhibiting early mitochondrial impairment, both culminating in apoptosis [108,109]. The exact *coup de grâce* will likely vary by cell type, reflecting differences in metabolic activity, mitochondrial content, and intrinsic sensitivity to oxidative or genotoxic stress. Ablation kinetics are similarly context-dependent: NTR expression levels, cell identity, developmental stage, and MTZ dose all influence how rapidly and completely cells are eliminated. Defining how these variables shape NTR/MTZ-induced cytotoxicity across tissues remains an important direction for future work.

NTR/MTZ in Regenerative Studies

The NTR/MTZ system for cell-specific ablation was first described in two 2007 studies. Pisharath et al. placed the *E. coli* nfsB gene (NTR) under the zebrafish insulin promoter to express an NTR-mCherry fusion in pancreatic β cells; treatment with 10 mM MTZ produced complete β-cell loss without affecting neighboring α cells or exocrine tissue [107]. Curado et al. used cell-specific promoters to express CFP-NTR in cardiomyocytes, hepatocytes, and β cells, likewise demonstrating that MTZ induced highly targeted cell death with no detectable bystander effects [106]. Of note, NTR functions robustly whether fused to the N or C terminus of a fluorescent reporter, facilitating flexible transgene design [104,110]. In both studies, tissues regenerated after MTZ withdrawal, establishing the NTR/MTZ approach as a versatile, specific, inducible, and reversible tool for regeneration studies.

Unlike acute physical injury, NTR-mediated ablation induces apoptotic cell death and predominantly recruits macrophages rather than neutrophils [108]. This is an important consideration, as the type of cell death shapes the regenerative response. In zebrafish, macrophages are not merely phagocytic responders but essential regulators of regeneration: they clear apoptotic debris, modulate the inflammatory milieu, and release factors that support appropriate tissue remodeling [111,112].

This controlled context of NTR/MTZ ablation provides a platform for identifying the cells responsible for regeneration, a goal that can be achieved by pairing ablation with lineage tracing. For example, following targeted ablation of pancreatic β-cells, researchers used Cre-based lineage tracing to track the origins of regenerated endocrine cells. These studies revealed that Notch-responsive ductal cells called centroacinar cells (CACs) act as facultative progenitors that

delaminate from the ducts, and replenish lost β-cells [113–115]. A parallel strategy applied to hepatocyte ablation uncovered an analogous regenerative mechanism in the liver where Notch-responsive biliary epithelial cells (BECs/cholangiocytes) delaminate, dedifferentiate, proliferate, and redifferentiate into new hepatocytes [116,117]. Combining single-cell RNA sequencing (scRNA-seq) of pancreatic ducts and hepatic ducts after β-cell/hepatocyte ablation has been used to map molecular mechanisms and identify intermediate progenitor states as new β-cells/hepatocytes are formed [118,119]. This integrated paradigm of targeted ablation, cre-based lineage tracing, and scRNA-seq provides a systematic framework for dissecting the mechanisms that drive tissue regeneration.

Combining Cre-based lineage tracing with NTR ablation can reveal the origins of regenerated cells. Another way to identify progenitors is to induce regeneration and then use NTR to ablate candidate progenitor populations, assessing whether regeneration is subsequently impaired. The Raible lab used two complementary ablation approaches to pinpoint the cells responsible for regeneration [40]. Neomycin, an aminoglycoside antibiotic, reliably ablates mature hair cells [39], which normally regenerate fully. In parallel, the NTR/MTZ system was used to selectively ablate dorsoventral (DV) support cells, which were suspected to act as progenitors. When both the mature hair cells and DV support cells were eliminated, regeneration was dramatically impaired [40]. This dual-ablation strategy demonstrates another way the NTR/prodrug system can be used to identify the cell populations that contribute to regeneration.

NTR/Prodrug – Dependent Ablation in Modeling Human Disease

The NTR/prodrug ablation system also lends itself well to modeling human diseases that are characterized by the specific and progressive loss of distinct cell populations. The core strengths of this chemogenetic approach include cell-type specificity, and temporal control which allows it to be a toolkit for recapitulating pathological events *in vivo*. This allows for the real-time dissection of disease initiation, progression, and complex cellular responses to injury. A diverse array of human pathologies has been modeled using NTR/MTZ in zebrafish, including chronic hyperglycemia (a symptom of diabetes) [120], acute liver damage [116], and cardiac injury [106,121,122]. Here, we analyze selected models in greater detail, focusing on kidney disease, retinal degeneration, demyelinating disorders, and neurodegeneration. (Figure 1 – orange boxes).

Kidney Glomerular Disease

Glomerular diseases stem from a common problem: progressive podocyte loss or dysfunction. These cells are crucial for maintaining the kidney's filtration barrier and their damage leads to proteinuria, the leakage of abnormal amounts of protein into the urine [123]. To model this pathology, a podocyte-specific NTR mouse line was generated, in which NTR expression under the *podocin* promoter enables inducible podocyte injury [92]. Administration of CB1954 triggered acute podocyte damage with proteinuria and leads to progressive focal segmental glomerulosclerosis, providing a mammalian proof-of-principle for NTR/CB1954-mediated podocyte ablation, though this approach was never widely adopted. Subsequent mouse studies of podocyte ablation instead used DTR-based approaches [124–126], even though DT alone can induce transient podocyte injury and proteinuria in wild-type mice [62].

Other investigators employed the NTR/MTZ to induce podocyte-specific loss in zebrafish. Researchers used the *nphs2* promoter to drive NTR in transgenic zebrafish (nphs2:NTR-GFP), enabling precise, inducible podocyte ablation.

[127,128] Administering MTZ triggered rapid podocyte apoptosis, resulting in classic features of human glomerular injury, including disruption of the filtration barrier, proteinuria, and edema. By mirroring these essential aspects, this model provides a direct and relevant system for studying the progression of human podocytopathies. Furthermore, the zebrafish pronephros allows live imaging of podocyte injury and subsequent regeneration [127]. After MTZ withdrawal, podocyte repopulation occurs through residual cells and local progenitors [128]. This makes the model an ideal platform for studying podocyte repair and uncovering pathways with therapeutic relevance.

Retinal Degeneration

Given the high conservation of eye structure between zebrafish and humans, the NTR/MTZ system provides an excellent platform for modeling inherited retinal degenerations such as retinitis pigmentosa and cone dystrophies, conditions in which progressive photoreceptor loss leads to vision decline [129]. By driving NTR expression under photoreceptor-specific promoters, distinct photoreceptor subtypes can be selectively ablated. For example, expression under the rhodopsin (rho) promoter enables targeted elimination of rod photoreceptors, providing a robust zebrafish model of retinitis pigmentosa [10]. Similarly, the use of cone opsin promoters such as *opnlswl* permits ablation of defined cone populations to study cone dystrophies [130]. This targeted ablation triggers apoptotic photoreceptor loss while sparing neighboring retinal cells [131]. A major advantage of the zebrafish system is its capacity for spontaneous retinal regeneration, driven by the dedifferentiation and proliferation of Müller glia that give rise to new photoreceptors [132,133]. The NTR/MTZ paradigm allows for precise initiation and synchronization of this regenerative process, enabling realtime dissection of the cellular and molecular programs underlying photoreceptor replacement and the contributions of innate immune signaling to retinal repair [83].

Demyelinating Disorders

Conventional autoimmune models of Multiple Sclerosis (MS), including Experimental Autoimmune Encephalomyelitis (EAE), often display substantial variability in the timing and severity of disease onset, alongside a highly complex and multifactorial immunopathology [134]. This inherent heterogeneity makes it difficult to disentangle the individual cellular and molecular events that specifically contribute to successful remyelination, thereby limiting the ability to clearly define the mechanisms required for effective tissue repair. To overcome these hurdles, NTR-MTZ based models have been utilized to ablate oligodendrocytes and their progenitors. Tg(*mbp:gal4-vp16*); Tg(*UAS-E1B:NTR-mCherry*) fish express NTR specifically in mature oligodendrocytes that myelinate CNS axons. Exposure to MTZ in these fish caused rapid and synchronized demyelination within 48 hours, characterized by the retraction of myelin sheaths and oligodendrocyte cell death [135]. Furthermore, subsequent regeneration resulted in myelin sheaths that restored normal length and thickness correlated to axon caliber [136]. This mechanistic parallel is highly relevant, as the failure to restore proper myelin architecture is a central hallmark of progressive disability in human demyelinating diseases [134].

Dopaminergic Neurodegeneration

Traditional genetic models of Parkinson's Disease (PD) often exhibit weak or late-onset phenotypes. Neurotoxin-based models frequently induce off-target neuronal loss, and the compounds themselves pose safety risks to researchers, restricting their use in scalable or high-content screening applications [37,38]. To overcome these hurdles, Kim *et al.* utilized a chemogenetic model in zebrafish to abate dopaminergic (DA) neurons [109] NTR expression was driven from the *tyrosine hydroxylase (th)* promo ter (the *th1* gene encodes an enzyme required for dopamine synthesis). *Th:NTR* fish express NTR1.0 in the DA neurons of the ventral forebrain, the zebrafish homolog of the mammalian substantia nigra.

Exposure to MTZ in *th:NTR* fish caused pronounced mitochondrial damage within DA neurons, including mtDNA damage, impaired mitochondrial function, reduced organelle motility, and altered morphology, ultimately resulting in neuron loss [109]. The finding that NTR/MTZ ablation kills dopaminergic neurons through mitochondrial dysfunction is particularly significant, as mitochondrial impairment is a central pathological hallmark of human PD [137]. By recapitulating this key feature, the *th:NTR* model moves beyond a simple cell-elimination system to one with strong disease relevance, providing a robust, scalable, and experimentally tractable platform ideally suited for screening small molecules that protect DA neurons or modulate PD-associated pathways. Furthermore, this work remains one of the few detailed mechanistic investigations of

NTR/MTZ-mediated cytotoxicity in a defined zebrafish neuronal population, indicating that mitochondrial injury, rather than early nuclear DNA damage, plays a major role in driving NTR/MTZ-induced dopaminergic neuron death.

NTR/Prodrug – Based Screening

The NTR/MTZ ablation system provides a reproducible and scalable platform for functional screening in zebrafish, combining cell-type specificity, quantitative imaging, and compatibility with both chemical and genetic perturbations (Figure 2).

Small-Molecule Screening

The NTR/MTZ ablation system has been adapted for high-content chemical screening in zebrafish, allowing quantitative evaluation of compound effects on cell death, protection, and regeneration across diverse tissues. In the context of retinal degeneration [138], Mumm and colleagues demonstrated the high-throughput capabilities of the system by screening 2,934 compounds using the *Tg(rho:YFP-NTR)* model of retinitis pigmentosa. By driving NTR specifically in rod photoreceptors, the lab induced targeted cell death and screened for small molecules that could preserve YFP-positive cells despite MTZ exposure. This large-scale effort identified 11 validated neuroprotectants (distinct from simple antioxidants) that were subsequently shown to have conserved efficacy in mouse retinal explant assays [138]. This cross-species validation confirms that the zebrafish NTR system effectively filters for compounds with relevant translational potential for human blindness.

Kim *et al.* (2022) utilized the *Tg(th:NTR)* model to perform a 1,403-compound screen for Parkinson's disease. By integrating automated imaging with rigorous statistical metrics including the Brain Health Score (BHS) and the Strictly Standardized Mean Difference (SSMD), the researchers identified 57 compounds that preserved dopaminergic neurons (Figure 2A-B). Importantly, the study advanced beyond simple measurements of cell survival and provided mechanistic validation that these compounds protected neurons by restoring mitochondrial function, which is a central hallmark of PD pathology. The predictive validity of these hits was further confirmed through cross-assay validation in a separate Gaucher disease behavior model, demonstrating the system's capacity to identify robust therapeutics for complex neurodegenerative conditions (Figure 2D) [109].

Another promising application of the NTR system lies in the identification of therapeutic agents that actively promote tissue regeneration. Lee *et al.* (2025) leveraged an optimized QF-based binary expression system (*mbpa:qf2;quas:epNTR-P2A-mCherry*) to perform a remyelination phenotypic screen for regenerative compounds. This transgenic line achieved greater than 85% oligodendrocyte loss following treatment with 2 mM MTZ for 18 hours, creating a highly reproducible regenerative baseline. Using this platform to screen a kinase-inhibitor library, the authors identified the TGF-β receptor I inhibitor AZ-12601011 as a potent driver of remyelination [139]. Mechanistic validation revealed that this compound promotes repair by modulating microglial and progenitor activation, thereby confirming the system's predictive validity for discovering clinically relevant restorative therapeutics that actively drive the reconstruction of functional tissue.

Similar regenerative screens have been successfully implemented in other tissues, such as the pancreas. Andersson *et al.* (2012) utilized the *Tg(ins:CFP-NTR)* line, crossed with a *Tg(ins:Kaede)* reporter to induce complete β-cell ablation and then monitor the formation of new β cells. This model was used in a high-content screen of approximately 7,000 small molecules to find compounds that would enhance regeneration of the insulin producing β cells [140]. This screen identified adenosine receptor agonists, specifically NECA, as potent stimulators of endocrine regeneration. Detailed mechanistic characterization revealed that NECA signals via the A2aa receptor to specifically enhance the proliferation of regenerating β-cells rather than neogenesis, a therapeutic pathway that was subsequently validated to restore normoglycemia in a streptozotocin-induced diabetic mouse model [140].

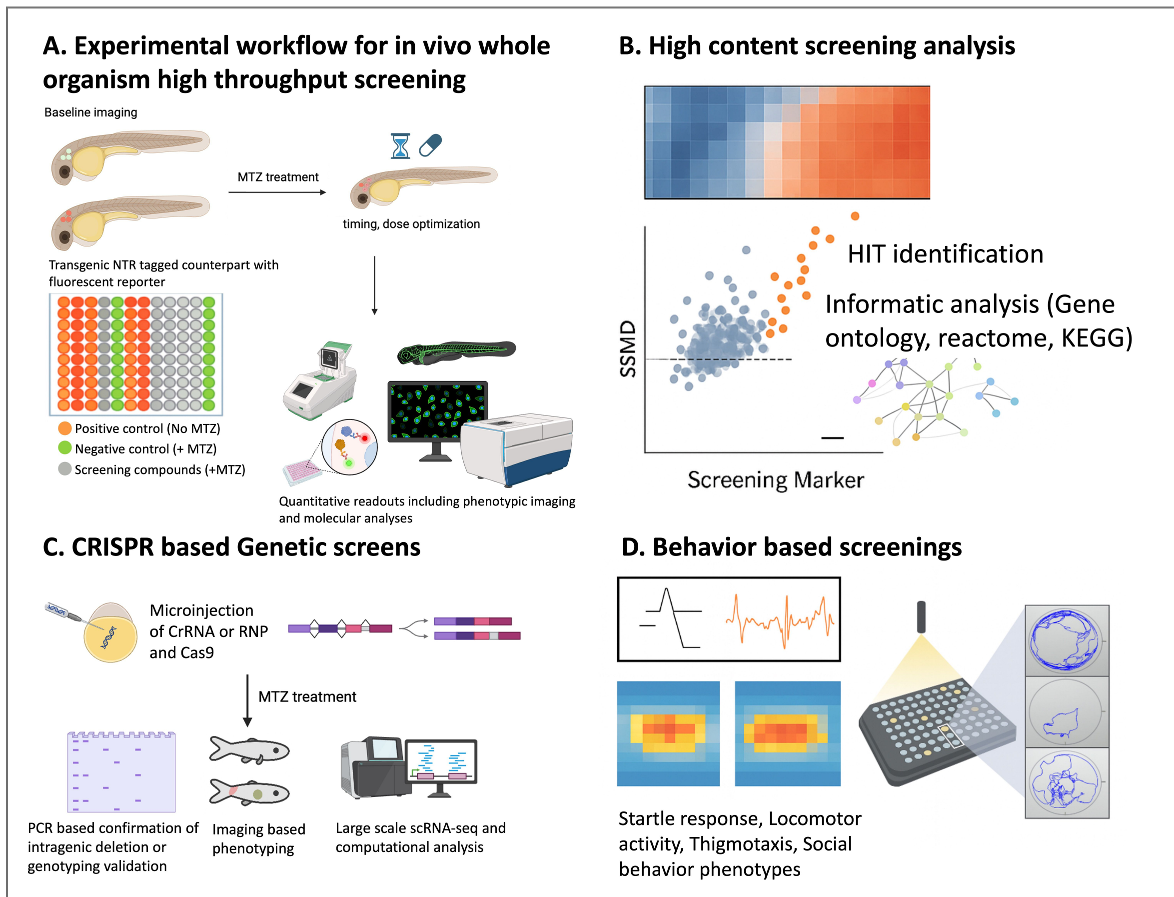

Figure 2. **NTR/MTZ-based screening platforms in zebrafish.**

Overview of the integrated chemogenetic screening workflow using nitroreductase (NTR)–mediated ablation. (A) Experimental design showing transgenic zebrafish expressing NTR in target tissues, baseline imaging, and subsequent metronidazole (MTZ) treatment to induce cell-type–specific ablation. The use of parallel transgenic controls and multiwell plate layout enables quantitative assessment of tissue loss and recovery. (B) High-content chemical screening pipeline integrating automated imaging, hit identification, and pathway-level analysis using standardized statistical metrics. (C) Genetic screening framework coupling sgRNA-based mutagenesis with imaging-based phenotype scoring to uncover modifiers of cell loss or regeneration. (D) Behavioral assays to quantify functional recovery or pharmacological response.

Genetic and CRISPR-Based Screening

Chemical screens can identify potential therapeutic reagents, though their molecular targets often remain unknown. A complementary approach is to perform reverse-genetic screens that integrate NTR-mediated ablation with CRISPR mutagenesis to identify genes affecting regeneration [141]. This mutagenesis is achieved by injecting Cas9 ribonucleoprotein (RNP) complexes multiplexed with several guide RNAs per target gene directly into NTR-transgenic embryos (Figure 2C). This FO 'crispant' strategy generates high-efficiency somatic mutations in the first generation [142], allowing researchers to induce cell-specific ablation with MTZ and immediately quantify the effect of gene disruption on regeneration without the delay of establishing stable mutant lines.

To identify regulators of Retinal Pigment Epithelium (RPE) repair, Lu *et* al. (2023) conducted a focused F0 CRISPR screen targeting 27 candidate genes in rpe65a:nfsB-eGFP larvae [143]. By injecting ribonucleoprotein (RNP) complexes containing three highly mutagenic guide RNAs per gene, they achieved high-efficiency somatic mutagenesis in FO injected fish. The NTR/MTZ system induced the synchronized, widespread degeneration of the RPE, which subsequently triggered the secondary loss of photoreceptors. This screen identified numerous regulators of regeneration and revealed a novel mechanism that regulates the infiltration of phagocytic cells required for clearance of debris and complete regeneration [143].

To find regulators of retinal ganglion cell (RGC) regeneration, Emmerich *et al.* (2024) performed a large-scale CRISPR screen on 100 genes. Using the isl2b:Gal4; UAS:YFP-NTR2.0 line for RGC ablation, they identified 18 effector genes comprising key transcription factors and signaling pathway components [144]. The screen revealed that inhibition of Ascl1a accelerated the regeneration of new RGC neurons.

Finally, the integration of F0 mutagenesis with automated imaging establishes a scalable framework for future genetic screens. The 'ZebraReg' platform utilizes a dual-transgenic line (tbx5a:CreERT2; myh7l:loxP-tagBFP-STOP-loxP-mCherry-NTR) that restricts NTR expression specifically to the heart ventricle [122]. Treatment with MTZ ablated approximately 97% of cardiomyocytes, triggering a robust regenerative response that typically restores the tissue within three days. By combining this precise injury model with F0 CRISPR mutagenesis followed by immediate phenotyping, the study demonstrates a proof-of-concept workflow to understand the genetic mechanisms of cardiac repair.

Caveats and Improvements to the NTR/MTZ System

The NTR/MTZ system is widely used for diverse applications, but its performance has varied between labs. Key issues include batch-to-batch and preparation variability of MTZ, the need for high MTZ doses (≈10 mM) that can cause off-target toxicity (e.g., developing brain [145], larval/adult intestine [120]), and differential susceptibility of some cell types to ablation [110]. Because ablation rate depends on both NTR activity and MTZ dose, researchers have pursued three complementary strategies to improve reproducibility and experimental interpretation: 1) increase NTR expression, 2) engineer higher-activity NTR mutants, and 3) identify more efficacious prodrugs that achieve effective killing at lower, less toxic concentrations. These iterative improvements are aimed at mitigating previous limitations and expanding the range of feasible experimental paradigms and are discussed next:

1. Increase NTR expression: Strong, well-characterized promoters/enhancers (e.g., the zebrafish insulin promoter) [107] can drive high NTR expression, especially when present in multiple copies via Tol2-mediated transgenesis [146]. However, many cell-specific regulatory elements are weak, and maintaining multiple insertions is challenging and prone to genetic drift and intergenerational variability. An alternative is to use a bipartite system such as Gal4/UAS, [147,148] which can produce robust, amplified NTR expression even from single genomic insertion. With this approach, a cell-specific promoter drives a Gal4 transactivator that binds UAS sites to strongly activates *NTR* transcription (Figure 3). For example, elements from the 14xUAS constructs of Köster and Fraser [148] were used to generate the transgenic line Tg(UAS-ElB:NTR-mCherry)^{c264}. [110,149] These fish were distributed by the

Zebrafish International Resource Center (ZIRC) and have been widely used by the zebrafish community: 10 of the 32 most-cited papers on zebrafish nitroreductase ablation use this line (Supplemental Table 1). A caveat is that Gal4/UAS DNA elements can be prone to epigenetic silencing, producing mosaic expression; the repetitive UAS contains multiple CpG sites susceptible to DNA methylation. [150,151] Silencing can be mitigated by using a less repetitive UAS (e.g., 4x) [152] or by using the QF/QUAS bipartite system (derived from Neurospora), [153] which has been reported to show reduced silencing [154] and has recently been adapted for NTR-based ablation (Figure 3). [139,155]

2. Higher-activity NTR mutants: Substantial effort has gone into engineering more active NTRs; first driven by their promise as cancer 'suicide-gene' therapies [156] and later to improve NTR-based ablation in basic research. [157] Two research groups independently engineered the same three substitutions into the wild-type *E. coli* enzyme (now termed NTR1.0), creating more efficient versions they named epNTR and NTR1.1. [156–158]. Cross-species screening identified a highly active nitroreductase (NTR) from *Vibrio vulnificus*. Using this enzyme as a scaffold, rational engineering yielded the second-generation variant NTR2.0, which exhibits a greater than 100-fold enhancement in activity over the original NTR1.0. [78]

The use of first-generation nitroreductase (NTR1) for chronic cell ablation was problematic, as the required 10 mM metronidazole (MTZ) dose induces intestinal pathology and approaches the LD50 in zebrafish. [120] However, the more active NTR2.0 variant enables effective ablation with far lower, better-tolerated MTZ concentrations. To demonstrate this, Tucker *et al*. developed a zebrafish model expressing NTR2.0 specifically in pancreatic β cells. [152] They found that efficient larval β cell ablation required only 100 μM MTZ, a regimen that could be maintained for 10 days without ill effects. In stark contrast, the NTR1 system required a toxic 10 mM MTZ dose, which is lethal to larvae (independent of NTR) within three days. In adult fish, a regimen of 5 mM MTZ for two days followed by two weeks at 1 mM was completely tolerated by wild-type fish with no ill effects but induced sustained hyperglycemia and weight loss in NTR2.0-expressing fish. This established a powerful model for studying chronic diabetic consequences, such as retinopathy, nephropathy, and impaired wound healing.

This well-tolerated ablation paradigm now makes it possible to model a range of other chronic conditions, including neurodegenerative, renal, and muscular disorders. This capability, in turn, facilitates the study of long-term disease progression and the evaluation of new therapeutic interventions.

3. More efficacious prodrugs: Metronidazole (MTZ) efficacy can vary across suppliers and batches. To ensure consistency, it is recommended to prepare fresh MTZ solutions for experiments. [110,120] To overcome MTZ's limitations, alternative prodrugs like nifurpirinol (NFP) have been tested. NFP is a more potent nitrofuran-based prodrug. [110,120] However, its structural class is distinct from the nitroimidazole-based prodrugs for which NTR2.0 was specifically engineered. [78] As a nitroimidazole prodrug, Ronidazole (RNZ) likely retains compatibility with newer NTR systems while offering significant practical advantages over MTZ, primarily its better potency. [80,145,159] For instance, in *Tg(fabpl 0:mCherry-NTRl*) fish, 2 mM RNZ achieved hepatocyte ablation comparable to 10 mM MTZ, a five-fold increase in potency. [159] This pattern of higher efficacy was replicated in a macrophage model, where a five-fold lower RNZ dose was as effective as MTZ. [145] Lai *et al*. also reported no bystander effects and demonstrated RNZ efficacy with the NTR1.1 variant. Recently, it has also been shown that RNZ functions with NTR2.0 to cause cell-specific ablation, [160] although a direct comparison of RNZ versus MTZ with NTR2.0 has not been reported. Whether RNZ shows batch-to-batch variability similar to MTZ has not yet been reported. Given the potential for variability, it would be prudent for researchers to titrate each new batch of RNZ or, alternatively, adopt a dosing strategy that exceeds the minimum effective concentration to ensure consistent ablation results. Nonetheless, it is anticipated that the NTR2.0/RNZ combination will further lower the required prodrug concentrations and minimize off-target activity.

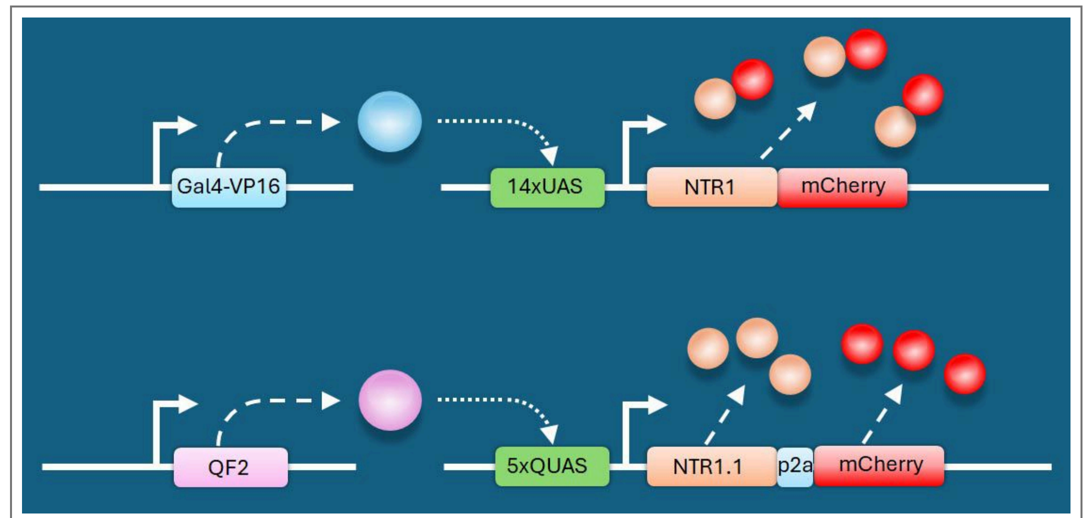

Figure 3. Schematic of bipartite systems to drive robust levels of NTR. On left, driver lines express (dashed arrows) transactivators (A) Gal4 (blue sphere), or (B) QF2 (purple sphere) under the control of a cis-regulatory element (CRE). These transactivators bind their respective upstream activating sequences (either UAS or QUAS, grey boxes) to achieve controlled and amplified NTR expression (tan spheres) in target cells. NTR expression can be monitored by co-production of mCherry (red spheres) either as a fusion protein or as separate proteins due to P2A dependent ribosome 'skipping' [162]. (A) redrawn from Pisharath & Parsons where the CRE was from ptf1a[110], and (B) redrawn from Lee et al. where the CRE came from mbpa[139].

Given this evolving landscape of prodrugs and enzymes, what are the critical factors a researcher must weigh when designing an NTR ablation experiment?

Experimental Design: Practical and Technical Considerations

A successful ablation experiment using the NTR/prodrug system requires careful consideration of three key components: (1) transgenic strategy, (2) optimal NTR activity, and (3) appropriate controls. The optimal design depends on the biological question.

- For regeneration studies, aim for complete ablation to clearly assess neogenesis.
- For functional studies, partial ablation may be sufficient to reveal a phenotype.

1. **Transgenic strategy:**
   - **Regulatory elements**: Select regulatory elements that ensure precise tissue or cell-type specificity.
     - Discrete regulation: When a single promoter is insufficient to achieve the desired tissue specificity, use intersectional approaches (e.g., Cre/lox) to restrict expression. [161]
   - **Fluorescent marker**: Include an independent fluorescent marker (fusion or 2A reporter [162]) to identify transgenic animals and confirm appropriate expression.
   - **Positional effects**: NTR transgenes, like any transgene, can exhibit positional effects (leakiness, mosaicism). To ensure reliable lines:
     - **Screen multiple founders**: Identify ≥5 FO founders and ideally establish 5 independent F1 lines.
     - **Compare stable F1 lines**: Confirm that fluorescent-marker expression matches expected regulatory-element activity.
     - **Prioritize F1 lines based on**:

       – Mendelian transmission, indicating a single-site insertion.

       – Consistent, non-mosaic expression of the fluorescent marker, indicating uniform NTR expression in all intended cells.

       – Robust and reproducible expression, independent of whether the transgene is

inherited maternally or paternally.

– Reliable and consistent ablation of target cell

2. NTR activity:

- **General principle**: Strong NTR expression generally yields faster and more complete ablation. [78,120]
- **NTR variant**: NTR2.0 is currently the most active NTR variant used in zebrafish and is recommended for future studies.
- **Prodrug choice**: For most applications, RNZ is the recommended prodrug to start with, due to its higher efficacy and lower required dosing relative to MTZ, which can improve both ablation efficiency and experimental consistency.
- **Enhancing expression**: If the promoter driving NTR2.0 is weak, amplify expression using binary systems such as Gal4/UAS or QF/QUAS (Figure 3). [139,149,155]

3. Controls:

- **Validating cell death**: To properly interpret ablation results, it is important to confirm that cell death occurred. Useful readouts include:
  - Apoptosis assays: TUNEL, cleaved caspase-3 immunostaining. [104,110]
  - Loss of fluorescent reporters: [106,107,110] If signal perdurance is a concern, use destabilized reporters to reduce fluorescence longevity. [163,164]
- **Cell-death kinetics**: As stressed cells may downregulate NTR and escape ablation, validating cell-death kinetics can be informative.
  Use endpoint analysis (serial time-point fixation + apoptosis markers).
  Or use cell-death biosensors, such as Hmgbl-GFP, which distinguishes necrosis (nuclear release) from apoptosis (nuclear retention). [165–167] Example: In larvae co-expressing *ins:mCherry-2A-NTR2.0* and *ins:hmgbl-eGFP*, [120,168] high-dose MTZ (1 mM) induced apoptosis within 4 hours with complete β-cell loss by 24 hours; a low dose (10 μM) produced slower dynamics (Figure 4).
- **Negative controls**
  - NTR transgene, no prodrug: Controls for effects of exogenous NTR expression.
  - No NTR transgene, prodrug: Controls for nonspecific MTZ/RNZ effects, including antimicrobial activity. For microbiome-associated studies, consider alternative ablation methods.

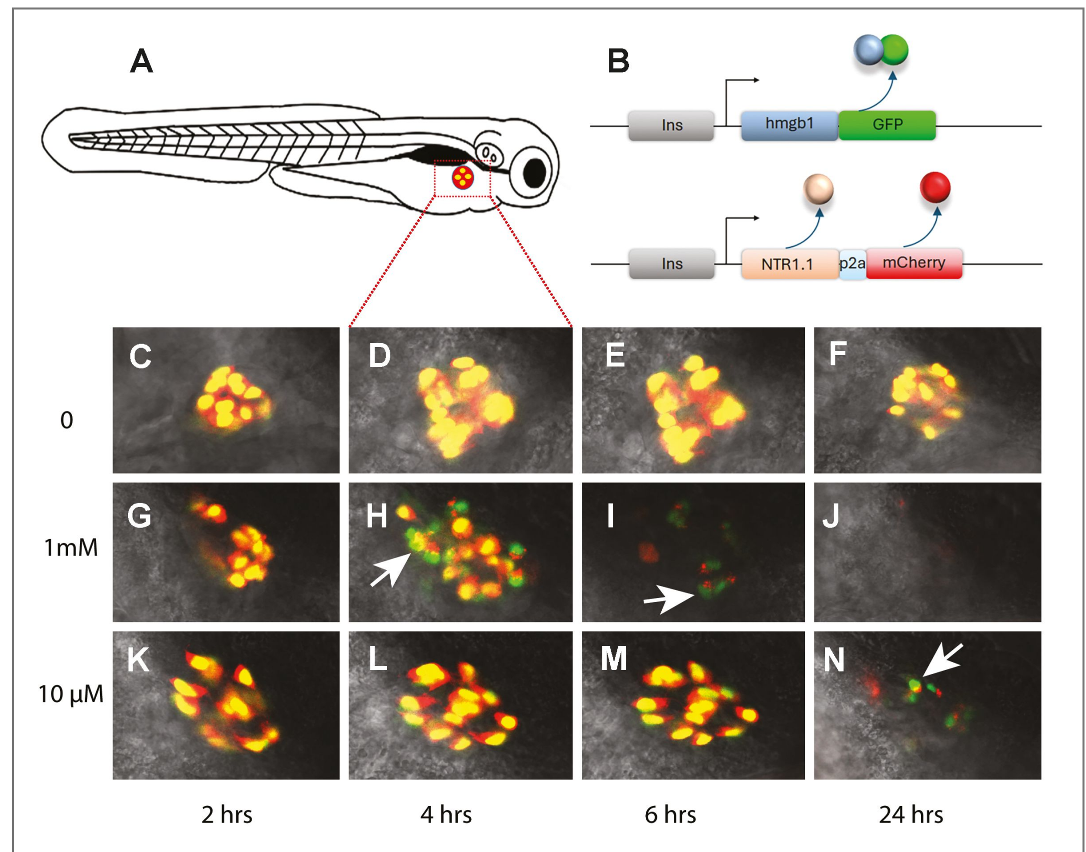

Figure 4. Live imaging of cell-death kinetics. (A) Schematic of 6 dpf larvae showing position of the pancreatic islet imaged in C-N (red/yellow). (B) Diagram of the two transgenes (ins:Hmgb1-GFP, ins:mCherry-2a-NTR2.0) in the fish. The insulin promoter (grey box) drives expression of the following: (B-above) an Hmgb1-GFP fusion protein and (B-below) NTR2.0 and mCherry [presence of the P2A (blue box) makes separate proteins]. (C-N) Confocal images of the islet of in three larval fish over a time course from 6 dpf to 7 dpf (times along the X axis). (C-F) negative control – no MTZ (0). (G-J) fish treated with high MTZ dose (1mM). (K-N) fish treated with a low dose MTZ (10μM). (G-N) Dying β cells first lose red fluorescence, revealing green nuclei (arrow heads). A higher dose shows appearance of green nuclei (H) earlier than the lower dose (N). (J) 24 hrs in 1mM and no debris remains.

Additional files

Supplemental file 1. Most Cited Publications Using the NTR/Prodrug System. This table lists the most highly cited publications employing the nitroreductase (NTR)/prodrug system for targeted cell ablation. Candidate studies were identified through a Web of Science search using the query “nitroreductase ablation,” and results were manually curated to include only those papers that directly used an NTR-expressing transgenic line or construct together with a prodrug to induce selective cell death. Entries are ranked by citation count at the time of data collection. For each study, the table reports the targeted cell type or tissue, the species and specific NTR transgenic line used, and the primary biological purpose addressed. The columns ad. (adult) and la. (larvae) indicate whether the transgenic system was used in adults, larvae, or both. The transgene shown in green correspond to a widely used UAS line that drives Gal4 dependent expression of NTR1, included here to aid identification of experiments utilizing this common effector line.

Additional information

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Peer reviews

Reviewer #1 (Public review):

Summary:

Kim and Parsons present a timely overview of the NTR/prodrug system and its applications in regenerative biology research, with particular emphasis on tissue-specific cell ablation. The system has substantially advanced the field by enabling non-invasive, conditional cell elimination, and has proven especially powerful in zebrafish, though applications in other classical model organisms are also noted. The review covers the historical origins of the NTR system, its use in regeneration studies, small-molecule screening, and genetic and CRISPR-based screening, as well as future directions including the development of the highly efficient NTR2 enzyme variant.

Strengths:

This is a useful and well-structured contribution. The manuscript is a valuable resource for the regeneration biology community.

Weaknesses:

The revised manuscript shows significant improvements; however, two points remain insufficiently addressed and should be resolved in the final version.

(1) The term 'suicide gene'

As noted in my first round of revisions, the term 'suicide gene' as applied to bacterial nitroreductase remains unaddressed in the revised manuscript, despite being scientifically inappropriate and a potential source of confusion regarding the NTR/Mtz mechanism.

'Suicide' implies an intrinsic, cell-autonomous programme of self-destruction. This is incompatible with the NTR/Mtz system, in which cell death is experimentally induced through exogenous administration of metronidazole (Mtz) by the investigator. While the 'suicide gene' framing may have utility in the cancer therapy literature, likely to aid communication with non-specialist and clinical audiences, however, it is not standard in the zebrafish field, where NTR is more accurately described as a conditional toxigene. Since this review focuses predominantly on zebrafish models, its terminology should reflect that of the relevant literature.

A further conceptual problem with the 'suicide gene' framing is that it obscures the pharmacological nature of Metronidazole. Mtz is a pharmaceutical agent with intrinsic baseline toxicity: extended exposure or modestly elevated concentrations cause toxic side effects and lethality even in non-transgenic (wild-type) zebrafish (PMID: 24428354). NTR-expressing cells do not self-destruct; rather, they are rendered selectively hypersensitive to Mtz relative to other eukaryotic cells by virtue of expressing the enzyme. This distinction is mechanistically important and should be reflected in the language used throughout the manuscript.

In summary, the term 'suicide gene' does not accurately capture enzyme-mediated bioactivation of an exogenous prodrug and should be removed from the manuscript.

(2) Barriers to using the NTR/Mtz system in non-aquatic model organisms

In response to my suggestion that the title should include "zebrafish" to accurately convey the scope of the review to prospective readers, the authors stated that "there is no intrinsic barrier to adopting this technique more broadly in other systems," citing the example that

"NTR was first developed in mice, but with a prodrug that proved difficult to use, and it was not widely pursued." These two statements are, however, contradictory: if the prodrug proved difficult to use, this constitutes precisely the kind of practical barrier the authors claim does not exist. The authors should clarify and reconcile this inconsistency, and provide a more thorough discussion of why the NTR/Mtz system has seen limited adoption in classical model organisms, such as mice and Drosophila.

https://doi.org/10.7554/eLife.110593.2.sa1

Reviewer #2 (Public review):

Summary:

Kim and Parsons reviewed the nitroreductase (NTR)/prodrug system: when engineered cells expressing the enzyme NTR are treated with prodrug (e.g. metronidazole), NTR converts the prodrug into cytotoxic compound which kill these cells. The review covers how the system has been developed, spatiotemporal control of targeted cell ablation, and its broad utility to study regenerative mechanisms, model human diseases, and screen chemicals to discover pro-regenerative and protective compounds. They further discussed the newer version of NTR, more potent prodrug, and experimental design, which not only expand the possible utility of the NTR/prodrug system, but allow the research community to develop a precise, reproducible and versatile platform.

Strengths:

The review summarized landmark work application of the NTR/prodrug system, and recent studies in model organisms, with focus on the model organism zebrafish. The review provides a good gateway to understanding the system and considering regenerative studies.

Weaknesses:

None.

Comments on revisions:

The authors have addressed the previous points, and the manuscript has been greatly improved.

https://doi.org/10.7554/eLife.110593.2.sa2

Reviewer #3 (Public review):

Summary:

This manuscript by Kim and Parsons presents an overview of the nitroreductase/metronidazole (NTR/MTZ) cell ablation system.

Strengths:

This manuscript nicely places the NTR/MTZ system in context of other cell ablation methods, with a discussion of their respective advantages and disadvantages. This review is particularly useful for highlighting the many ways the NTR/MTZ system has been applied to study regeneration of multiple cell types and to model different degenerative human diseases. The review concludes with a discussion on recent improvements made to the system and practical considerations and "best practices" for NTR-based experiments. This review could be a helpful resource, especially for researchers new to regeneration or cell ablation studies.

Comments on revised version:

I thank the reviewers for revising the manuscript to expand their discussion of using the prodrug/NTR system in other model organisms while also revising the abstract to make it clear this review will be zebrafish focused. With these revisions, this review provides an informative overview of how the prodrug/NTR system has not only been an important tool for regeneration studies and but also for elevating the zebrafish as a regeneration model. That said, including other model organisms could have been a nice addition to the last section on experimental considerations, especially in the context of discussing potential barriers to wider adoption of the NTR system. However, given that the vast majority of studies using the NTR system are in zebrafish, the current scope of this review is understandable.

https://doi.org/10.7554/eLife.110593.2.sa3

Author response:

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *Kim and Parsons present a timely overview of the NTR/prodrug system and its applications in regenerative biology research, with particular emphasis on tissue-specific cell ablation. The system has substantially advanced the field by enabling non-invasive, conditional cell elimination, and has proven especially powerful in zebrafish, though applications in other classical model organisms are also noted. The review covers the historical origins of the NTR system, its use in regeneration studies, small molecule screening, and genetic and CRISPR-based screening, as well as future directions, including the development of the highly efficient NTR2 enzyme variant.*
>
> *Strengths:*
>
> *This is a useful and well-structured contribution. The manuscript is a valuable resource for the regeneration biology community.*
>
> *Weaknesses:*
>
> *The impact and scientific value of this paper could be meaningfully enhanced by addressing several points outlined below. The concerns centre on completeness, conceptual precision, and the depth of mechanistic discussion.*
>
> *(1) Title: Species specificity.*
>
> *Given that the review's primary focus is the zebrafish model, it would be appropriate to include the species name in the title. This would improve discoverability and accurately set the scope of the article for prospective readers.*

Thank you for this suggestion. In revising the review, we have substantially expanded the content to address the reviewers' comments, including adding more detail on the use of NTR in other species. We agree that the majority of published work, and the research we cover, has been conducted in zebrafish, and we have clarified this in the abstract and introduction. However, our aim in writing the review was also to highlight that there is no intrinsic barrier to adopting this technique more broadly in other systems. Notably, NTR was first developed in mice, but with a prodrug that proved difficult to use, and it was not widely pursued. In mouse models, the development of DTR offered an alternative, though that approach carries risks of kidney toxicity and is incompatible with chronic ablation due to immunogenicity. Given this context, we would prefer to retain a title that does not limit the scope exclusively

to zebrafish, so as not to discourage readers working in other model systems who might benefit from considering the NTR system.

> *(2) Subchapter: Physical injury.*
>
> *The subchapter enumerates different types of physical injury models but would benefit from a more substantive comparative discussion. In particular, the authors are encouraged to address the following:*
>
> *(2.1) Outcome comparison: Surgical and other invasive approaches cause damage to entire tissue structures comprising multiple cell types, whereas tissue-specific genetic ablation eliminates a defined cell population while leaving the surrounding architecture largely intact. This fundamental distinction has direct implications for the interpretation of regenerative outcomes and should be clearly articulated.*

We appreciate the reviewer raising these important points, as well as those noted in Section 2.2. We addressed the concerns from Sections 2.1 and 2.2 throughout multiple parts of our review, specifically in the following sections:

• Physical injury – where we highlight the importance of precisely characterizing the nature and extent of tissue damage in order to appropriately interpret subsequent biological responses.

• Chemogenetic cell-specific ablation – where we expand on this theme by discussing the advantages of selectively eliminating discrete cell populations and how this improves mechanistic interpretation of regeneration.

• Development of NTR as a suicide gene – where we examine apoptotic pathways and their relevance to nitroreductase-mediated cell ablation.

• NTR/prodrug systems in regenerative studies – where we compare what is currently known about immune activation and inflammatory responses across different NTR-based ablation paradigms.

> *(2.2) Inflammatory response: Invasive injuries typically trigger a robust inflammatory response, which itself can be a potent driver of regeneration. By contrast, genetic cell ablation may elicit a qualitatively different inflammatory reaction. A comparative discussion of this distinction would help readers appreciate a critical limitation of genetic ablation systems relative to models of natural, accidental tissue damage.*

Please see above response 2.1

> *(3) Subchapter: Cell-specific toxins.*
>
> *This subchapter would benefit from several targeted expansions:*
>
> *(3.1) Off-target effects: The authors should include evidence that the exemplified drugs have known off-target activities, with a discussion of how these confounded the interpretation of experimental data. At least a few concrete published examples should be cited.*

Thank you very much for the comments. We have strengthened the discussion of off-target effects by adding concrete published examples. We now note that MPTP/MPP^+ can affect noradrenergic and serotonergic systems in addition to dopaminergic neurons, that aminoglycoside antibiotics can damage support cells and afferent neurons at higher concentrations with compound-specific differences in ototoxicity, and that streptozotocin exhibits hepatotoxicity beyond pancreatic β-cells.

> *(3.2) Completeness of the toxin list: The current list appears illustrative rather than comprehensive. A more complete enumeration would be valuable, particularly for neurotoxins and drugs targeting sensory cells, as these are highly relevant to the zebrafish regeneration field.*

We have now consolidated the toxins discussed throughout the review into Table 1, which includes additional entries alongside the previously listed agents. We have explicitly noted that this list is representative rather than exhaustive, as the full range of cell-specific toxins used across species is extensive.

> *(3.3) Interspecies differences: It would be informative to specify whether drug specificity differs across species, as this is a practical consideration for researchers working in organisms other than zebrafish.*

We appreciate the reviewer's question regarding potential interspecies differences in prodrug performance. Early work using NTR in mammals was conducted in mice, and all five published mouse studies relied exclusively on CB1954. No other NTR-activating prodrugs have been reported in mouse models, so direct comparisons are not available. Likewise, all published Xenopus studies used MTZ and thus do not provide internal comparisons across prodrugs. The Nematostella study employed NFP (citing rationale from a zebrafish study) and the approach yielded effective ablation.

The only non-zebrafish study that directly compared prodrugs is the Drosophila work, which evaluated MTZ, RNZ, and NFP and reported lower activity for MTZ relative to the other compounds. Because it is not clear whether the authors were aware of the batch variability of MTZ or the need for freshly prepared solutions, interpreting this specific comparison is difficult.

To address the reviewer's comment, we have expanded the section on non-zebrafish organisms to clearly state which prodrug was successfully used in each species. However, given the limited number of studies, the absence of titration experiments, and the lack of standardized conditions across laboratories, we do not feel that the available evidence supports drawing conclusions about interspecies differences in prodrug performance.

Consistent with our original discussion and based on the broader biochemical and empirical data available, we continue to recommend RNZ as the starting point for new experiments.

> *(4) Subchapter: Optogenetic cell ablation.*
>
> *The authors note that optogenetic cell ablation has not yet been applied in conventional regeneration studies. It would strengthen this section to include a discussion of the underlying reasons for this gap, whether technical or biological, so that readers can appreciate the barriers and potential for future adoption.*

We thank the reviewer for this helpful suggestion. As recommended, we have added a concise, explicitly speculative statement discussing potential technical factors that may explain why optogenetic cell ablation has not yet been widely applied in regeneration studies. Specifically, we note that KillerRed-based ablation requires localized light delivery and ROS generation, making it best suited for discrete, optically accessible cells and less practical for targeting large or deep tissues. We also highlight that the dependence on microscopy-based illumination inherently limits throughput. This new text clarifies possible barriers to broader adoption while acknowledging that these points remain speculative.

> *(5) Terminology: "Suicide gene".*
>
> *The use of the term "suicide gene" to nitroreductase is conceptually imprecise and merits reconsideration. Strictly speaking, a suicide gene is one whose expression alone is*

> *sufficient to kill the cell, as in the case of genes encoding direct triggers of apoptosis or the catalytic A subunit of diphtheria toxin (DTA). NTR does not meet this criterion: it requires the exogenous administration of a prodrug (e.g., metronidazole) to produce a cytotoxic metabolite and is therefore only conditionally lethal.*
>
> *It is worth noting that nitroreductases evolved in bacteria and fungi as enzymes involved in chemoprotection and detoxification, converting potentially toxic and mutagenic nitroaromatic compounds into less harmful metabolites (PMID: 18355273). This biological context further underscores that NTR is not inherently a lethal protein. The authors are encouraged to replace or qualify the term "suicide gene" and instead adopt terminology that more accurately reflects the conditional, prodrug-dependent nature of the system.*

We appreciate the reviewer's thoughtful attention to terminology. We agree that, in its most classical and stringent sense, a suicide gene is one whose expression alone is sufficient to induce cell death. We also recognize that NTR does not meet this strict criterion.

At the same time, we note that the term has broadened in contemporary usage, particularly within applied and translational contexts, to encompass prodrug-dependent systems. For example, the National Cancer Institute Thesaurus defines a suicide gene as "a gene which will cause a cell to kill itself, typically through interaction with a prodrug," and Taber's Medical Dictionary likewise states that it is "a gene that causes a cell to kill itself, usually by encoding an enzyme that converts a nontoxic prodrug into a toxic metabolite." Under these widely used definitions, NTR is included within the scope of suicide gene systems.

Nevertheless, we appreciate that terminology in this area is not universally standardized. To ensure clarity for all readers, we have added a brief definition in the revised manuscript explicitly noting the conditional, prodrug-dependent nature of NTR-mediated ablation. We are grateful to the reviewer for prompting this clarification.

> *(6) NTR/MTZ in regenerative studies: Mechanistic depth.*

While the review catalogues several studies employing the NTR/MTZ system, it lacks mechanistic depth regarding the cellular basis of ablation. The following questions should be addressed, where evidence exists in the literature:

> *(6.1) Temporal dynamics of cell death: What is known about the kinetics of NTR/MTZ induced lethality across different tissue types in larval and adult zebrafish, as well as other organisms? Are there age- and tissue-specific differences in the speed or completeness of ablation?*

Thank you for this important question. We have added text noting that the kinetics and completeness of NTR/prodrug-mediated ablation vary across experimental contexts, including with differences in NTR expression, enzyme/prodrug pairing, dose, cell type, and developmental stage. Published studies illustrate that the time course of ablation can differ substantially between models. Because most studies were designed to optimize ablation within individual tissues rather than for direct side-by-side comparison, the literature does not yet support broad quantitative conclusions about age- or tissue-specific differences across systems.

> *(6.2) Mechanism of cell death: What is the cellular basis of NTR/MTZ-induced cytotoxicity in zebrafish? In particular, do the toxic metabolites preferentially cause mitochondrial damage or nuclear DNA damage, and what downstream death pathways are engaged?*

Thank you for the comments. We have added text discussing the mechanism of NTR/MTZ-induced cell death. We now note that NTR-mediated reduction of MTZ generates reactive intermediates that cause DNA damage and oxidative stress, with cell death occurring

predominantly through apoptosis. We have also more strongly emphasized that in dopaminergic neurons, mitochondrial damage was identified as the primary cytotoxic mechanism. We acknowledge that the relative contribution of these pathways is likely to vary by cell type and remains an important area for future study.

> *(6.3) Proliferative versus post-mitotic cells: Are proliferating and non-proliferating cells equally sensitive to the NTR/MTZ system, or does the proliferative status of a cell influence susceptibility? This is a practically important question for researchers designing ablation experiments in tissues with mixed cell populations.*

We appreciate the reviewer's insightful question. We have now added a brief clarification to this section explaining that the NTR/MTZ system has been shown to act in a cell-cycle–independent manner, and both proliferating and post-mitotic cells can be ablated effectively.

> *(6.4) Ablation of progenitor cells: Are there published examples demonstrating that co-ablation of differentiated functional cells and organ-specific progenitor cells abolishes regenerative capacity? Such examples would be highly informative in illustrating the system's power to dissect the cellular requirements for regeneration.*

To our knowledge, the zebrafish lateral line currently provides the clearest example in which NTR-mediated ablation of progenitor populations results in a loss of regenerative capacity. In this system, targeted ablation of support-cell progenitors severely reduces hair-cell regeneration, illustrating how NTR enables direct testing of cellular requirements for tissue repair.

> *Addressing the points above, particularly the comparative discussion of injury models and inflammatory responses, the clarification of terminology, and the mechanistic discussion of NTR/MTZ-induced cell death would substantially strengthen the review's scientific contribution and utility.*
>
> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *Kim and Parsons reviewed the nitroreductase (NTR)/prodrug system: when engineered cells expressing the enzyme NTR are treated with prodrug (e.g. metronidazole), NTR converts the prodrug into a cytotoxic compound that kills these cells. The review covers how the system has been developed, spatiotemporal control of targeted cell ablation, and its broad utility to study regenerative mechanisms, model human diseases, and screen chemicals to discover pro-regenerative and protective compounds. They further discussed the newer version of NTR, a more potent prodrug, and experimental design, which not only expands the possible utility of the NTR/prodrug system, but also allows the research community to develop a precise, reproducible and versatile platform.*
>
> *Strengths:*
>
> *The review summarized landmark work application of the NTR/prodrug system, and recent studies, with focus on the model organism zebrafish. The review provides a good gateway to understanding the system and considering regenerative studies.*
>
> *Weaknesses:*
>
> *No weaknesses were identified by this reviewer.*
>
> ***Reviewer #3 (Public review):***
>
> *Summary:*

> *This manuscript by Kim and Parsons presents an overview of the nitroreductase/metronidazole (NTR/MTZ) cell ablation system.*
>
> *Strengths:*
>
> *This manuscript nicely places the NTR/MTZ system in the context of other cell ablation methods, with a discussion of their respective advantages and disadvantages. This review is particularly useful for highlighting the many ways the NTR/MTZ system has been applied to study the regeneration of multiple cell types and to model different degenerative human diseases. The review concludes with a discussion on recent improvements made to the system and practical considerations and "best practices" for NTR-based experiments. This review could be a helpful resource, especially for researchers new to regeneration or cell ablation studies.*
>
> *Weaknesses:*
>
> *Although the NTR/MTZ system has been used in other model organisms, this review is primarily focused on its uses in zebrafish. While this is understandable given the wide adoption of NTR/MTZ in the zebrafish field, discussion of the unique considerations and/or challenges for non-zebrafish systems would be an interesting addition and could broaden the potential audience for this review. Additional minor revisions, as suggested below, could also improve readability.*
>
> ***Recommendations for the authors:***
>
> ***Reviewer #2 (Recommendations for the authors):***
>
> *Since the lab mouse is an important mammalian model system, with certain tissues harbouring some regenerative capabilities, including the peripheral nervous system (e.g., sciatic nerve regeneration after crush), and myelin, etc., it would be great if a section could be included to discuss the potential adoption of the NTR/prodrug system in future mouse studies.*

We appreciate the reviewer's suggestion to discuss the potential future use of the NTR/prodrug system in mouse models. In surveying the literature, we identified only five mouse studies employing NTR, all of which used CB1954. These early studies were conducted primarily as proof-of-principle work in the context of gene-directed enzyme prodrug therapy (GDEPT) for cancer, rather than for regenerative or lineage-specific ablation applications. We added this point to the text.

Since those reports, we have not found additional examples of NTR use in mice. We do not know the precise reasons for this limited adoption, but it may reflect the availability of alternative ablation systems that are widely established in mouse research, such as the diphtheria toxin receptor (DTR) system.

We agree that certain mouse tissues exhibit regenerative capacity and that targeted ablation tools can be valuable in such contexts. To address the reviewer's point, we have added text noting the very limited historical use of NTR/CB1954 in mouse. We have no explanation as to why no one moved onto using NTR/MTZ in the mouse but note in two places in the text that DTR is preferred method to use in mouse ablation experiments (even though DT does cause kidney damage and is incompatible with chronic studies!).

> *Minor:*
>
> *(1) Line 174-176, the sentence was repeated.*
>
> *(2) Figure 1, for the transgenic line, please be consistent with the line name in italics.*

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) In the abstract as well as in the main text, the authors note that the NTR/MTZ system has been used in multiple model systems. Yet, most of the review, and especially the practical advice given at the end, is very zebrafish-focused. Although this is understandable given the wide adoption of NTR/MTZ in the zebrafish field, the authors might consider revising the abstract to make it clearer that this review is primarily concerned with the use of the NTR/MTZ system in zebrafish.*

Thanks for the suggestion. We have changed last half of first paragraph in abstract

> *That said, a brief discussion of any unique considerations and/or challenges for non-zebrafish systems would be an interesting addition and could broaden the potential audience for this review.*

Agreed and we have expanded in several places in the text to discuss more about the NTR system in non-zebrafish. We especially expanded our discussion about NTR in the mouse.

> *(2) Line 176: There is a repetition of the sentence, "NTR/MTZ-mediated ablation has also been adapted for other model organisms."*

Found and deleted. Thank you!

> *(3) Line 177: To improve clarity, the authors should include species names to prevent confusion. For example, both Xenopus laevis and Xenopus tropicalis are commonly used model organisms. Similarly, multiple Drosophila species are used by researchers.*

Added melanogaster and laevis to text.

> *(4) Can the authors address whether alternatives to MTZ (RNZ, etc.) have the same issues with batch-to-batch variability? That might be an important consideration for potential users. It would also be useful to include practical guidance for accounting for batch variability, for example, how to determine optimal prodrug concentrations, whether effective concentrations need to be determined for every batch/replicate/experiment, etc.*

Added text that discusses that, it is not yet known whether RNZ exhibits batch-to-batch variability similar to MTZ, as this has not been systematically reported. Given the potential for variability, it would be prudent for researchers to titrate each new batch of RNZ or, alternatively, adopt a dosing strategy that exceeds the minimum effective concentration to ensure consistent ablation results.

> *(5) For the last section ("Experimental design: Practical and technical considerations"), readability would be improved by applying a consistent bullet point format.*

Made the changes as requested.

> *(6) Figure 1: Asterisks are not defined.*

The asterisks where to link to two boxes depicting the same transgene without rewriting the name of the transgene. Clearly, this wasn't clear, so we have added explanation to legend too.

> *(7) Figure 3: Given that the schematics specify expression of NTR1 and NTR1.1, I assume this figure is adapted or based on previous published report(s). If so, the reference(s) should be noted in the figure legend or on the figure itself (as done for Figure 1). If the schematic is meant to depict only in general terms how binary expression vectors can be used, a more inclusive "NTR" label might be less confusing.*

Changed figure legend and figure

> *(8) Figure 4: To improve readability and accessibility, the authors should consider modifying panels C-N to use a more colorblind-friendly palette (e.g., green/magenta) or to present each channel as separate grayscale images.*

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