Promoting oncolytic vector replication with switches that detect ubiquitous mutations

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Abstract:
Most existing cancer therapies negatively affect normal tissue as well as cancerous tissue. A potentially effective strategy for treating cancer that precludes off-target damage and could be an option for most patients would involve targeting one or more mutations that are ubiquitous in the given patient's tumor(s). To effect this strategy, one would employ multi-region sequencing of a patient's primary tumor and metastases to seek out mutations that are shared between all or at least most regions. Once the target or targets are known, one would ideally rapidly generate a molecular switch for at least one of said ubiquitous mutations that can distinguish the mutated DNA, RNA, or protein from the wild-type version and subsequently trigger a therapeutic response. I propose that the therapeutic response involve the replication of an oncolytic virus or intracellular bacterium, as any mutation can theoretically be detected by a vector that enters the cell - and automatic propagation could be very helpful. Moreover, the mutation "signal" can be easily enhanced through transcriptional and translational (if the target is an intracellular protein) enhancement. Importantly, RNA may make the best target for the molecular switches in terms of amplification of the signal and ease of targeting.

Graphical abstract:
A.

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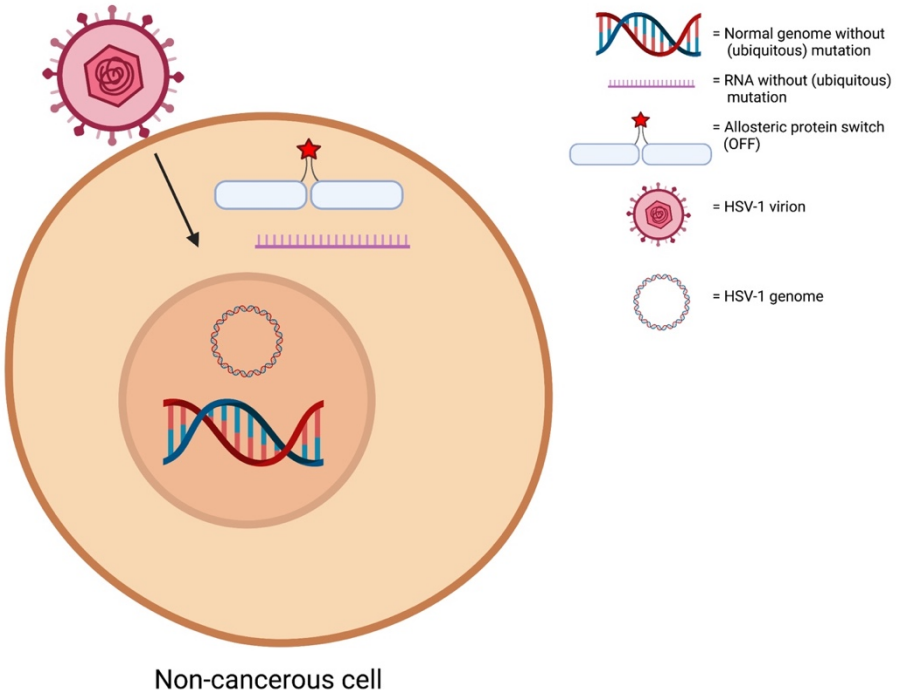

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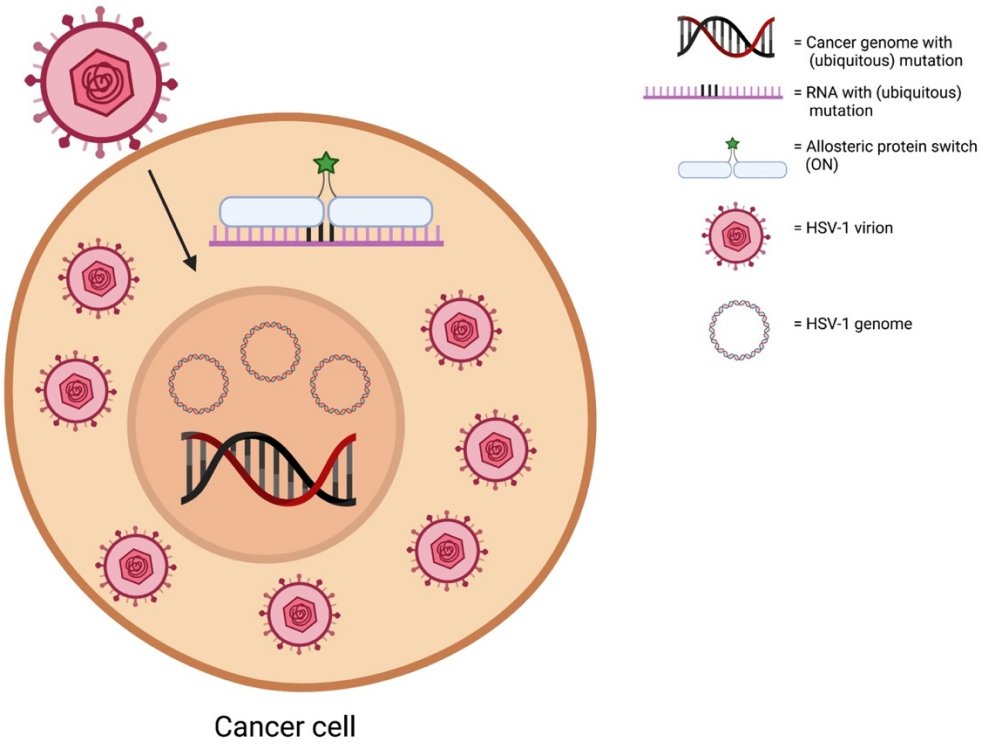

Graphical abstract: A) If a non-cancerous cell is transduced by the ubiquitous mutation detection-restricted HSV-1 vector, it will not sense the target mutation and thus will not replicate. B) If a cancer cell is transduced by the aforementioned HSV-1 vector, it will sense the ubiquitous mutation and replicate, spreading to neighboring cells and ultimately lysing the original host cell. *Instead of 'triggering' replication of an otherwise replication-

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incompetent vector via ubiquitous mutation detection, one could also potentially use an attenuated vector and simply enhance its replication via ubiquitous mutation detection.

Keywords:
Molecular switches, oncolytic vectors, patient-specific ubiquitous mutations, targeted therapy, multi-region sequencing, and molecular biology

Introduction:

Recent studies have shown that sometimes there are "ubiquitous" mutations found in every sequenced region of a given cancer patient's primary tumor and/or metastases[1,2,3,4]. The more samples that are taken and found to harbor a particular mutation, the more likely it is that the mutation is actually ubiquitous throughout a patient's cancer (i.e., truly ubiquitous, or "TU", mutations). TU mutations are likely almost always mutations that occurred very early on in the development of the primary tumor, i.e., truncal mutations. TU mutations would make excellent markers for a treatment aimed at targeting a patient's cancer cells while sparing his/her normal cells. One should always sequence multiple regions of all of a patient's tumors when trying to identify a candidate TU mutation, in case one or more tumors contain large quantities of cells that lack it (if the mutation was not truly truncal) or have lost it (through further mutation).

I argue that these ubiquitous mutations in cancer patients can be exploited best by using intracellular "switches" in the context of promoting the replication of an oncolytic vector. Switches here refers to elements that involve a detection component and an effector component, wherein the effector is in the "OFF-state" when the detection element is not bound to its target and in the "ON-state" once the detection element binds its target. Some switches are permanently activated following binding, whereas others (i.e., transcriptional regulation switches and some allosteric switches) require continuous binding of the target for continuous activation.

At certain times in the past, tumor regressions have coincided with natural viral infections. It was evident that in some cases, viral infections could target cancerous growths and largely spare normal tissue[5]. Since then, there has been a huge amount of research done to increase their selectivity for and potency against cancer cells. One notable success in the field of oncolytic virotherapy is Talimogene laherparepvec (T-VEC). T-VEC, an oncolytic herpes virus that lacks infected cell protein 34.5, has shown efficacy in treating melanoma in certain cases[6,7]. Relapse, however, is still possible[6], and there are side effects with T-VEC, albeit usually rather minor[8]. Additionally, there are many cancers that do not respond well to this treatment. It is a bit unclear why melanoma responds so well to T-VEC vs. many other types of cancers[9]. Perhaps this is because epithelial cells have tight cell-to-cell junctions, which allows facile spreading of the vector[10].

Another striking example of oncolytic virotherapy working well is cited here[11]. In this case, the combination of oncolytic virotherapy and checkpoint inhibition often led to the most potent results. Before "stealth" vectors are possible which evade the immune system, carrier cells could provide a platform for initial vector replication in tumor locales.

However, despite certain successes, achieving a cure in all cases may require conditionally enhancing or triggering oncolytic vector replication/hyper-virulence through the detection of patient-specific mutations. Mutation targeting can be used to enhance the replication of attenuated vectors - or replication solely based on mutation detection may also be feasible.

Certain researchers have employed intracellular switches to target cancer cells based on particular mutations[12,13,14,15]. For example, Phelps *et al*. utilized CRISPR/Cas9[14] – which allosterically activates upon binding the target DNA sequence and subsequently cleaves at a site within the target sequence[16]. The CRISPR/Cas9 elements were encoded by an oncolytic myxoma virus (which is highly attenuated in humans, as rabbits are its natural host). However, cleavage of the target sequence does not necessarily kill the cell. Kim *et al*. utilized a trans-splicing ribozyme to target a point-mutated KRAS mRNA sequence[15]. Trans-splicing ribozymes are RNA molecules; 3'-acting trans-splicing ribozymes can recognize a U in a given RNA sequence and replace the downstream segment of the target with another sequence. In this experiment, the mutant KRAS that they studied had sustained a point mutation leading to the existence of a novel T, and therefore a U in its mRNA sequence – allowing it to be targeted specifically by a trans-splicing ribozyme. A conditional toxin was expressed when the mutant sequence was detected. The conditional toxin they selected was the herpes simplex thymidine kinase type 1 (HSV1-TK), which can convert ganciclovir into the toxic ganciclovir triphosphate (GCV-TP). GCV-TP can travel between cells, and therefore can incur a bystander effect (i.e., kill neighboring cells), but only if they are replicating; it becomes incorporated into the genomic DNA. Unfortunately, some cancer cells may lie dormant until environmental conditions change; thus, they may degrade or expel the GCV-TP before it endangers them[17]. Kim *et al*. used a non-replicating adenovirus to deliver the trans-splicing ribozyme to cancer cells.

In contrast to strategies which have been tried previously with intracellular switches targeting specific mutations, inducing oncolytic vector replication upon ubiquitous mutation detection may be much more effective. The aforementioned strategies require delivery of the gene vector to all or at least a large proportion of the cancer cells in the patient's body via intravenous (IV) and/or intratumoral (IT) injections. (With regard to the oncolytic myxoma virus, this is still essentially true, as the virus is attenuated in humans, which limits its amplification in tumors.) This is not feasible with current biotechnology, as large vectors cannot efficiently extravasate in most regions throughout the body after IV injection. Similarly, large vectors do not diffuse much when injected directly into parenchymal tissue[18]. It is true that extracellular matrix (ECM)-remodeling proteins can be injected with the vector to enhance its distribution throughout the tumor, but automatic replication and intercellular spreading based on mutation detection would still be helpful to minimize the number of injections required to make complete coverage a more likely outcome. The tumor vasculature is often leaky in certain locales at least[19]; if a single copy of the oncolytic vector were to reach a tumor after IV injection, it then could self-amplify (and secrete ECM-remodeling factors from infected host cells that could make intercellular spreading more facile[20,21]).

Despite the fact that certain adeno-associated viral (AAV) vectors can extravasate to a substantial extent, have broad tropism, and transduce a variety of cells efficiently, there is

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still much room for improvement with regard to the extensive transduction of the central nervous system (CNS) and most peripheral organs after IV injection – as evidenced by studies with juvenile/adult non-human primates (NHPs)[22,23,24]. With regard to cancer therapy, if one does not transduce at least the vast majority of a patient's cancer cells with a non-replicating vector, even with a truly effective bystander effect[25], the cancer may grow back[26,27]. Along those lines, extremely high IV doses of AAV may be genotoxic[28]. Additionally, it is not very convenient to target AAVs to a multitude of ubiquitous cell surface receptors, which is likely necessary to prevent cancer cells from escaping the treatment through mutation or silencing of a single or small number of receptors. (AAV9, which seems to have the broadest tropism, primarily targets terminal *N*-linked galactose; in addition to the fact that it is clearly only a single receptor, it is only found on the surface of certain cell types[29].) And as with larger vectors, they also may remain confined to the injection site when injected intraparenchymally[30] (or in this case intratumorally). It appears as though AAVs may be more suited to gene therapy of inherited disease rather than curative or very effective cancer therapy.

Patient-specific ubiquitous mutations, molecular switches, and oncolytic vectors: In this article, I argue that oncolytic vectors may be the best way to exploit any ubiquitous mutations that a cancer patient might have; molecular switches encoded by the oncolytic vectors could enhance or trigger their replication upon detection of the target mutation(s). The benefit of using oncolytic vectors (i.e., viruses or intracellular microbes) instead of CAR T/NK-cells or immunotoxins is that any mutation can be targeted with such vectors – as opposed to the latter entities which can only target mutations affecting extracellular antigens. Furthermore, with an intracellular vector, the signal intensity of mutations in gene regions and non-coding DNA that is transcribed into non-coding RNA molecules can be amplified by virtue of CRISPRa (of a given promoter)[31,32,33], which would upregulate the target transcript. Exons can be forcibly retained to help prevent alternative splicing in some of a patient's cancer cells from removing the target mutation site from the transcript[34]. Introns can potentially be retained as well via high-affinity RNA-binding proteins (RBPs) targeting specific sites that possess additional functional domains[35]. dCas13-sgRNA could suffice here as the RNA-binding component; it is programmable. Alternatively, perhaps phage-assisted continuous evolution (PACE)[36] or eventually viral evolution of genetically actuating sequences (VEGAS)[37], once negative selection is incorporated into this system, can create high-affinity, site-specific RNA-binding domains. Furthermore, AG-dependent introns can possibly be retained in a straightforward manner via a dCas13-methyltransferase fusion protein (with an sgRNA)[38,34]. And for RNA molecules at least, 5'- and 3'-untranslated regions (UTRs) can also be targeted. With CRISPRa, activation is substantially improved by "tiling" the target promoter with multiple sgRNAs[39].

Chromatin remodeling with regard to one[40,41] or multiple enhancers[42] may also be important for particular targets. Notably, it was recently shown that for some genes, activation of the gene's enhancer will only help in certain cell types when the gene's promoter is also activated[43]. Thus, a larger vector like an intracellular oncolytic bacterium may be the best option for treatment here – as packaging space is essentially unlimited with such vectors. (As little is currently known about RNA secretion in bacteria[44], TALE

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DNA-binding domains fused with potent transcriptional activators could be utilized for now instead of CRISPRa when such vectors are employed[45].)

With regard to the exact mechanisms of promoting vector proliferation, if a mutated cytoplasmic RNA molecule or protein is targeted by an oncolytic nuclear virus, initiation of viral replication could be made dependent on a transcription factor (TF) that is tethered to the endoplasmic reticulum (ER). Detection of the target mutation(s) would activate an orthogonal protease, e.g., the TEV protease, that liberates many copies of this TF, which could then travel to the nucleus and promote replication[46]. Cyclic TALEs (cycTALEs[47]) can also potentially be utilized instead of ER-tethered TFs. (Lastly, TALEs with orthogonal protease recognition sites can serve as repressors of viral replication – which would then be degraded following activation of the orthogonal protease[48].) For intracellular bacterial oncolytic vectors, there are other considerations.

Non-replicating intracellular bacteria can be rapidly generated with a technique involving the deletion of the *dnaA* gene from the bacterial genome and introduction of a plasmid encoding the *dnaA* gene that has a temperature sensitive origin of replication[49]. Thus, when the temperature is increased, the bacteria will lose the plasmid and become non-replicating – ampicillin can then be added to the culture to lyse the dividing cells while sparing the non-dividing cells. However, it is unclear if those non-replicating bacteria can resume replication normally if *dnaA* expression is induced later on, as those bacteria elongate substantially and may have other metabolic abnormalities. (Moreover, it is plausible that bacteria that are so elongated could potentially have trouble entering certain cancer cells.) As another option, RelA overexpression induces the large scale production of pppGpp and ppGpp. These two molecules are known together as (p)ppGpp, and they are known to greatly slow bacterial growth[50,51]. Thus, (perhaps truncated) RelA overexpression could be the baseline state in the intracellular bacterial oncolytic vector[52], whereas RelA could be silenced to some degree upon mutation detection. (Some amount of RelA may be necessary for the vector to survive in host cells[53].)

In order to transmit a switch detection signal to the bacterial genome and elicit replication, a switch could be utilized with SepM as the response element, which would cleave competence-stimulating peptide (CSP) precursor molecules into mature CSP molecules and activate a two-component regulatory system (i.e., for Gram-positive bacteria like *Listeria monocyogenes*)[54]. Alternatively, nitric oxide (NO) can be used as the signal to promote intracellular bacterial replication[55,56]; the effector component of the secreted switches could be the inducible nitric oxide synthase (iNOS). iNOS, however, must form a homodimer in order to function, so an allosteric switch that requires continual binding of the target mutation for sustained activation of its effector domain may not be ideal here. Additionally, secretion of Bcl-2[57] may be necessary to prevent toxicity to the cancer cells prior to sufficient replication of the bacterial vector. (Another issue is that macrophages at times generate large quantities of NO[58].) Finally, regulated intramembrane proteolysis (RIP) can possibly be exploited[59]. Secreted switches would search for the target mutation(s); if found, an orthogonal protease component of the switches would activate and cleave a protein domain from the bacterial outer membrane. Then, an intramembrane bacterial protease would cleave a segment of the same protein within the membrane,

releasing an intracellular effector domain that initiates replication. I am not aware of any RIP systems in place in the outer membrane of Gram-negative bacteria, but to transmit the signal to their cytoplasm, two rounds of RIP would be required (i.e., one on the outer membrane and one on the inner membrane). It may be more facile for Gram-positive intracellular bacteria (e.g. *Listeria monocytogenes*), which lack an second, outer membrane[60] – although it is unclear if a relatively large protein such as an orthogonal protease can travel through the cell wall to the membrane of Gram-positive bacteria.

It is important to remember that cancer cells could still evolve resistance to vector entry (via downregulation of various cell surface receptors) and even lysis. However, one could theoretically incorporate numerous transgenes that allow the vector to bind to a multitude of ubiquitously-expressed cell surface markers[61,62], such as *Slc20a1* and *SCAMP2*, to increase the chances of entry (if there is sufficient packaging space). (Fusing the cell-penetrating peptide, Tat, to a protein on the surface of the vector could also help with resistance to entry[63].) Of course, entry into all cells, including non-adherent cells, would be ideal. But if one chooses receptors that are present on circulating white and/or red blood cells – larger and/or multiple doses of the vector would be necessary to overcome the sequestration effect with regard to IV administration. In certain circumstances, one may wish to employ a cell-penetrating peptide like Xentry[64], which only binds to adherent cells. Additionally, one could make the vector hyper-virulent in terms of its replication and lytic capacity – of course only when a mutation is detected. Methods of making oncolytic vectors more formidable are discussed in an accompanying piece.

The next topic is slightly unclear: a bystander effect. A toxin with a bystander effect could be utilized to destroy any nearby cells that lack or have lost the target mutation(s). The loss of a few normal cells due to the bystander effect may be worth it to destroy neighboring cancer cells without the target mutation(s). However, if a bystander effect is desired, vector should perhaps first be given time to spread throughout the tumor(s) as much as possible before the toxin is induced automatically (e.g., through quorum sensing) or via small molecule administration. This is important to ensure maximal destruction of the tumor as well as a gradual tumor destruction process that precludes tumor lysis syndrome. HSV1-TK-based imaging can be utilized to visualize vector spreading throughout the tumor(s)[65]. It may be advisable to secrete a toxin like *Staphylococcus aureus* a-Hemolysin[25], a pore-forming toxin, or perhaps diphtheria toxin (DT) from infected cancer cells to ensure that even non-replicating cancer cells in the surrounding area are eliminated. (If one wishes to utilize secreted DT for the bystander effect, a truly "non-leaky" inducible system must be utilized[66,67] or the toxin must be mutated to make it somewhat less toxic.) Employing a bystander effect is similar to the concept of surgically removing more tissue than may appear necessary around a tumor – i.e., increasing the surgical margins. Importantly, if large doses of the vector are administered intravenously, a time-delayed self-destruction gene circuit should be included so that prior to toxin induction, the vector is destroyed in non-cancerous cells. (The timer would be reset upon mutation detection.) Self-destruction would be effected by a late promoter-inducible CRISPR/Cas9 system or meganuclease[68] for viruses – or, for intracellular oncolytic bacterial vectors, an *actA*-inducible[69] TF cascade involving early, intermediate, and late

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promoters that results in xenophagy[70]. (Manual induction of vector self-destruction in cells lacking the target mutation is also possible through small molecule administration[71].)

The 'kindling' strategy mentioned in my 2018 *Gene Therapy* article may be applicable here in addition to or instead of the toxin bystander effect[72]. I may favor the latter approach, as cycles of non-specific enhancement of replication (potentially interspersed with time-delayed vector self-destruction in non-cancerous cells) could give the vector a boost that may be necessary to destroy cancer cells wherein the mutation-targeting switches are less effective for some reason (e.g., if there are endogenous, high-affinity RBPs that attach to the target sequence and interfere somewhat with switch binding.)

As a side note, with regard to a bystander effect, some cancer cells may be temporarily dormant, but it is also possible that some cells that contain the targeted ubiquitous mutation(s) may become permanently arrested with regard to division through the loss of at least some of the genes required for cell division, but then still contribute to an aberrant microenvironment in a way that may lead to oncogenic effects in nearby cells - similar to the senescence-associated secretory phenotype[73].

Even if a hyper-virulent vector is utilized and a bystander effect is incurred, some cancer cells may survive the treatment (i.e., if they are particularly robust with regard to expressed anti-viral and anti-toxin machinery). In a certain sense, a bystander effect may be counter-productive, as it could increase the toxicity of the treatment and perhaps not increase the probability of completely destroying the tumor(s); even a single surviving cancer cell could seed another tumor. Perhaps a bystander effect could be tried at first, and if the cancer recurs, further treatments could be applied without it. Alternatively, perhaps with time, clinicians will be able to tell which patients would be best served by incorporating a bystander effect into this treatment. Finally, if the bystander effect is made to be somewhat mild, it could be employed in general. The reason that this topic is important is because if one or more tumors grow back, they can simply be retreated by re-enacting the aforementioned strategy (i.e. thorough resequencing, molecular switch generation, and vector engineering and delivery), as the specificity of the treatment would make it non-toxic and therefore suitable for repeated administration if a substantial bystander effect is not incorporated into the treatment.

Crucially, a multitude of small molecule-inducible kill switches should ideally be added to each vector in case off-target switch activation occurs. The reason that a plethora of kill switches would be helpful is because the vector may evolve to disable one or more of them. If an automatic (or manual) self-destruction circuit is not added to the vector so that it dies in non-cancerous cells, the kill switches will be important to preclude any mutagenic or cytotoxic effects the vector may have on host non-cancerous cells through prolonged transcriptional/translational upregulation. However, even if the self-destruction circuit is utilized, kill switches would still be necessary in case the switch or switches activate in some of the patient's non-cancerous cells due to specific cellular conditions - or if the vector evolves to no longer require mutation detection for the promotion of replication. (The kill switches should not kill the host cell, but rather cleave up the viral vector genome or cause xenophagy of the intracellular bacterial vector. Actually, in both the case of the

self-destruction circuit and the kill switches, it would be ideal if viral vector DNA in the nucleus could be expelled through exocytosis or vesicle release and then autophagocytosed – to limit the possibility of insertional mutagenesis[74].)

One possible drawback to this ubiquitous mutation detection-based oncolytic vector strategy at present is that immune suppression may be required to allow successful propagation of the oncolytic vector within the tumor(s)[75,76]. In the context of repeated treatments, this would be very inconvenient and clearly at least somewhat dangerous for the patient. If cyclophosphamide (CP), or cycles of CP, are necessary for sufficient immunosuppression during the treatment, fasting beforehand could help limit genotoxic damage – at least in leukocytes and bone marrow cells[77]. (Transient fasting may even enhance the efficacy of oncolytic viral treatment[78].) With hyper-virulent vectors especially, the tumor(s) may be eliminated rapidly, which would preclude the need for a large number of CP cycles. However, it may currently be possible to imbue certain vectors with gene modules that make them "stealth" vectors, so that they can replicate in tumor tissue unhindered by the immune system[79,80]. (Additionally, stealth vectors would be able to circulate for a prolonged amount of time[81,82] without being neutralized[83] after IV injection or shedding from an infected tumor, making it more likely for any micrometastases that might exist to be destroyed.)

Switches targeting RNA:
Importantly, unique targeting of DNA/RNA within the human genome requires that one recognize at least 16 sequential nucleotides when statistically assuming random base distribution, but in reality, targeting at least 18 sequential nucleotides would be more ideal[84]. RNA can be directly targeted with regard to many types of mutations in non-coding DNA and coding DNA. For example, mutant long non-coding RNA (lncRNA) molecules can be detected. Additionally, mutations in the 5'- or 3'-UTRs, exons, and potentially introns of any mRNA molecule can be sensed. Any large mutation in a target transcript (i.e., when there are more than three mismatches between the mutant and original sequences) can be targeted by programmable RNA-binding switches - based on Pumby modules[85]. Additionally, with RNA (as well as DNA), as opposed to protein, even synonymous mutations can be targeted – which may sometimes be ubiquitous mutations in a patient's cancer[86]. Another important point is that proteins can get modified to be sent to all sorts of cellular locales; in general[87], RNA is only found in the nucleus or the cytoplasm. (Two different switches targeting the same transcript, one with a nuclear localization sequence and one with a nuclear export sequence, can be encoded by the vector. With regard to the former case, a second transcription factor driving vector replication could be tethered to the inner leaflet of the inner nuclear membrane.)

Types of switches:
There are four main types of molecular switches that one may wish to focus on with regard to detecting cancerous mutations using oncolytic vectors: dual, proximity-based switches, epigenetic/transcriptional, post-transcriptional, and translational regulation switches, allosteric switches, and (ribo)nucleotide editing-based switches.

Dual, proximity-based switches

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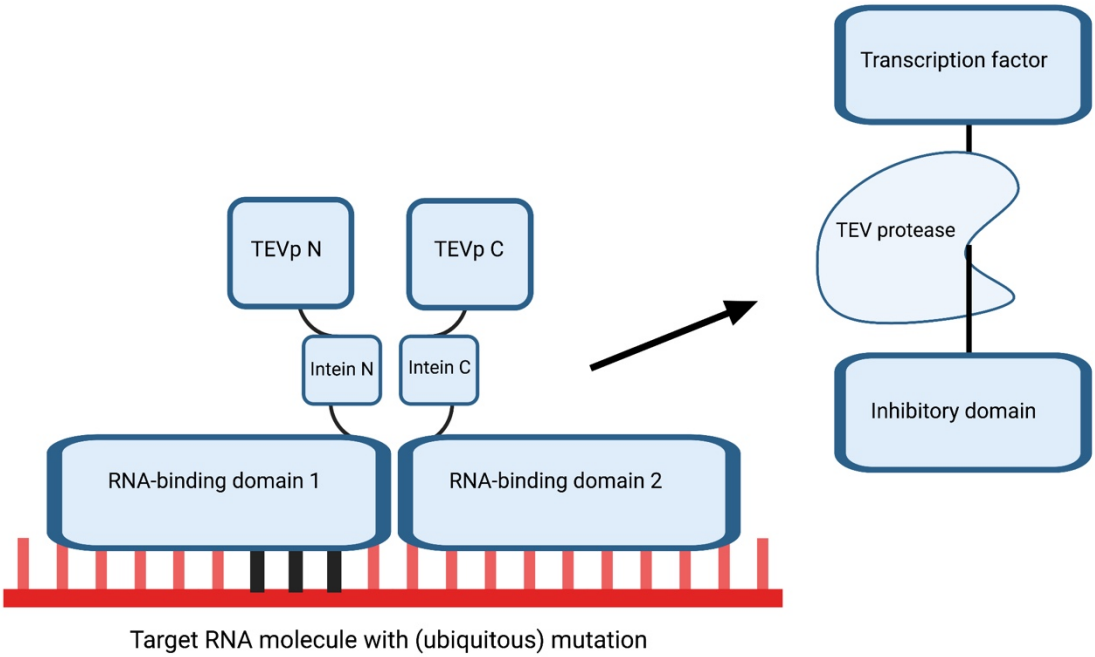

Figure 1: Dual module 'proximity' switches. Here, two proteins, each with an RNA-binding domain and half a protease domain, would be designed to dock next to each other only on a mutant sequence. Then, the split protease exteins would be fused together and released from their RNA-binding domains.

Figure 1 depicts dual, proximity-based switches, which involve two RNA aptamers or proteins binding next to each other on a molecular target, leading to split protein assembly (and possibly liberation due to the formation of DUB+UBLP recognition sites[88]) or split intein-based generation of an effector protein. One may target mutated DNA[88,89], RNA[85], or proteins[90] with such switches. A protease cascade could amplify the signal if DNA must be directly detected[91,92]; the switch response element could be an orthogonal protease (e.g., the TEV protease) – which would then activate an orthogonal protease zymogen (e.g., the TVMV protease). Many activated, TVMV proteases, would then liberate many more transcription factors tethered to the inner leaflet of the inner nuclear membrane. However, such a cascade might increase off-target activity.

With regard to using dual, proximity-based switches on protein targets, there are two possibilities. First, RNA aptamers generated through systematic evolution of ligands by exponential enrichment (SELEX) may be very helpful[93]. Evolved RNA aptamers can even selectively target a mutant protein over the original based on a single amino acid difference[94]. In the article cross-referenced here[90], the researchers designed a system where a TF tethered to the inner leaflet of the plasma membrane would be liberated when

the intrabody domains of two fusion protein bound different regions of a certain protein target. In the case of this therapy, the intrabody domains would be replaced with RNA aptamers that are connected to the effector domains via genetically-fused RNA-binding domains[95]. However, the aforementioned researchers found, as one might expect, that the best operating conditions were achieved when they employed a low-affinity TEV protease cleavage site and ensured low sensor concentration[90]. One could alternatively utilize a split TEVp construct (potentially with DUB+UBLP recognition sites) or TEV protease split intein construct in the context of two RNA aptamers (with no tethering to the plasma membrane). Notably, it is also theoretically possible that SELEX could be used to create an RNA aptamer that binds a point-mutated dsDNA, or more suitably, ssRNA molecule selectively over the original sequence. Second, OrthoRep could potentially be harnessed here to generate antibodies against different domains of a protein target[96].

With regard to detecting a target RNA molecule, "Pumby" modules have recently been developed that allow for the straightforward design of proteins that recognize any given RNA sequence[85]. It was shown that with Pumby-based proteins, three or more mismatches from the target sequence precluded binding. It is unclear if point mutations can be distinguished from the original sequence by Pumby-based proteins if PACE or eventually VEGAS (once negative selection is incorporated) is used to increase their specificity. Importantly, CRISPR/Cas13 can target RNA molecules. It was shown that it can be made sensitive to point mutations in the RNA if a second, synthetic mismatch is introduced by altering the crRNA slightly – although this is with regard to cleavage; whether mere binding is as sensitive is unclear. (Using truncated crRNAs [23 nt] can also impart single-base mismatch-level specificity with regard to cleavage[97].) If simple binding occurs with regard to the original sequence, even of a transient nature, it may be enough time for split protein assembly or intein trans-splicing to occur – which would be an issue here. It has been shown, however, that FnCas9 can discriminate between RNA sequences at single-nucleotide resolution even in the absence of an allosteric shift related to cleavage, and it could be utilized here instead (with inactivating mutations in its nuclease domains to prevent any risk of it cleaving the host cell genomic DNA if it somehow enters the nucleus even with a nuclear export signal [NES] or is sent to the nucleus to target RNA that is retained there)[98].

If point mutation sensitivity is possible with dCas13 or dFnCas9, one dCas13/dFnCas9 protein and one Pumby-based protein could be used together. dCas13- or (d)FnCas9-gRNA pairs could also work here, but I am unsure if they could land close enough to one another to be used as dual, proximity-based switches. It is also possible that RNA-targeting ZFPs[99] can be made more specific (via rational design and/or PACE/eventually VEGAS) in terms of distinguishing point mutations than Pumby-based proteins based on differing modes of RNA recognition[100].

Epigenetic/transcriptional, post-transcriptional, and translational regulation switches:

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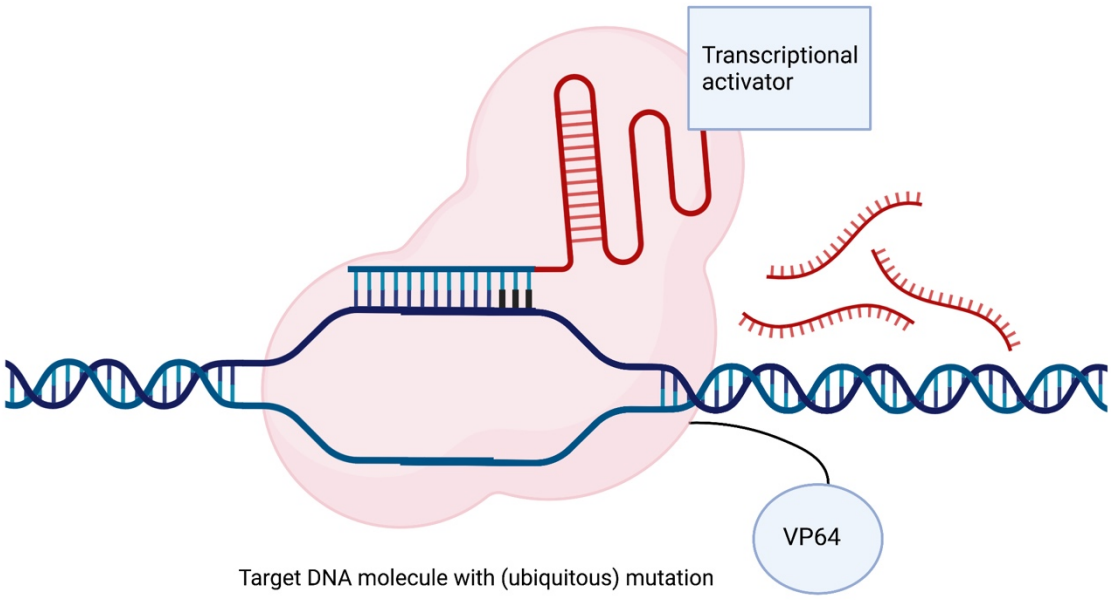

Figure 2: Transcriptional regulation switch based on dCas9. If there is a mutation in the promoter region of a gene, the mutation can be selectively recognized by a particular kind of switch based on CRISPR/Cas9, TALEs, or ZFPs that are fused to a protein domain that upregulates the production of the given transcript. (For dCas9, the sgRNA can also be extended and used as a docking site for transcriptional activators as shown here[31].) This is facile when large mutations are targeted, but perhaps slightly more complicated when point mutations are targeted. The red, floating strands are transcribed mRNA molecules that are generated as a result of the targeted transcriptional upregulation process.

Currently, some mutations in promoters can be targeted. Very large mutations in a promoter region, i.e., those that affect many nucleotides, can be targeted by multiplexed dCas9 or multiple TALE DNA-binding domains fused to transcriptional activators (i.e., tiling to enhance activation[39]). The target transcript can be downregulated, too, in non-cancerous cells by virtue of CRISPRi or TALE DNA-binding domains fused to transcriptional inhibitors. This discrepancy in expression levels can then be the basis for promoting replication of the oncolytic vector solely in cancer cells. Relatively large mutations in promoters, i.e., those that affect enough nucleotides to still allow some degree of tiling, should be easily exploitable as well (with regard to sufficient upregulation in cancer cells and downregulation in non-cancerous cells).

Even point mutations can sometimes be targeted, if one generates a known PAM or perhaps if a PAM is situated at an appropriate distance from the point mutation so that a

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mismatch would occur in part of the seed region of the relevant sgRNA[101]. Ideally the mutation would change one known PAM to another, so that CRISPRa can be applied in the cancer cells and CRISPRi can be applied in noncancerous cells. Alternatively, TALE DNA-binding domains fused to transcriptional activators can selectively upregulate a transcript in cancer cells when a nucleotide in a promoter region is changed to a T or a G[102]. If the nucleotide changes from a G to a T or a T to a G, the original nucleotide can be used for selective downregulation of the transcript in non-cancerous cells. (A TALE that is somewhat selective for a 5' A, C, or G over T [i.e., a ~4-fold specificity change] has been generated as well, and could be of use here[103].) If an oncolytic intracellular bacterial vector is utilized, it would have to secrete a TALE or ZFP-based transcriptional activator rather than CRISPRa for the time being, as the mechanisms of bacterial RNA secretion are still relatively mysterious (although there is evidence that they do so through microvesicles as well as at least one microvesicle-independent pathway)[44].

However, in the case of targeting a point mutation in the promoter of a given gene (i.e., in the absence of tiling), an enhancer activation-related strategy may be necessary as well to sufficiently upregulate a target transcript or protein in general or at least in a timely manner. This would involve repression at the promoter in non-cancerous cells; simply activating an enhancer may not always help if the promoter is not also activated[43]. (Figure 2 illustrates a scenario similar to targeting a point mutation where extensive tiling is not possible - in this case there are three nucleotides mutated in a row.)

Ideally, for DNA that is not endogenously transcribed, it would be possible to detect a point mutation with a switch that leads to transcription of a relatively short RNA sequence - possibly using dCas9 roadblocks as a way of enabling transcriptional termination[104]. This would potentially amplify the target RNA "signal" without leading to off-target effects like a protease cascade.

Of course, once the transcript is upregulated, it must be detected in some fashion by another set of switches. This would be rather facile, as Pumby-based dual proximity switches could certainly be applied here (as one is not seeking to target a single nucleotide difference in the transcript).

Small interfering RNA (siRNA) has been shown to be able to distinguish between genes that differ by a single nucleotide with regard to post-transcriptional silencing[105]; siRNA could silence the non-mutated RNA in normal cells, thus leaving that transcript available for binding by RNA-binding switches only in cancerous cells. With regard to post-transcriptionally amplifying a mutation signal, a 3'-acting trans-splicing ribozyme could selectively add the promoter sequence for a RNA-dependent RNAP (RDRP) with proofreading capacity[106] to a mutant transcript that has a novel U (but perhaps only when the novel U lies close to the 3' end)[107]. This RDRP would amplify the signal by transcribing large quantities of the complementary strand that could also be targeted by switches. (dsRNA-binding switches might be helpful here.) This approach could also be used to amplify the signal of a transcript regardless, i.e., when the targeted mutation is further upstream in the transcript.

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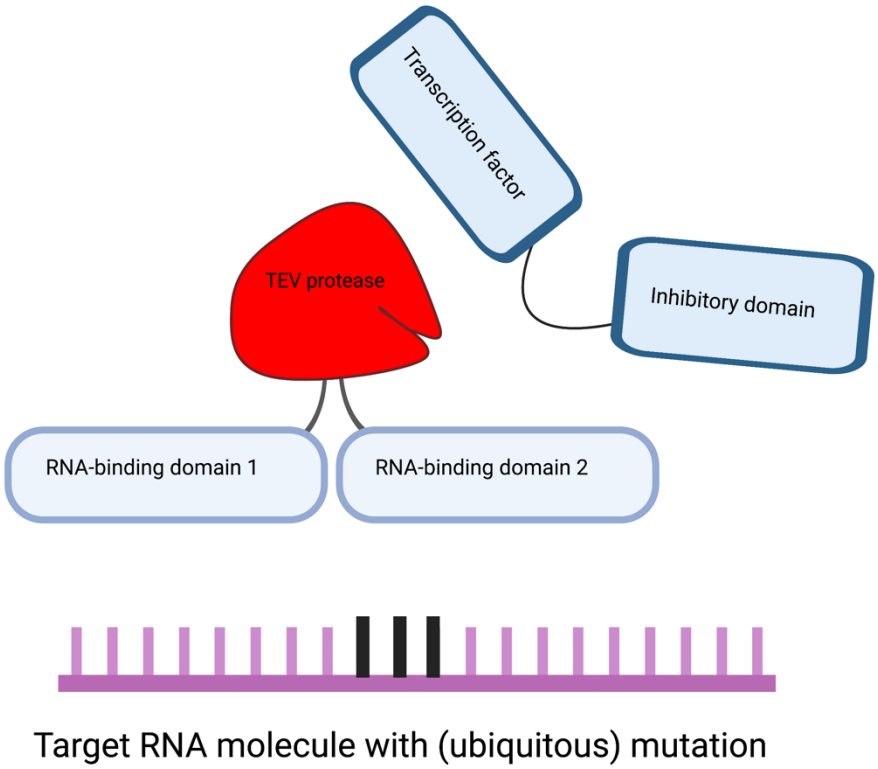
For translational regulation switches, one option may be to use a 5'-acting trans-splicing ribozyme[108] with a potent, cap-independent translation enhancing element[109,110] - to target mutations wherein a novel G is generated close to the 5' end of the transcript. These strategies could also, of course, enhance translation of a transcript in general (i.e., when the targeted mutation is further downstream in the transcript). One can detect a protein that is translationally upregulated via RNA aptamers generated through SELEX that are utilized as part of dual, proximity-based switches (as mentioned in the previous section).

Allosteric switches:

A.



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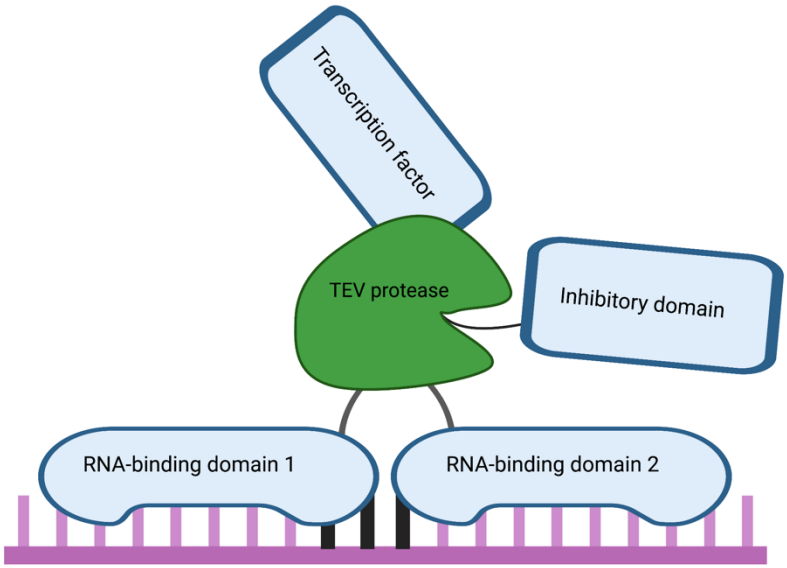
504 B.



505
506 Figure 3: A) Allosteric switches. Here, an allosteric protein switch with two or three
507 domains could be obtained through rational design and/or directed evolution. It may be
508 the case that allostery is more readily achieved via domain insertion and subsequent
509 optimization than simple end-to-end connection followed by optimization, meaning that
510 the original RNA-binding domain will be split into two smaller domains. Thus, perhaps in
511 most cases, the N-terminal and C-terminal domains will bind to the target RNA sequence
512 (possibly as Pumby-based domains) – and the third domain could be an orthogonal
513 protease domain (e.g., the TEV protease). Initially, without binding the correct target
514 sequence, this orthogonal protease domain would be inactive. B) Upon binding the target
515 RNA sequence, a conformational change will occur in the bipartite RNA-binding domain
516 that is propagated via linkers to the orthogonal protease domain, making it go from the
517 OFF-state to the ON-state – although its activity would be dependent on continued binding
518 of the target sequence.
519
520 Figure 3 depicts an allosteric switch. An allosteric switch here would likely be a single-
521 component agent with an N-terminal and C-terminal domain that together recognize the
522 target mutation in an RNA molecule, e.g., Pumby-based domains, which are connected via
523 linkers to an inserted orthogonal protease domain. The orthogonal protease domain would
524 be in the OFF-state when the flanking Pumby-based domains are not bound to the target
525 RNA molecule (i.e., mutant RNA). The orthogonal protease domain could also be fused to
526 either end of a single, merged Pumby-based domain, but this might not generally be

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feasible for allosteric switches – as insertion of the effector domain into a sensor domain might typically be required for substantial allosteric interactions to occur[111]. After binding to the target sequence, a conformational change in the Pumby-based domain(s) would propagate via linker(s) to the orthogonal protease domain, thereby changing it to the ON-state (as long as binding is maintained).

There are two systems of directed evolution which may be able to generate allosteric switches within a therapeutically-relevant timeframe[112,37]. I referenced a preliminary version of the first system in my first paper on the topic of oncolytic virotherapy based on mutation detection, which was published in *Gene Therapy*[72]. However, the second system, VEGAS, designed by Dr. English *et al.*, may be the best option. It is more rapid than the other system and also involves insertion and deletion mutations in the evolution scheme, so the compositions/lengths of both linkers connecting the orthogonal protease domain to the RNA-binding domains could be varied automatically to some extent at least. Once negative selection is incorporated into VEGAS, it may be a perfect system for the evolution of allosteric switches, provided the schema detailed in my *Gene Therapy* article works. One would still wish to run multiple VEGAS experiments simultaneously for each desired switch, though, to evolve separate viruses that encode the orthogonal protease domain inserted at different points in the RNA-binding domain. Moreover, VEGAS could be made even more effective if a suspension culture system with an inflow and outflow could be created (like PACE) – as Dr. English *et al*. mentioned in the conclusion of their paper.

(Ribo)nucleotide editing-based switches:
(Ribo)nucleotide editing-based switches include trans-splicing ribozymes, RNA base editors, RNA endoribonucleases/RNA ligases working together[113], and a pair of proteins or an enzyme that can edit the DNA based on zinc-finger nucleases (ZFNs), TALE nucleases (TALENs), or CRISPR/Cas9.

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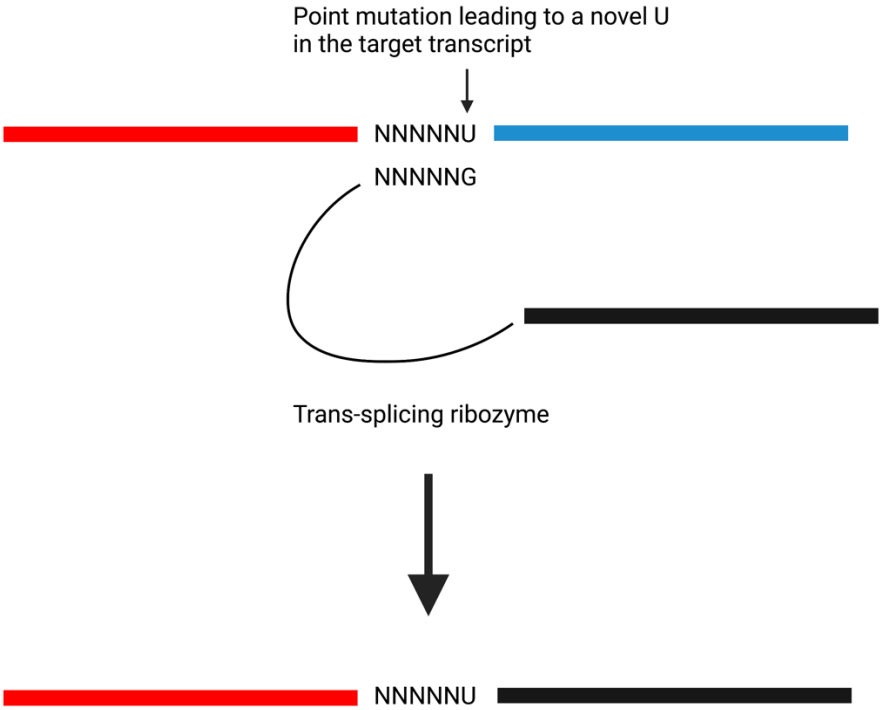

Figure 4: Here is an example of a ribonucleotide editing switch. This image depicts a trans-splicing ribozyme that can replace the 3'-portion of its target RNA molecule with its own 3'-exon when a U is present in the target. A trans-splicing ribozyme, if designed in other ways, can also replace the 5'-portion of a target RNA molecule with its own 5'-exon, insert a sequence into the target RNA molecule, or remove an internal sequence from the target RNA molecule.

Figure 4 illustrates a trans-splicing ribozyme. Trans-splicing ribozymes are RNA molecules that, when designed in various ways, can replace the 3'-portion of its target RNA molecule with its own 3'-exon, replace the 5'-portion of a target RNA molecule with its own 5'-exon, insert a sequence into the target RNA molecule, or remove an internal sequence from the target RNA molecule[114]. However, they may only be usable when a novel uridine (3'-acting trans-splicing and trans insertion-splicing ribozymes[115]), a novel guanosine (5'-acting trans-splicing ribozyme[108]), or both (trans excision-splicing ribozyme[116]) are introduced into a target transcript, i.e., via mutation.

With these switches, the target mRNA molecule could be altered to encode a protein driving oncolytic viral replication.

A dCas13-related RNA base editor could selectively change a C to a U at a point mutation site[117]. The U could then be targeted by a 3'-acting trans-splicing ribozyme. Additionally, it may be possible, by employing the same synthetic mismatch tactic mentioned before, to have dCas13 bind a point-mutated sequence (i.e., wherein an A is generated) with a mismatch introduced elsewhere to change a C to a U (which is how the base editor "knows"

which base to edit). In non-cancerous cells, there would be a total of two mismatches, and potentially a lessening of base editing efficiency.

Oncolytic vectors

Possible vectors that can harness such switches to trigger their own replication are described herein. The herpes simplex virus type 1 or 2 (HSV-1 or HSV-2), *Listeria monocytogenes*, and *Shigella flexneri* may make excellent vectors.

Viruses:

HSV-1 has evolved to enter and replicate within a multitude of human cell types efficiently[118] and may be able to carry multiple therapeutic transgenes without compromised replication. With regard to HSV-1's packaging capacity, only ~2 kb of foreign DNA has been added to the full-length 152 kb genome[119,120,121]. To my knowledge, no one has experimentally determined the maximum amount of DNA that can be added to the HSV-1 genome – although they have done so for the Epstein-Barr virus (a γ-herpesvirus)[122] and the guinea pig cytomegalovirus (a β-herpesvirus)[123]. Approximately half of HSV-1's genes were found to be non-essential for growth in culture, which seemed to indicate that at least 40 kb of foreign DNA could be accommodated within the genome after certain sequences are deleted[124].

However, what is non-essential for growth in culture may actually be essential for growth *in vivo*. Moreover, recently it was shown that deleting multiple genes from HSV-1, which were each deemed to be non-essential when deleted singularly, can still lead to replication defects even in culture[125]. *In vivo*, certain non-essential genes may in fact be important to ensure maximal virulence (which, of course, is desirable when made contingent upon ubiquitous mutation detection). Gene products related to immune evasion[126] may be truly non-essential, as the patient can be immunosuppressed. (It is possible, though, that some or all gene products that have immune evasion functions may serve multiple purposes – one or more of which could be indispensable[127].) It would also be much better to use stealth vectors rather than immunosuppress the patient - if at all possible.

The HSV-1 capsid appears to be very full, and is under high pressure[128,129]. This seems to indicate that one cannot add too much additional DNA to its genome. However, the related β-herpesvirus, human cytomegalovirus, has a capsid which is not much larger (~17% larger) and still packages a much larger genome into it (>50% larger)[130]. It is possible that if the HSV-1 capsid is bolstered with certain stabilizing proteins or elements that a substantial amount of extra DNA could fit within. Another strategy would be to do some vector engineering to remove certain dispensable regulatory elements, or add in IRESs, 2A sequences, or connect multiple proteins together into polypeptides that can be separated into their constituent parts by an orthogonal protease (thus removing the need for various promoters)[131]. Also, certain proteins can perhaps be truncated[132].

HSV-1 should avoid neutralization in the bloodstream. Mutation of the HSV-1 gD protein has been shown to aid with this[83]. Additionally, targeted plasmapheresis[133] or the administration of empty capsid-filled envelopes (or even just empty envelopes) could assist in depleting circulating antibodies against the vectors[134]. CD47 is useful to prevent

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macrophage uptake, increasing circulation time as well as persistence at the tumor sites - and decreasing inflammation[81,82].

Hyper-virulence modules should only be activated upon detection of the ubiquitous mutation(s). With regard to intratumoral spreading[135], one can add transgenes that encode proteins such as MMP9[136,21], heparanase[137], elastase, collagenase[138], hyaluronidase[139], and relaxin[140] (to be secreted and/or displayed on the host cell surfaces/envelopes of the viral particles). However, one does not necessarily want to add too many extracellular matrix-degrading transgenes; otherwise, cells may break off and seed metastases. On the other hand, with the expression of relaxin alone, an inhibition of metastatic seeding was observed[140]. P19 expression could also be helpful for oncolytic HSV-1[141]. GALV is another protein that can be expressed for hyper-virulence - it causes cell-cell fusion and enhances the spread of oncolytic vectors throughout tumors[142]. If there is limited packaging space, however, as there certainly might be with HSV-1, the vector cannot be made truly (but conditionally) hyper-virulent.

Intracellular bacteria:
If one wishes to target a plethora of mutations simultaneously or program in truly extensive hyper-virulence upon detection of one or more mutations, one could utilize an intracellular bacterial vector such as *Listeria monocytogenes* – as its packaging space is essentially unlimited - and it is already adapted to survival and replication inside human cells. *S. flexneri* is a Gram-negative bacterium that is similar in many ways to *L. monocytogenes*. The main difference between using these two vectors is their mode of cellular entry. *L. monocytogenes* uses a system called zippering[143], which for said bacterium sometimes involves specific cellular machinery associated with the E-cadherin and c-Met receptors[144]. In contrast, *S. flexneri* uses a system called triggering, which involves a type 3 secretion system (T3SS) - wherein it injects effectors across the cell membrane of non-professional phagocytes, inducing membrane ruffling and the uptake of the bacterium[143]. This theoretically could allow entry into a wider variety of target cells, as (given adhesion) the target cell's machinery can be manipulated externally - whereas zippering may require that certain proteins are already expressed by the target cell. To increase the chances of entry, though, until all the necessary effectors for inducing cell entry into a wide range of target cells are known, *S. flexneri* could be bioengineered to also induce zippering.

Finally, *Vibrio natriegens* has an extremely fast growth rate; it has a doubling time of <10 minutes[145]. Notably, it only takes the expression of two proteins to allow a normally extracellular bacterium to enter a host cell and escape to the cytoplasm[146]. If the division rate of *V. natriegens* can be sustained inside a host cell[147,148], this would be ideal with regard to rapid cytotoxicity in cancer cells and intercellular spreading.

Decorating the outside of the bacterial outermost membrane with USP30[149,150] and a fragment of ActA[151] could be effective (at least in certain cell types)[152] could help to prevent xenophagy prior to mutation detection. The latter protein does not apply to *L. monocytogenes*, as it already expresses the full version of ActA on its surface. For intracellular bacteria - it is important to make sure that the multiple, small molecule-

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inducible kills switches are chromosomally-encoded[153]. With regard to making bacterial vectors "stealthed"[79], there are a few considerations. CD47 on the surface of the bacteria and host cells (when bacterial RNA secretion can be effected) would be ideal. In terms of hyper-virulence, some of the same proteins mentioned in the **Viruses** section are applicable. Additionally, host cell-cell fusion can be induced by a type 6 secretion system[154].

Discussion:

There is no guarantee that enhancing oncolytic vector replication through the detection of ubiquitous mutations will be curative, as some of a patient's cancer cells may lack or have lost the targeted mutation and divide to replenish tumors or grow tumors at new sites. However, with all the therapeutic modules discussed in this article, the aforementioned anti-cancer strategy should be at least somewhat effective in shrinking a given patient's tumor(s) – and, most critically, this strategy can be repeated indefinitely and therefore may at least keep the cancer from ever killing the patient. (Notably, if a patient has no ubiquitous mutations, targeting multiple subclonal mutations could work as well.) Imaging via HSV1-TK in combination with multiple small molecule-inducible kill switches would help to make sure a given oncolytic vector is safe for use in patients.

Another important issue is cost. Importantly, whole genome sequencing (WGS) costs have greatly decreased in recent years[155]. Nebula Genomics offers $299 deep whole genome sequencing for customers[156]. Moreover, the Beijing Genomics Institute (BGI) Group stated in 2020 that they can sequence whole genomes for $100 per genome with newly-developed technology[157]. Cheaper WGS makes it more clinically-feasible to implement multi-region sequencing of a patient's primary tumor and metastases. Some studies indicate that truly cheap prices may only be realized in practice if sequencing is scaled up in terms of samples; if this (ubiquitous) mutation-targeted strategy is adopted for all cancer patients, however, that may not be an issue[158,159]. However, another issue is the vectors themselves. Especially if one has to treat a single patient with multiple oncolytic viruses targeting different mutations (due to limited packaging space), this could be an issue. Growing multiple oncolytic viruses for a single patient to high titer and properly purifying them could be prohibitively expensive[160]. Culturing facultative intracellular bacteria would be much cheaper.

It would be helpful to perform a proof-of-concept experiment showing that targeting a (large) mutation in a transcript with dual, proximity-based switches could lead to replication of an oncolytic vector in such a way that it eliminates tumors in mice. It would be ideal if one did not have to worry initially about chromatin status or cells that lack or have lost the targeted mutation. To surmount those issues, one could utilize a molecular trick that was described in 2015. One would simply implant human tumor cells that chromosomally encode a monomeric iRFP protein[161] or enhanced luciferase enzyme[162] next to a diphtheria toxin resistance gene into immunocompromised mice[163]. Thus, if one periodically administers diphtheria toxin to the mice, it would select for tumor cells that contain the target transcript, which would also serve to illuminate the tumor cells. One would then administer an oncolytic HSV-1 vector, for example, that encodes Pumby-based proximity switches targeting the iRFP or enhanced luciferase transcript. Then, the extent

of tumor destruction could be measured by near infrared fluorescence-based imaging or AkaLumine-HCL-based imaging.

Conclusion:

Hopefully this oncolytic strategy will prove fruitful with regard to curing or effectively, repeatedly treating cancer. I call this ubiquitous mutation-targeted oncolytic vector strategy "Oncolytic Vector Efficient Replication Contingent on Omnipresent Mutation Engagement" (OVERCOME). Of course, there is also a variation of OVERCOME that involves detecting multiple subclonal mutations (i.e., if a patient has no ubiquitous mutations). It may take a fair amount of bioengineering of particular vectors, but eventually stealth vectors that are conditionally hyper-virulent could be generated wherein the particular switches could simply be swapped out for new patients. As OVERCOME and its variation (when no ubiquitous mutations are present) might be the best way to treat cancer, we should probably put some effort into testing these types of cancer treatment.

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