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SPOTLIGHT

Transcriptional adaptation: a mechanism underlying genetic robustness

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ABSTRACT

Mutations play a crucial role in evolution as they provide the genetic variation that allows evolutionary change. Although some mutations in regulatory elements or coding regions can be beneficial, a large number of them disrupt gene function and reduce fitness. Organisms utilize several mechanisms to compensate for the damaging consequences of genetic perturbations. One such mechanism is the recently identified process of transcriptional adaptation (TA): during this event, mutations that cause mutant mRNA degradation trigger the transcriptional modulation of so-called adapting genes. In some cases, for example when one (or more) of the upregulated genes is functionally redundant with the mutated gene, this process compensates for the loss of the mutated gene's product. Notably, unlike other mechanisms underlying genetic robustness, TA is not triggered by the loss of protein function, an observation that has prompted studies into the machinery of TA and the contexts in which it functions. Here, we review the discovery and current understanding of TA, and discuss how its main features appear to be conserved across species. In light of these findings, we also speculate on the importance of TA in the context of human disease, and provide some recommendations for genome-editing strategies that should be more effective.

KEY WORDS: Transcriptional adaptation, Gene expression, Genetic compensation, Genetic robustness, Genetic variation

Introduction

Genetic compensation is a process that buffers against the otherwise deleterious loss of a genetic element. It manifests itself as altered gene/protein expression or function, leading to a wild-type-like phenotype in homozygous mutant or heterozygous individuals that would be predicted to exhibit clear defects (reviewed by El-Brolosy and Stainier, 2017). The increased use of genome-editing technology has resulted in multiple reports of substantial differences between the phenotypes induced by gene knockdown (e.g. antisense) and gene knockout (i.e. mutation) (Kok et al., 2015; Stainier et al., 2015; Morgens et al., 2016; Housden et al., 2017). Several hypotheses have been put forth to account for these differences, including the low specificity and/or high toxicity of the knockdown reagents, as well as the hypomorphic nature of the mutant alleles generated (Schulte-Merker and Stainier, 2014; Stainier et al., 2017; Lai et al., 2019). However, recent findings have revealed another process as a potential reason for the differences between gene knockdown- and knockout-induced phenotypes. This process, termed transcriptional adaptation (TA), modulates the expression of potentially compensating gene(s) independently of protein feedback loops, thereby preventing, or at least reducing, the manifestation of harmful phenotypes (Rossi et al., 2015).

TA was first identified in zebrafish while studying the developmental role of Egfl7, an extracellular matrix protein highly expressed in endothelial cells (Rossi et al., 2015). This study confirmed previous observations (Parker et al., 2004) that zebrafish *egfl7* morphants (morpholino-injected animals) exhibit a severe vascular phenotype, as was also observed in *Xenopus laevis* morphants (Charpentier et al., 2013) and in human endothelial cells in culture (Huang et al., 2014). However, it also revealed that most zebrafish *egfl7* mutants, unlike the morphants, do not display an obvious phenotype, with less than 5% exhibiting brain hemorrhage (Rossi et al., 2015). Similarly, most mouse *Egfl7* mutants do not exhibit an obvious phenotype either (Schmidt et al., 2007; Kuhnert et al., 2008). Based on these and other observations (Rossi et al., 2015), it was hypothesized that the phenotypic differences between *egfl7* mutants and morphants were due to genetic compensation in the mutants but not the morphants. Mass-spectrometry analyses were used to test this hypothesis and revealed that another extracellular matrix protein, Emilin3a, which like Egfl7 contains an EMI domain, is upregulated in *egfl7* mutants but not morphants, suggesting a potential compensating role. Gene expression studies further showed that *emilin2a*, *emilin3a* and *emilin3b* are upregulated in *egfl7* mutants compared with wild types, but not in morphants or CRISPRi-injected embryos. Moreover, injections of wild-type mouse *Emilin2* and *Emilin3* mRNAs into early stage zebrafish embryos showed that these genes could, at least in part, compensate for the loss of Egfl7 in *egfl7* morphants. Notably, *egfl7* heterozygotes also display a TA response, albeit one that is less pronounced than that observed in homozygous mutants, indicating that TA is a dominant phenomenon with a semi-dominant effect (Rossi et al., 2015). Similar observations were reported for *vegfaa* mutants, which upregulate *vegfab* (Rossi et al., 2015). In another example of TA, zebrafish embryos homozygous for a premature termination codon (PTC) mutation in *actc1b* display a milder phenotype than that observed in *actc1b* morphants, due to the upregulation of an *actc1b* paralogue, *actc1a*, which restores skeletal muscle function (Sztal et al., 2018). Similarly, Zhu et al. (2017) reported a milder phenotype in zebrafish *nid1a* mutants than in *nid1a* morphants due to the upregulation of *nid1b* and *nid2a*. Notably, these early documented cases of TA (Rossi et al., 2015; Zhu et al., 2017; Sztal et al., 2018) suggested that the upregulated genes can be paralogous to the mutated gene (*vegfaa/ab*; *nid1a/1b*, *2a*; *actc1b/1a*) or encode proteins with common domains (*egfl7/emilin2a*, *3a*, *3b*).

In addition to these initial papers, a few other zebrafish studies (Wei et al., 2017; She et al., 2019; Ye et al., 2019; Rothschild et al., 2020) made similar observations and reported that potentially compensating genes are transcriptionally upregulated in mutants but not morphants. Notably, many of these initial studies (Rossi et al., 2015; Zhu et al., 2017; Sztal et al., 2018) reported that all

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TA-displaying mutants exhibit mutant mRNA degradation. Following up on this observation, it was shown that mutant mRNA degradation plays a crucial role in activating TA in zebrafish embryos and mouse cell lines (El-Brolosy et al., 2019). Indeed, not all mutations activate TA. For example, zebrafish *hbegfb* and *vcla* mutant alleles that exhibit mutant mRNA degradation display TA, whereas other mutant alleles of the same genes that do not exhibit mutant mRNA degradation do not display TA (El-Brolosy et al., 2019). Intriguingly, a mutant allele of *hbegfa* that exhibits only a 15% reduction in mutant mRNA levels does not display TA either, suggesting that a certain amount of mutant mRNA degradation products is required to trigger TA (El-Brolosy et al., 2019). Similar observations were made while studying zebrafish *capn3a* mutants: only the PTC mutation leading to a 90% reduction in mutant mRNA levels causes TA (Ma et al., 2019). Furthermore, whereas mutant alleles of zebrafish and mouse genes that exhibit mutant mRNA degradation display TA, RNA-less alleles (e.g. promoter-less alleles or full locus deletion alleles) do not (El-Brolosy et al., 2019), indicating that mutant mRNA degradation is required for TA. However, Ma et al. (2019) reported that TA, which they termed genetic compensation response, can be initiated solely by the presence of a PTC without the need for mutant mRNA degradation.

Although these studies together have documented the occurrence and importance of TA, they raise several questions with regard to how TA occurs and how prevalent it is. Here, we summarize our current understanding of the TA process, drawing from studies in zebrafish, mouse cell lines and *Caenorhabditis elegans*. We also speculate on the importance of TA in the context of human disease.

Is mutant mRNA degradation required for transcriptional adaptation?

Although Ma et al. (2019) proposed a TA model that does not involve mutant mRNA degradation, the El-Brolosy et al. (2019) study provides several lines of evidence indicating that mutant mRNA degradation is indeed required for TA. In addition to the evidence presented above, it was reported that wild-type zebrafish embryos injected with uncapped RNAs, which are rapidly degraded, could display TA (El-Brolosy et al., 2019). Furthermore, injecting wild-type embryos with capped mRNAs or with uncapped transcripts containing an upstream sequence that blocks endonuclease-mediated decay, did not induce TA, indicating that RNA degradation is required to trigger this response. Ma et al. (2019) also injected zebrafish embryos with uncapped RNAs but failed to see evidence of TA in their experiments. This discrepancy could be explained by the fact that their analysis was performed at 1.5 days post-fertilization (dpf), as opposed to 6 h post-fertilization as used in the El-Brolosy et al. (2019) study. Given the instability of uncapped RNAs, and the data mentioned earlier indicating that a certain amount of RNA degradation products is required to trigger TA (El-Brolosy et al., 2019), it is possible that the amount present at the 1.5 dpf stage was not sufficient to trigger TA.

To further test the requirement of mutant mRNA degradation in triggering TA, the nonsense-mediated mRNA decay (NMD) pathway was blocked using both pharmacological and genetic means. These experiments revealed that knocking out the NMD factor Upf1 in several zebrafish mutants leads to a reduction in mutant mRNA degradation, and a loss of the TA response (El-Brolosy et al., 2019). However, Ma et al. (2019) failed to see a role for Upf1 in their TA models. Instead, they found a role for Upf3a, an NMD machinery component that represses or only modestly promotes mutant mRNA degradation (Shum et al., 2016). Notably, TA is also observed in mutant alleles predicted to exhibit non-stop decay (*Fermt2* and *Actg1*) (El-Brolosy et al., 2019), indicating that mutant mRNA degradation and not NMD per se is required for TA.

Is transcriptional adaptation observed across metazoans?

The first examples of TA were identified in zebrafish (Rossi et al., 2015; Zhu et al., 2017; Sztal et al., 2018) and in mouse cell lines (El-Brolosy et al., 2019). However, discrepancies between gene knockout- and knockdown-induced phenotypes have been observed in other organisms, including yeast (Jost and Weiner, 2015; Chen et al., 2016a), *Drosophila* (Yamamoto et al., 2014; Chaturvedi et al., 2016; Tiebe et al., 2018), mouse (De Souza et al., 2006; Young et al., 2009; McJunkin et al., 2011; Daude et al., 2012) and *Arabidopsis* (Braun et al., 2008; Chen et al., 2014; Gao et al., 2015). These observations suggest that TA, or other forms of genetic compensation, might also be at play in these organisms. In fact, TA was recently reported in worms: it was found that *C. elegans act-5(ptc)* mutants, which exhibit mutant mRNA degradation, upregulate *act-3* and display no severe phenotype (Serobyan et al., 2020). Notably, *act-5* mutant alleles that exhibit no significant changes in *act-5* mRNA levels do not upregulate *act-3*, and they display growth defects and sterility (Serobyan et al., 2020). Similar observations were made when examining mutant alleles of *unc-89* (a *titin*-like gene), which upregulate *sax-3* (a *titin*-related gene) (Serobyan et al., 2020).

To test whether NMD factors are involved in regulating TA in *C. elegans*, Serobyan et al. (2020) knocked down several NMD genes, including *smg-2* (the *C. elegans* orthologue of *Upf1*), *smg-4* (*Upf3*) and *smg-6* (*Smg6*), and found that knockdown of *smg-2* and *smg-4* blocks the TA response in all three *unc-89(ptc)* alleles but not in the *act-5(ptc)* allele (Serobyan et al., 2020). Conversely, knockdown of *smg-6* blocks the TA response in the *act-5(ptc)* allele but not in the three *unc-89(ptc)* alleles, suggesting that the requirements for mutant mRNA degradation, and consequently TA, may vary in different tissues for different genes and/or even different alleles of the same gene (Serobyan et al., 2020). Moreover, the knockdown of several proteins involved in small RNA (sRNA) biogenesis and transport, including the argonaute proteins ERGO-1 and NRDE-3, the RNA-dependent RNA polymerase RRF-3, as well as the ribonuclease DCR-1, blocks the TA response without affecting mutant mRNA degradation (Serobyan et al., 2020). Importantly, several of these knockdown data were confirmed by analyzing double-mutant worms (Serobyan et al., 2020). Together, these results indicate that both mutant mRNA degradation and sRNA biogenesis are required for TA, thereby confirming and extending data obtained in zebrafish and mouse cell lines (El-Brolosy et al., 2019; Ma et al., 2019).

What are the potential mechanisms underlying transcriptional adaptation?

Epigenetic remodelling is tightly associated with transcriptional modulation (Weber et al., 2007), and it is likely to also play a role in promoting TA. Some long non-coding RNAs (lncRNAs) are known to activate gene expression by directly binding to the COMPASS (complex proteins associated with Set1) complex and increasing H3K4me3 marks on target gene promoters (Khalil et al., 2009). Accordingly, it was reported that Upf3a can interact with key components of the COMPASS complex including Wdr5 (WD Repeat Domain 5) and Rbbp5 (Retinoblastoma-binding protein 5) to induce TA (Ma et al., 2019). Notably, Wdr5 was observed to be enriched specifically at predicted promoter regions of upregulated genes in zebrafish *capn3a* mutants and, using knockdown and knockout approaches, Wdr5 was found to be required for TA. Based on these and other data, Ma et al. (2019) suggested that TA occurs

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when Upf3a interacts with the PTC-bearing mRNA and the exon junction complex to recruit Wdr5-COMPASS components; the PTC-bearing mRNA then guides these proteins to the promoter region of homologous gene(s) to increase H3K4me3 marks and, consequently, promote transcription. However, if Upf3b joins the complex instead of Upf3a, Upf1 and Upf2 are recruited to initiate the degradation of the PTC-bearing transcript.

Consistent with these observations, enrichment of WDR5 and H3K4me3 is also observed at the transcription start site of upregulated genes in mouse cell line models (El-Brolosy et al., 2019). Moreover, knockdown of WDR5, or of decay factors including UPF1, leads to reduced levels of H3K4me3 at the adapting locus in *Rela* knockout cells and dampens the TA response, further indicating the importance of these factors in TA (El-Brolosy et al., 2019). These findings are in line with previous studies (Haimovich et al., 2013) reporting that cytoplasmic mRNA decay factors are required for efficient transcription, and suggest a model in which mRNA decay factors translocate to the nucleus and interact with histone modifiers to modulate gene expression (Fig. 1). Similar observations were reported in an earlier study which showed that the transfection of small dsRNAs in human cells leads to a long-lasting and sequence-specific induction of the genes targeted by these dsRNAs (Li et al., 2006). Additional data indicated that the 5′ end of the antisense strand of the dsRNAs, the so-called ‘seed’ sequence, is needed for the reported gene induction and that this dsRNA-induced gene activation is associated with loss of inhibitory H3K9 methylation marks at the dsRNA-target sites and requires the function of Argonaute 2. This phenomenon, which can involve exogenous or endogenous triggers, was termed RNA activation (RNAa), and its underlying mechanisms remain under investigation (Li et al., 2006; Portnoy et al., 2011; Seth et al., 2018).

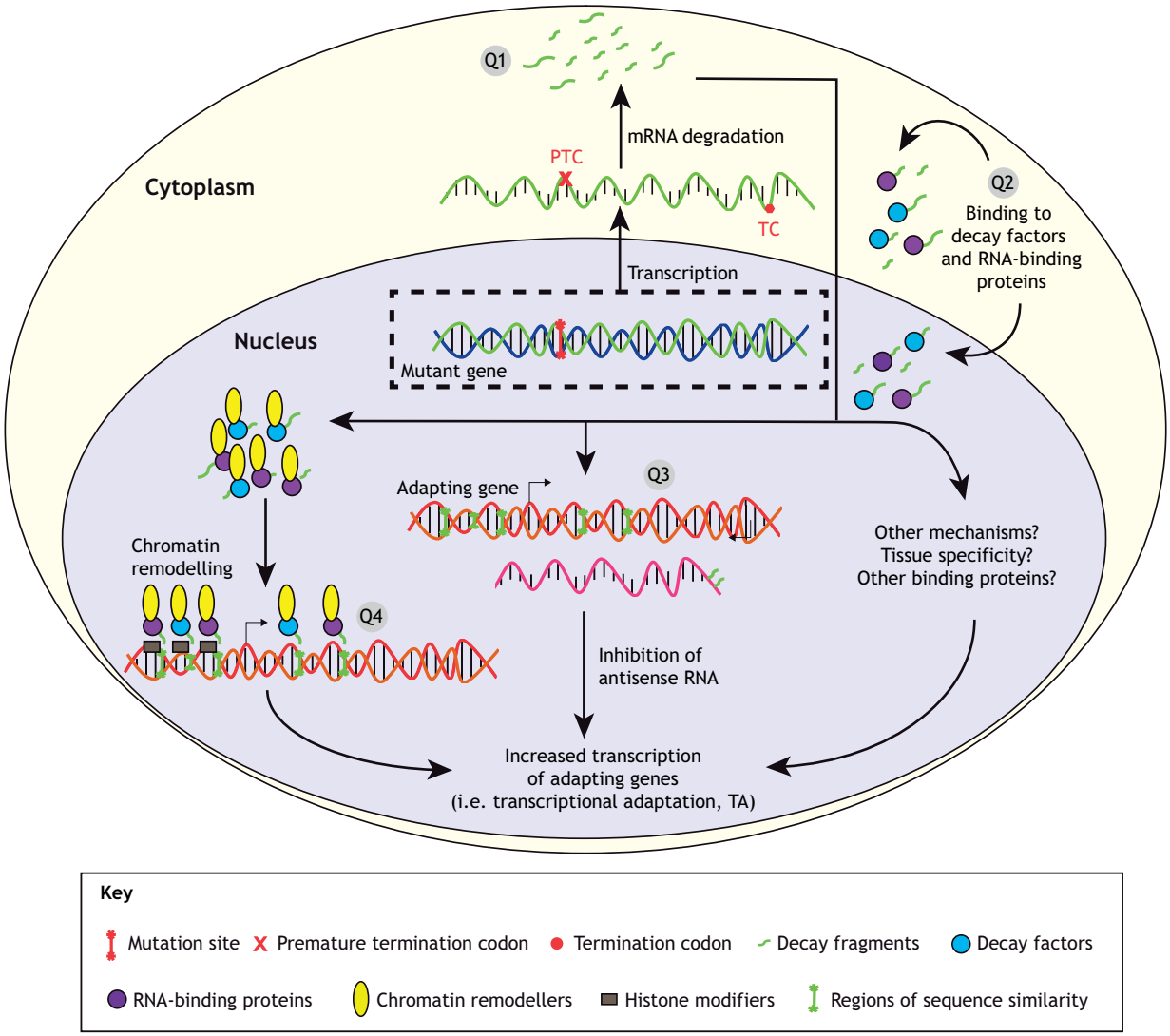

Fig. 1. Working model of transcriptional adaptation. Mutant mRNA degradation fragments translocate to the nucleus where they modulate the expression of specific genes. The mechanisms that underlie this response are currently being investigated and include: the modification of chromatin marks, the inhibition/degradation of antisense RNAs, and the modulation of expression of intermediary players including lncRNAs. Open questions include: Q1. What is the nature (e.g. size, composition, modifications) of the mutant mRNA degradation fragments, and what proteins are they associated with? Q2. Do the mutant mRNA degradation fragments get into the nucleus and, if so, how does this occur (or is there an intermediary step of amplification to generate other sRNAs)? Q3. What determines which genes get modulated by the mutant mRNA degradation fragments or their derivatives? Q4. How do these genes get modulated by the mutant mRNA degradation fragments or their derivatives?

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Antisense RNAs might also be involved in TA. Previous studies have reported that the transfection of short fragments of *Cdk9* or *Sox9* mRNA leads to increased expression of these genes in mouse embryonic stem cells (mESCs) (Ghanbarian et al., 2017). In addition, these mRNA fragments were found to downregulate the native antisense transcripts that normally function as negative regulators of *Cdk9* and *Sox9* expression (Ghanbarian et al., 2017). Another study reported the upregulation of sense *BDNF* transcripts after the knockdown of *BDNF* antisense transcripts in HEK293T cells transfected with siRNAs or complementary locked nucleic acid (LNA)-modified DNA oligonucleotides (Modarresi et al., 2012). Furthermore, this increased transcription correlates with a decrease in inhibitory H3K27me3 histone marks at the *BDNF* locus (Modarresi et al., 2012). These observations were extended to study the control of *Cdk9*, *Sox9* and *BDNF* expression using uncapped RNAs transfected into mESCs, MEFs and HEK293T cells, also resulting in the upregulation of these genes (El-Brolosy et al., 2019). In this context, a downregulation of antisense transcripts is observed at the *hbegfb* and *vclb* loci in *hbegfa* and *vcla* zebrafish mutants, respectively (El-Brolosy et al., 2019). Together, these data indicate that mutant mRNA degradation products might induce TA by inhibiting antisense transcripts at specific loci.

How important is sequence similarity for activating transcriptional adaptation?

Another related question is how much sequence similarity is required to transcriptionally activate homologous genes? Transcriptomic analyses of mouse *Fermt2*, *Actg1* and *Actb* knockout cells found that genes with sequence similarity to the mutated gene's mRNA are much more likely to be upregulated. In fact, at least 50% of similar genes are significantly upregulated in the different knockout cell models, compared with a maximum of 21% for all non-similar genes (El-Brolosy et al., 2019). Furthermore, whereas injections of uncapped transcripts of the coding strand of *hif1ab* or *vegfaa* induced TA, injections of uncapped transcripts of the non-coding strand did not (El-Brolosy et al., 2019), further highlighting the importance of sequence similarity. Synthetic transcripts containing *hif1ab* sequences similar to the promoter, exons, introns or 3′ untranslated region (UTR) of *epas1a* were also analysed, and it was found that only those that exhibit sequence similarity with exons or introns induced TA (El-Brolosy et al., 2019). These latter data are consistent with transcriptomic analyses of *Fermt2*, *Actg1* and *Actb* knockout cells, which found that genes exhibiting sequence similarity with the mRNA of the mutated gene in their 3′UTR or promoter regions displayed mild upregulation or were not upregulated (El-Brolosy et al., 2019). Based on these and other data, we propose a model that relies on a certain level of sequence similarity to induce TA (Fig. 1). In this model, mutant mRNA degradation fragments containing specific sequences upregulate the transcription of homologous genes by bringing histone modifiers and/or chromatin remodellers to specific loci, or by binding to antisense RNAs and thereby repress their function, or even by acting indirectly, via lncRNAs for example. However, several additional models of TA will need to be investigated in detail in order to determine the importance of each of these mechanisms, and possibly others, in modulating the expression of homologous genes.

Is transcriptional adaptation important in the context of human disease?

The current dogma is that pathogenic missense mutations may be common in affected individuals because they lead to constitutively active or dominant-negative proteins. In addition, genetic variants known as homozygous loss-of-function (HLoF) variants are considered to be rare in the general population because they presumably abolish the function of protein-coding genes and have a high probability of causing severe disease. Based on this hypothesis, it is assumed that strong selection likely acts on 'essential' genes with severe heterozygous or lethal homozygous loss-of-function phenotypes to ensure that potentially deleterious alleles do not increase to a high frequency in the population (Lenz et al., 2016; Minikel et al., 2020). However, this notion may not be entirely straightforward. Recent studies have shown that many common nonsense variants and mutations induced by CRISPR-Cas9 do not disrupt protein function significantly, and therefore some essential genes may have different selective pressures imposed upon them (Jagannathan and Bradley, 2016; Smits et al., 2019).

A 2015 study (Sulem et al., 2015) reported that nearly 8% of the Icelandic population carry complete loss-of-function mutations and suggested that PTC-containing alleles may be more common in the general population than previously suspected. We propose that this latter observation can be explained, at least partly, by the fact that nonsense mutations have the potential to trigger mutant mRNA degradation and the upregulation of homologous genes, which could bypass the deleterious effects of the mutations. Previous sequencing analyses have also reported that each human genome contains approximately 100 genuine HLoF variants with roughly 20 genes completely inactivated (Ng et al., 2008; Genomes Project et al., 2010; MacArthur et al., 2012). More recent analyses have reported that HLoF mutations are tolerated within outbred human populations, and exist at a frequency of approximately 18% in genes that do not show any disease association (Karczewski et al., 2020; Minikel et al., 2020). HLoF variants have also been identified in genes that affect disease risk (Karczewski et al., 2020), e.g., *ACTN3*, which is associated with ageing (Yang et al., 2003), *HAO1*, which is associated with kidney dysfunction (McGregor et al., 2020), and *LRRK2*, which is associated with Parkinson's disease (Whiffin et al., 2020). Similarly, in a recent analysis of 219 populations from 64 countries across Asia, it was reported that 43% of all protein-coding genes contain at least one protein-truncating variant, suggesting that HLoF variants can be well tolerated (Wall et al., 2019). In addition, a study of more than 500,000 human genomes examining 874 genes reported 13 individuals with disease-causing mutations in eight different genes, with two individuals carrying HLoF mutations leading to PTCs, although with no reported clinical manifestation of disease (Chen et al., 2016b).

There is also evidence for TA in some forms of nemaline myopathy, a congenital muscle disease often resulting in early death. In the approximately 10% of recessive cases that lead to a complete loss of the skeletal muscle α-actin gene (*ACTA1*), one of its paralogues, cardiac muscle α-actin (*ACTC1*), is upregulated, leading to a milder phenotype (Nowak et al., 2007). Similarly, and as mentioned earlier, zebrafish *actc1b* (the *ACTA1* orthologue) mutants display a mild myopathy phenotype compared with that observed in morphants, due to the upregulation of *actc1a* (the *ACTC1* orthologue) (Sztal et al., 2018). In addition, a study on Marfan syndrome reported that the mildest form of the disease was observed in an individual who displayed very low mutant *FBN1* transcript levels owing to an out-of-frame indel that leads to a PTC in the *FBN1* coding sequence (Dietz et al., 1993). Activation of TA may also be influenced by the variability in NMD efficiency in distinct cells, as observed in 13 different murine tissues for *Men1* transcripts carrying PTCs (Zetoune et al., 2008). Overall, TA holds the potential to act as a phenotypic modifier and to affect disease severity when mutations lead to a PTC and/or mutant mRNA degradation.

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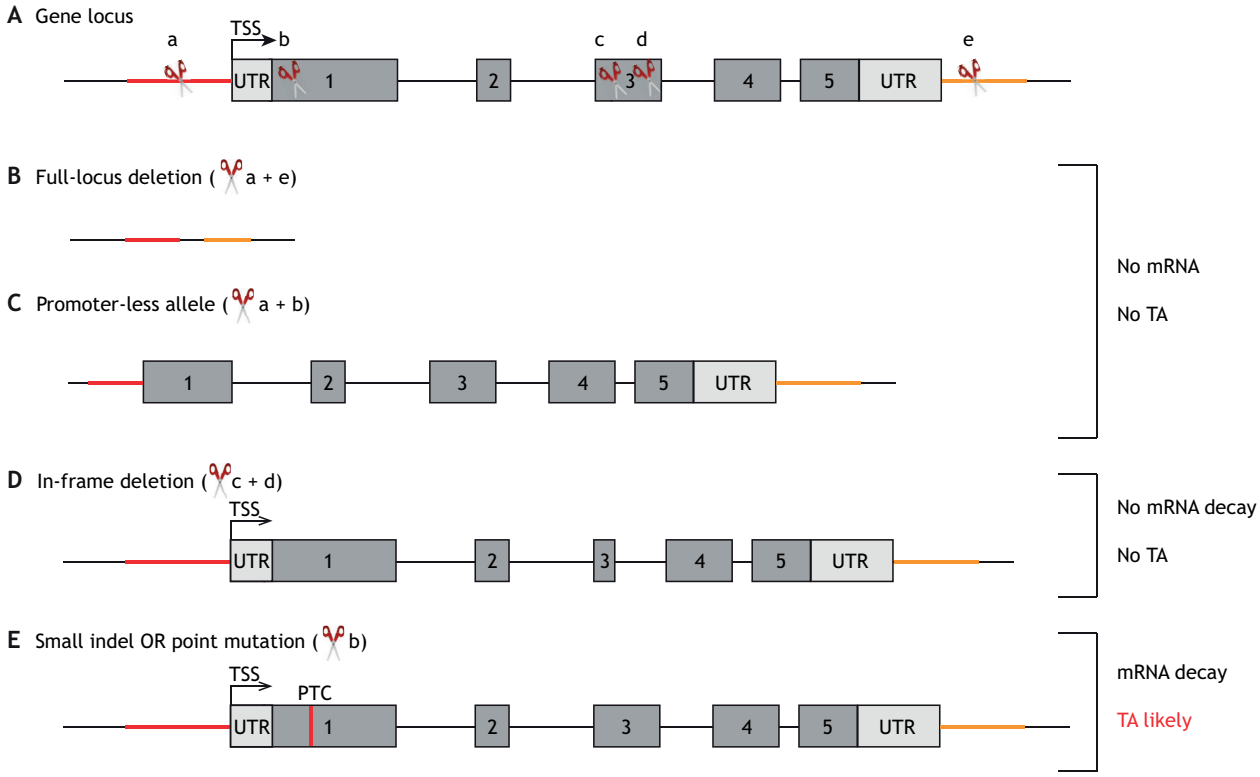

Fig. 2. How to avoid TA when designing loss-of-function alleles. (A-D) When designing loss-of-function alleles for a given locus (A), several approaches can be used to avoid TA, including full-locus deletion (B), RNA-less alleles through deletion of promoter and/or regulatory elements (C), and in-frame deletions of important functional domains (D). (E) By contrast, small indels (insertions or deletions) or PTC-causing point mutations introduced early in the coding sequence often cause mutant mRNA decay (Lindeboom et al., 2016; Hoek et al., 2019), and thus are more likely to induce TA. Note that in-frame deletions (D) can cause No-Go decay.

Concluding remarks

We hypothesize that mutant mRNA degradation products, once they reach a yet-to-be-defined threshold, modulate the expression of adapting genes. This phenomenon has clear implications when designing mutant alleles and also when comparing the transcriptomes of mutant versus wild-type cells and organisms. To avoid, or at least to minimize, genetic compensation due to TA, we recommend generating RNA-less mutant alleles (e.g. promoter-less alleles or even full-locus deletions, the latter being much easier to design and validate). If full-locus deletion alleles exhibit interesting phenotypes, one would of course need to establish more refined alleles (e.g. small deletions or point mutations) to evaluate the possible loss of additional important genetic information in the full-locus deletion allele (Fig. 2). Alternatively, when the protein of interest has clearly defined and well-studied domains, small in-frame deletion alleles could be very informative as they are expected to escape mutant mRNA degradation, thus avoiding TA (Fig. 2).

Several important questions need to be answered to understand the full impact of TA-induced genetic compensation on phenotypic variability and its relevance to genome editing in humans. For example: (1) Can TA also directly cause reduced transcription? (2) Is TA only observed in developing tissues? (3) Does TA occur in earlier arising eukaryotes such as yeast? It is equally important, of course, to gain a detailed understanding of the mechanisms underlying TA. For example: (1) What is the nature of the mutant mRNA degradation products? (2) Which proteins are involved in the process of TA, starting with mutant mRNA degradation and culminating with transcriptional modulation? (3) What are the sequence similarity requirements for TA?

As more sophisticated genome editing strategies emerge, we can attempt to answer some of these questions in order to uncover the mechanistic basis of TA. By comparing TA responses in various tissues and in different organisms, we can begin to understand why certain loss-of-function mutations elicit genetic compensation and others do not. Further insight into compensatory responses in human cells will represent a new frontier in the study of genetic diseases and mechanisms affecting genetic and phenotypic robustness in humans. Accordingly, exploiting these compensatory pathways will have important therapeutic implications with regard to the development of more effective treatments for various genetic disorders.

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