ANNUAL REVIEWS

Annual Review of Genetics

Genotype–Phenotype Relationships in the Context of Transcriptional Adaptation and Genetic Robustness

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Annu. Rev. Genet. 2021. 55:71–91

First published as a Review in Advance on July 27, 2021

The *Annual Review of Genetics* is online at genet.annualreviews.org

https://doi.org/10.1146/annurev-genet-071719-020342

Keywords

knockdown, knockout, genetic compensation, transcriptional adaptation, genotype, phenotype

Abstract

Genetic manipulations with a robust and predictable outcome are critical to investigate gene function, as well as for therapeutic genome engineering. For many years, knockdown approaches and reagents including RNA interference and antisense oligonucleotides dominated functional studies; however, with the advent of precise genome editing technologies, CRISPR-based knockout systems have become the state-of-the-art tools for such studies. These technologies have helped decipher the role of thousands of genes in development and disease. Their use has also revealed how limited our understanding of genotype–phenotype relationships is. The recent discovery that certain mutations can trigger the transcriptional modulation of other genes, a phenomenon called transcriptional adaptation, has provided an additional explanation for the contradicting phenotypes observed in knockdown versus knockout models and increased awareness about the use of each of these approaches. In this review, we first cover the strengths and

limitations of different gene perturbation strategies. Then we highlight the diverse ways in which the genotype–phenotype relationship can be discordant between these different strategies. Finally, we review the genetic robustness mechanisms that can lead to such discrepancies, paying special attention to the recently discovered phenomenon of transcriptional adaptation.

GENETICS RELIES ON MUTANT GENERATION

Sequencing the human genome was arguably the most pivotal work in genetics in the last few decades. It unlocked not only the sequence of every human gene but also the sequence of the vast intergenic regions that account for the majority of the transcriptional output of our genome and contain important regulatory elements, including promoters and enhancers as well as noncoding RNAs. In addition, this project led scientists to lower the estimated number of human genes and provided critical information about their structure and organization, thereby laying the foundation for the omics revolution as well as for the identification of candidate genes for many diseases (75, 104). Nevertheless, 20 years after the initial publication of the project, up to a third of human genes remain poorly characterized (139), and we know little about their physiological importance. Similarly, despite great improvement in sequencing technology, approximately 20% of proteins across all eukaryotic species remain functionally unassigned (152).

Introducing mutations in an organism's genome and investigating the resulting phenotypes have helped uncover the function of many genes. For decades, the deciphering of gene function was based on forward genetics: The identification of phenotypes came first, after which each phenotype of interest was linked to a specific mutation and locus (105, 128). A reverse genetics approach started to emerge in the 1980s. While numerous eukaryotic genomes were being sequenced, the field still lacked tools to specifically target loci of interest. A major breakthrough was the discovery of RNA interference (RNAi) by Fire, Mello, and colleagues (37), which followed the identification of the first miRNA, *lin-4* (80). Their findings in *Caenorhabditis elegans* showing that exogenous double-stranded RNAs (dsRNAs) can specifically silence genes with sequence identity to the injected dsRNA were a major step to quickly decipher gene function in a targeted manner. However, RNAi often leads to partial loss-of-function phenotypes and, as with other antisense approaches (32), causes a certain probability, from 5% to 80% in a range of organisms, of off-target effects (59, 120). For these reasons, scientists continued developing tools to modify the genome in a targeted manner.

Making site-specific changes to the genome has been a long-standing goal of many scientists. The discovery of DNA repair mechanisms in the late 1970s to early 1980s (85, 89, 125) suggested that inducing DNA breaks could pave the way for targeted genome engineering. A long path, which started with the use of chemical DNA recognition (complementary oligonucleotides, peptide nucleic acids, and polyamides coupled to cleavage reagents), self-splicing introns, and homing endonucleases, led to the discovery of sophisticated techniques such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (7, 20, 30). The latter two approaches enabled site-specific modifications by inducing double-strand breaks that often result in small indels with frameshifts (17). However, ZFNs, and to some extent TALENs, require fairly labor-intensive design, synthesis, and validation. Thus, the field of mutant generation was further invigorated by the discovery of type II CRISPR-Cas systems, which gave rise to the CRISPR-Cas9 technology. Originally discovered in 1987 (58) and later recognized as a bacterial adaptive defense system against viral infections (96), clustered regularly interspaced palindromic repeats with associated helicase and nuclease domain-containing proteins, or CRISPR-Cas systems, were shown to be guided by mature CRISPR RNAs (crRNAs) (16) and to belong to at least three

different types (I, II, and III) that use distinct molecular mechanisms to achieve nucleic acid recognition and cleavage (95). Landmark papers were published in 2012 showing that CRISPR-Cas9-crRNA complexes can be universally programmed for specific sequence recognition and protein-mediated DNA cleavage (43, 62). Since then, CRISPR-Cas9 has become the tool of choice for genome editing studies, and over the subsequent years, the CRISPR genome-engineering toolbox has been complemented with several Cas enzymes, including Cas3, Cas12, Cas13, and the Cascade complex, as well as their derivatives, offering a range of target recognition sequences as well as cutting properties, thereby allowing nearly unlimited genome editing possibilities. In addition, a super-precise CRISPR, composed of cytosine and adenine base editors fused to a catalytically impaired Cas9 protein, has recently been engineered to recognize noncanonical NRNH protospacer adjacent motifs (PAMs) (where R is A or G and H is A, C, or T) (102) and minimize off-target mutations (28), greatly expanding the range and precision of targeted genome editing.

This large number of gene targeting tools offers an extensive control over DNA and RNA changes. However, and perhaps to no surprise, manipulating the same gene with different approaches can lead to a range of phenotypic outcomes (24, 77, 124, 153). In this review, we first describe and analyze the range of methods to perturb gene function, then we discuss intriguing discrepancies between knockdown and knockout models. Finally, we highlight the discovery of transcriptional adaptation (TA) and the new practices, guidelines, and directions its investigation thus far has brought to the field of mutant generation.

GENE FUNCTION DISRUPTION BY RNA INTERFERENCE AND ANTISENSE APPROACHES

Among the range of approaches to disrupt gene function, the knockdown approach usually refers to transcriptional or translational silencing, without introducing modifications to the genome. The most widely used technique for this purpose is RNAi, which relies on an evolutionarily conserved pathway in which small noncoding RNAs and their associated proteins regulate gene expression (18). The three main modes of RNAi are (*a*) microRNAs (miRNAs), which are widespread across eukaryotes and control most, if not all, biological processes including cell growth and proliferation, tissue differentiation, and disease onset and progression (151); (*b*) small interfering RNAs (siRNAs), which are endogenous or exogenous RNAi inducers used for target gene repression (136); and (*c*) animal-specific PIWI-interacting RNAs (piRNAs), which differ significantly from miRNAs and siRNAs in the sense that they are produced independently of the endoribonuclease Dicer and are expressed specifically in the germline (69) but still participate in gene regulation by promoting heterochromatin assembly and DNA methylation, thereby silencing transposable elements and fighting viral infections (113).

All three modes of RNAi form a ribonucleoprotein complex with proteins of the Argonaute (AGO) family. AGO clade proteins are guided by 20- to 35-nucleotide-long miRNAs and siRNAs, whereas piRNAs associate with PIWI clade AGOs (PIWI proteins) (113), and they all target specific genes via complementary base-pairing interactions. In the case of miRNAs and siRNAs, this structure is called an RNA-induced silencing complex (RISC). RNAi is used to disrupt gene function primarily with siRNAs or short hairpin RNAs (shRNAs) (103). These synthetic dsRNA molecules, which feature full sequence complementarity to the target gene messenger RNA (mRNA), are processed by the Dicer enzyme, which loads the guide (antisense) strand onto the RISC, while the passenger (sense) strand is degraded (151). The RISC then performs cellular surveillance, binds the mRNA in the region complementary to the AGO-bound guide strand, and cleaves it or induces nonendonucleolytic repression, followed by mRNA deadenylation and degradation (151).

A major advantage of siRNA is that the silencing machinery is present in many eukaryotic cells (133). Further, in plants and *C. elegans*, RNAi silencing is heritable: siRNA-treated nematode hermaphrodites give rise to progeny with both maternal and zygotic target mRNAs depleted (47), and in *Drosophila*, siRNAs are often used for tissue-specific screens and analyses (27). In addition to the fact that the cells of interest do not require any prior modifications before RNAi treatment, the delivery of RNAi agents is fairly straightforward, and their specificity can be precise enough to target distinct mRNA isoforms or disease-specific alleles (1). Furthermore, the ability to generate partial loss-of-function phenotypes allows one to study the function of genes required for viability.

miRNAs play an important role also during zebrafish development, as shown by the phenotype of *dicer* mutants, which stop developing at 10 days post-fertilization (149). However, the siRNA approach was never widely adopted in the zebrafish field, in part due to the interference between the injected siRNAs and the biogenesis and activity of essential endogenous miRNAs (156), as well as the activation of the innate immune response (130). Rather, the most widely used method of gene knockdown in zebrafish utilizes morpholino antisense oligomers. Morpholinos are chemically synthesized oligomers, composed of a chain of 25 nucleotides that possess a morpholine instead of a ribose ring. They target mRNAs via a complementary interaction and prevent their splicing or translation (21). The unique structure of morpholinos protects them from nuclease activity, improving stability in comparison to other antisense reagents. In addition, because they do not carry a negatively charged backbone, morpholinos are less likely to interact with other components of the cell, thus reducing their toxicity to some extent (32). However, as morpholinos do not usually lead to the degradation of their target RNA, it becomes difficult to assess knockdown efficiency, especially when translation-blocking morpholinos, which do not create changes to the splicing pattern, are used (116).

Antisense oligonucleotides (ASOs) are another option in the gene knockdown toolbox. They are synthetic DNA (or RNA) oligomers or single-stranded RNA-DNA hybrids (gapmers) that induce the catalytic degradation of complementary cellular RNAs via ribonuclease H (RNase H). ASOs are typically designed to contain phosphorothioate bonds to increase their stability and 2′-*O*-methyl RNA modifications to provide resistance to nuclease activity (57). The central phosphorothioate-modified DNA activates RNase H, whereas the flanking ribonucleotides increase the binding affinity with the target RNA. ASOs have been used in gene function studies and also as therapeutic agents for disorders caused by toxic gain-of-function mutations. Their efficacy was recently shown in patients with Ullrich congenital muscular dystrophy, where gapmer ASOs successfully silence the *COL6A3* mutant allele, thereby allowing the increased deposition of collagen VI into the extracellular matrix (2).

A major issue with antisense technology is the off-target effects. siRNA-associated off-target effects are categorized as sequence specific, which are caused by the complementary binding to non-target mRNAs, or non-sequence specific, owing, for example, to the converging miRNA and siRNA processing pathways. Flooding cells with exogenous dsRNAs can displace endogenous miRNAs from the RISC and interfere with gene expression modulated by these miRNAs (67). In addition, theoretical calculations indicate that injected morpholinos exceed the target mRNAs at a ratio of thousands to one (129). Therefore, inevitably, morpholinos also cause significant off-target effects, resulting in phenotypic artifacts (11, 45), as well as p53-dependent apoptosis (121). Although p53 activity can be suppressed, such drastic measures can hinder the evaluation of the resulting phenotypes (129). In addition, certain morpholino-injected animals display a dose-dependent upregulation of the interferon-stimulated genes *isg15* and *isg20*, the cell death pathway gene *casp8*, and certain cellular stress response genes (74). Another issue is the variable efficiency of different knockdown reagents, which often results in variable phenotypes even when the same gene is targeted, thus making the unified interpretation of results challenging.

INTRODUCING MUTATIONS USING GENOME EDITING

The knockout approach refers to the abrogation of gene function by introducing deleterious mutations in the genome. For a mutation to be considered deleterious, it has to negatively impact gene or regulatory element function (107). Deleterious mutations can arise from single base changes or larger modifications such as insertions or deletions. Single base changes are often neutral and produce a protein variant that acts as the wild-type protein, unless they cause the formation of a premature termination codon (PTC). Currently, the favored approach to introduce deleterious mutations is the induction of double-strand breaks into the genome, which triggers the DNA damage response. During this response, endogenous cellular mechanisms detect double-strand breaks via the ATM kinase and the MRE11-RAD50-NBS1 (MRN) complex, and a phosphorylation cascade activates cell cycle checkpoints and DNA repair (144). Double-strand break repair is achieved in one of two ways: by homologous recombination (HR) or non-homologous end joining (NHEJ).

HR requires a homologous DNA sequence template, for which the sister chromatid, rather than the homologous chromosome, is most often used (63). Indeed, recombination between homologous chromosomes can result in loss of heterozygosity or other chromosomal aberrations, and so HR most often occurs during the S and G2 phases of the cell cycle and is suppressed in the G1 phase when sister chromatids are unavailable for repair (41). In addition, newly formed postreplicative chromatin favors HR by recruiting an HR Tonsoku-like–Methyl methanesulfonate-sensitivity protein 22-like (TONSL-MMS22L) complex to DNA lesions (127). HR is accurate and leads to the precise repair of the damaged locus, which makes this pathway undesirable when trying to generate gene knockouts; however, it can be employed to produce small knock-ins, which can also lead to null alleles. NHEJ, on the other hand, directly reseals the two broken ends, which can lead to insertion and/or deletion events, resulting in frameshifts, premature termination, and loss-of-function (19). It should be mentioned that a recent study reported that NHEJ can also be accurate, as occurs in *Drosophila Mcm*^{5A7} mutants (49). In these mutants, the repair of programmed meiotic double-strand breaks is initiated by HR but is completed by NHEJ with precise repair results.

A frameshift mutation typically leads to an altered protein sequence or a PTC, which can subject the mutant mRNA to an endogenous mRNA quality surveillance pathway, termed nonsense-mediated mRNA decay (NMD) (72). Although NMD plays a significant role in endogenous gene regulation (91), its function to degrade mRNAs with PTCs is of particular importance in mutant generation using the sequence-specific nucleases. Precursor mRNAs (pre-mRNAs) go through several steps of maturation, including polyadenylation, capping, and splicing. The latter two have a major role in subjecting mRNAs to NMD. The cap binds to the cap-binding protein (CBP) heterodimer CBP80-CBP20 (CBC), which supports the pioneer round of mRNA translation (97), while splicing results in the assembly and deposition of exon junction protein complexes (EJCs) at the junctions of joined exons (10). EJCs comprise several core and peripheral components. The core proteins serve as a binding platform for peripheral factors, among which are the NMD proteins Up-frameshift 2 (UPF2) and UPF3 (78). Furthermore, EJCs serve as a mark for newly synthesized CBC-bound mRNAs and typically dissociate from mRNAs during the pioneer round of translation, unless a ribosome encounters a PTC, which then leads to the termination of translation and leaves the downstream EJCs bound to the mRNA (72). In a subsequent series of reactions, CBP80 interacts with the NMD factor UPF1, which then associates with EJC-bound UPF2 and UPF3, to trigger the rapid degradation of PTC-bearing transcripts (88, 97).

Frameshift mutations leading to NMD can generate strong mutant alleles; however, other modes of mRNA quality surveillance can also lead to such alleles. No-go decay (NGD) and non-stop decay (NSD) are additional cotranslational mRNA quality control pathways that degrade

aberrant mRNAs with stacks of ribosome complexes stalled internally midway through the mRNA (NGD) or terminally at the poly(A) site of stop codon–lacking transcripts (NSD) (134). Ribosome stalling that triggers NGD can result from stable intramolecular or intermolecular secondary RNA structures, enzymatic cleavage, the presence of rare codons, or ribosome collisions (108). RNAs form complex secondary structures through intramolecular base-pairing, and in-frame insertions and deletions as well as point mutations can alter an RNA's base-pairing pattern and subject it to NGD. Typically, single-nucleotide variants result in RNA hairpin formation, which decreases the translation rate or completely impedes ribosome progression (147). Recently, a point mutation was reported to cause hypertrophic cardiomyopathy in humans by altering the secondary structure of *MYH7* mRNA (122). NSD substrates include truncated mRNAs where ribosomes stack up at the end of the template or full-length mRNAs lacking a stop codon but containing a poly(A) tail. In such cases, ribosomes do not translate through the poly(A) sequence but usually stall shortly after incorporating in the nascent polypeptide several positively charged amino acids (lysines or arginines) that electrostatically interact with the negatively charged exit channel of the ribosome, thereby causing charge-dependent ribosome pausing and inhibition of their progression (90, 134). NSD can be utilized to generate a strong allele, e.g., by introducing *loxP* sites flanking the exon that contains the stop codon and triggering Cre-mediated recombination (142) or by deleting the last exon with CRISPR-Cas targeting (33).

A concern about the sequence-specific nuclease technology is its off-target effects, which can cause genomic instability and disrupt the function of non-target loci, impeding the phenotypic interpretation or even mutant viability. Although initial studies attributed the targeting specificity of Cas9 to 7–12 base pairs (bp) 5′ of the PAM of the 20-nucleotide single guide RNA (sgRNA) sequence (60, 126), more recent data suggest that guide RNA (gRNA)-Cas9 specificity exceeds this seed motif and that all 20 bases account for the specificity to a varying degree (54, 115). Nevertheless, the seed sequence remains the most important, and only two base mismatches in the PAM-proximal region are usually sufficient to make the guide nonfunctional (4). At the same time, the 5′ end of the sgRNA is far more tolerant, and it can bind targets with even five base mismatches, thereby directing Cas9 to unintended genomic loci and inducing off-target DNA cleavage (54).

DISCREPANCIES BETWEEN KNOCKDOWN AND KNOCKOUT PHENOTYPES AND THE CONCEPT OF GENETIC ROBUSTNESS

The multitude of gene knockout tools, especially the CRISPR-Cas9 technology, has allowed the generation of a plethora of mutants, and in many cases the equivalent knockdowns exhibit stronger phenotypes (70). Some documented examples include *Ppara* knockout and knockdown mice, where *Ppara* mutants display hypoglycemia and hypertriglyceridemia after fasting while siRNA-treated animals present the same abnormal blood glucose and triglyceride profile in the absence of fasting (24), and *klf2a* knockout and knockdown zebrafish, where *klf2a* mutants display unaffected cardiovascular development (110) while morpholino-injected embryos (morphants) exhibit gross cardiovascular defects (79, 109). Additional cases of discrepancies between knockout and knockdown phenotypes are reported in *Arabidopsis* (42), *Drosophila* (153), *C. elegans* (3, 38), and human cell lines (36, 106; for a review, see 34, 52).

Some of these phenotypic differences are possibly due to dose-specific off-target and toxic effects of the antisense reagents, e.g., a high dose of *foxc1a*-targeting morpholino caused a complete loss of somite boundaries in zebrafish embryos, whereas a lower dose produced a weaker phenotype (143), one more comparable with *foxc1a* mutants, which display only mild somite defects (82). Zimmer and colleagues (157) reported that a 4-nanogram dose of *rhcgb* ATG morpholino

caused the same phenotype as CRISPR-Cas9-generated *rhcgb* mutants without additional morphological defects; however, higher doses of *rhcgb* morpholino caused an additional curving of the trunk and tail and body axis deviation, which was also present in high-dose-morpholino-injected *rhcgb* mutants. Similarly, by performing an extensive comparison of zebrafish *tek* morphants and mutants, Jiang and colleagues (61) showed that, although the morphants exhibited severe vascular defects, as previously reported (84), *tek* mutants, regardless of the mutation type, including a full locus deletion, reach adult stages with no obvious cardiovascular malformations. In this case, additional experiments showed that the *tek* morpholino induced the same phenotype in wild types and in *tek* mutants lacking the morpholino binding site, thereby clearly demonstrating off-target effects of the morpholino. Another potential explanation for morphants exhibiting a more severe phenotype than mutants is the contribution of maternal mRNA: Zygotic mutants will express the target protein from maternal stores, whereas translation-blocking morpholinos and siRNAs can knock down both maternal and zygotic transcripts, thereby revealing phenotypes only observed in maternal zygotic mutants (157).

In addition, it is probably very rare to reach a 100% knockdown efficiency, and it is also reported that the abundance of target mRNAs affects siRNA efficacy (51, 55, 76). One of the few assessments of morpholino efficacy concluded that the most efficient morpholinos can cause reductions of more than 80% in target protein expression, and a simultaneous use of two morpholinos can cause reductions of more than 90% (64). Residual target activity can be problematic when one tries to knock down proteins with high affinity/efficiency or non-rate-limiting metabolic enzymes that maintain physiological function even when their expression is markedly reduced. For example, a greater than 80% reduction of α-aminoadipic-semialdehyde dehydrogenase (Aldh7a1) via morpholino did not affect lysine catalysis, likely due to the high catalytic efficiency of the enzyme for its substrate. By contrast, *aldh7a1*-null mutants exhibit a clear accumulation of toxic lysine intermediates (118).

However, not all cases of phenotypic discrepancy have such an explanation. Rather, genetic robustness also plays a significant role in gene perturbation studies. Genetic robustness refers to the invariance of the phenotype in the face of genetic perturbations. In 2007, Hiroaki Kitano (68) defined it as a fundamental characteristic that describes an organism's ability to withstand internal and external perturbations, anticipating, perhaps accidentally, that the dawning era of genome engineering and mutations-on-demand would lead to a renewed interest in robustness. Biochemical and network-level mechanisms responsible for robustness include dosage compensation (15), redundancy in the form of gene duplicates (98), and changes in gene regulatory network architecture—e.g., redundant wiring of transcription factors (TFs) (93) or regulatory compensation within gene networks (119).

A classic example of robustness due to dosage compensation is sex chromosome dosage compensation, which in *Drosophila* is achieved by transcriptional upregulation of X chromosomal genes in males (44), whereas in *C. elegans* it is achieved by a downregulation of genes on each of the two X chromosomes in hermaphrodites (100). In both cases, the aim is, of course, to equalize gene expression between the sexes. In mammals, sex chromosome dosage compensation was thought to be achieved solely by the complete inactivation of one of the two X chromosomes in females; however, recent reports indicate that a twofold upregulation of some X chromosomal genes also occurs in males (25, 26).

Robustness by paralogous genes, caused by the upregulation of a family member with similar function in response to gene loss, is a widespread observation reported in mouse (112), *C. elegans* (87), zebrafish (29), and other model organisms. Robustness due to gene network rewiring is a much more complex phenomenon and does not necessarily involve gene duplicates. For example, robustness can be facilitated when TFs converge onto different genes via redundant shadow

enhancers, which are distant from primary enhancers; when multiple TFs from one family bind the same regulatory DNA element; or when TFs from different families interact with a regulatory module that contains several elements (93). In such cases, targeting a single regulatory element to disrupt its function may not cause a distinct phenotype. Protein network architecture also facilitates compensation even when mutations occur in genes encoding structurally or functionally unique proteins: In yeast, enzymes that catalyze different chemical reactions can compensate for recessive mutations in genes encoding functionally unrelated enzymes, thereby sustaining an optimal turnover of metabolites (145), and in yeast as well as in other organisms, Hsp90 can buffer against mutations in multiple unrelated genes (155). In addition, large metabolic networks are able to buffer even against the complete loss of function of one or more enzymes by exploiting alternative metabolic routes (31).

The field of genetic robustness appeared to be relatively quiet for many decades, with new examples in animal models arising regularly, yet all falling under already-known underlying mechanisms. However, the switch from morpholino-based approaches to generating and analyzing mutants, especially in zebrafish, reawakened the field by revealing a novel mechanism conferring robustness. Since this new mode is not triggered by the loss of protein function, unlike the previously established mechanisms of genetic robustness, and manifests itself as the transcriptional upregulation of specific genes, it was termed transcriptional adaptation (TA) (124). Scientists discovered TA while studying the role of the endothelial enriched epidermal growth factor-like 7 (*egfl7*) gene during vascular development, using both knockdown and knockout approaches. While zebrafish and *Xenopus egfl7* morphants displayed vascular tube malformations (114), zebrafish and mouse *egfl7* mutants do not exhibit any vascular phenotype (124). Initially, it was not clear whether this discrepancy was due to another case of morpholino-induced off-target effects, hypomorphic mouse and zebrafish mutant alleles, or another reason. Several lines of evidence indicated that the zebrafish mutant was a severe allele and that the phenotypic differences between mutant and morphant animals were not caused by off-target effects (124). The major insight into the mechanism by which zebrafish overcome the genetic alteration, but not the antisense reagent, came after performing proteomic and transcriptomic analyses of mutant and morphant embryos. A single extracellular matrix protein Emilin3a was found to be upregulated in mutants but not morphants; transcriptomic analysis extended this observation by showing that several *emilin* genes were upregulated in mutants but not morphants. The ability of increased Emilin expression to compensate for the loss of Egfl7 was supported by experiments in which *egfl7* morphants, which do not upregulate *emilin* genes, displayed only mild vascular phenotypes after wild-type *Emilin* mRNA injections (124). As noted above, this phenomenon of transcriptional upregulation of specific genes in a mutant was called TA, and it was subsequently reported in several additional studies (141).

The next major questions were: What triggers TA and how widespread is this phenomenon? Why was it discovered only recently, given that genetic robustness has been under investigation for several decades (46, 48, 53, 98, 111)? El-Brolosy et al. (33) analyzed several different mutants in zebrafish and in mouse cells in culture and observed a correlation between TA, PTCs in the mutant mRNAs, and NMD. The authors further found that mutant mRNA degradation was required to trigger TA by showing that RNA-less alleles do not display TA and by knocking out, or down, mRNA processing factors involved in NMD, namely UPF1, SMG6, and XRN1. Furthermore, they observed epigenetic remodeling during the TA response, as indicated by the enrichment of WDR5, a constituent of the histone H3K4 methylase COMPASS complex, and of its generated H3K4me3 histone mark, indicating accessible and active chromatin, at the transcription start site (TSS) of the upregulated genes in several mouse cell line TA models (33). Notably, Ma et al. (92) reported a similar phenomenon they termed genetic compensation response (GCR). They first observed that zebrafish *capn3a* mutants increased mRNA levels of related genes, primarily

capn8 and *capn12*. Then, by knocking down, and out, various NMD factors, they reported a major role for Upf3a but not Upf1, Upf2, or Upf3b in mediating GCR. Based on previous reports that UPF3a suppresses NMD in certain conditions (135), that it can directly interact with the WDR5-COMPASS complex, and that WDR5 is required for the GCR response (92), Ma et al. proposed that PTC-bearing mRNAs, and not mutant mRNA degradation intermediates, trigger GCR by guiding these components to the TSS region of related genes to promote their transcription. TA was also called nonsense-induced transcriptional compensation (NITC) in a commentary on the El-Brolosy et al. (33) and Ma et al. (92) papers (150); however, this term implies that TA and GCR are triggered only by mutations that generate PTCs. But, given that multiple forms of mRNA degradation (NMD, NGD, NSD) can trigger the upregulation of adapting genes (33), the term NITC is not correct.

Later, TA was also reported in *C. elegans* (131), indicating that it is an evolutionarily conserved phenomenon that should be considered when generating knockout alleles in various model organisms. The work in *C. elegans* confirmed the involvement of several NMD genes [*smg-2* (ortholog of *Upf1*), *smg-4* (ortholog of *Upf3*), and *smg-6* (ortholog of *Smg6*)] in the TA response; it also uncovered a role for several factors involved in small RNA biogenesis, including the AGO proteins ERGO-1 and NRDE-3 as well as Dicer, suggesting, to a certain extent, potentially overlapping mechanisms between TA and RNAi (131). In addition, the authors observed a requirement for the RNA-dependent RNA polymerase RRF-3, which, together with the involvement of ERGO-1, NRDE-3, and Dicer, suggests a potential role for the amplification of mutant mRNA degradation fragments, or the generation of small RNA derivatives, such as the so-called 26G RNAs (131) in the TA response.

The relatively late discovery of TA, at least in comparison with other modes of genetic robustness, can be attributed to the recent technological breakthrough in generating mutants and the substantial shift from knockdown to knockout approaches. TA differs markedly from other modes of genetic robustness primarily because it is activated upstream of protein function; it is not a mechanism triggered by the loss of protein function, nor does it necessarily lead to functional compensation. Furthermore, only select mutations, particularly those that cause mutant mRNA degradation (33) or lead to mutant transcripts with PTCs (92), trigger TA.

In addition, the upregulated or so-called adapting genes in TA are not necessarily paralogs (124). One property that appears to determine which genes will be modulated in their transcription during the TA response is their sequence similarity to the mutant mRNA. Using thresholds of *E*-value (a parameter that describes the probability of a random alignment between the input sequence and a target genome) corresponding to local sequence alignments ranging from 24 to 1,901 nucleotides in length with 75% to 96% identity, El-Brolosy and colleagues (33) found that at least 50% of the similar genes were significantly upregulated in three different knockout cell line models, compared with only 21% of the non-similar genes. In addition, injection of uncapped RNAs, which are prone to rapid degradation, synthesized from the noncoding strand of *hif1ab* and *vegfaa*, did not upregulate the related genes *epas1a* and *vegfab*, respectively, whereas the injection of coding strand–derived uncapped transcripts triggered TA (33). These data provide further evidence for the importance of the mutant mRNA sequence itself. Likewise, using different alignment parameters, Ma et al. (92) showed that the upregulated genes in zebrafish *capn3a* mutants were 31% to 51% identical to the mutant gene. However, it should not be excluded that sequence similarity to noncoding regions, e.g., promoters, enhancers, or other regulatory elements, can also play a role in determining which genes get upregulated. It is also worth noting that long noncoding RNAs (lncRNAs) are present at or near the adapting genes in some of the TA models in nematodes (*act-3*), zebrafish (*bbegfb* and *vclb*), and mouse cell lines (*Actg1* and *Actg2*). Furthermore, adapting

genes do not necessarily need to be upregulated; a model of TA-mediated downregulation is also plausible (112).

It was also shown that zebrafish *vegfaa* mutants, despite upregulating *vegfab* via TA, are not able to compensate for the loss of Vegfaa and exhibit morphant-like vascular defects. Accordingly, *vegfab* mRNA injections did not rescue the phenotype in *vegfaa* morphants or mutants (123). Thus, TA may have remained hidden for many years because its phenotypic outcome in the context of genetic robustness is sometimes counterintuitive by not always leading to functional compensation and not always involving paralogous genes. Many technological advances, including genome editing and transcriptome sequencing, had to become more broadly available for clear examples to emerge and be identified as such.

HOW GENETIC COMPENSATION IS AFFECTING OUR UNDERSTANDING OF GENOTYPE–PHENOTYPE RELATIONSHIPS

To know or accurately predict what molecular or macroscopic traits (phenotypes) will emerge from a certain nucleic acid sequence (genotype) is a long-standing goal of genetics. Currently, massively parallel sequencing and deep mutational scanning allow the merging of a large number of mutations and their associated phenotypes, yielding predictive models and helping to distinguish harmful from benign genetic variants (66, 86). To date, the majority of such studies have been performed in yeast and on single genes; for example, Hietpas et al. (50) evaluated the fitness landscape for all possible point mutations in a nine-amino-acid region of Hsp90. Another example comes from a study in which a complementary DNA (cDNA) library encoding all possible 9,595 single amino acid substitutions in PPARγ, variants of which are associated with lipodystrophy and type 2 diabetes, was introduced into human macrophages, allowing the authors to measure the level of tolerance for each amino acid substitution (94).

Deep mutational scanning at the whole-genome level in single cells, together with high-throughput phenotyping, is the next frontier, facilitated by advances in CRISPR-Cas9-based methods and improvements in single-cell sequencing technology, ultimately enabling the detection of mutations and their association with various traits (66). It is of particular interest because completely understanding the relationships between genotype and phenotype does not mean solving a simple single-variable equation, as phenotypes typically result from multiple gene interactions or combinations of different mutations. Often, the phenotypic outcome of a mutation depends on the identity of the other variants in the genome (81). A classic example in human genetics concerns the single-gene disorder cystic fibrosis, which is caused by a mutation in *CFTR*, but whose clinical phenotypes vary because of at least seven different modifier loci (5).

Another reason why genotype–phenotype relationships are particularly complex is the time-dependent effects of genetic robustness on evolvability, which is defined as a biological system's ability to produce heritable and adaptive phenotypic variation through genetic mutations (35, 117). In short, mutational robustness produces cryptic genetic variation, which, in certain environmental conditions, can reveal itself as novel, heritable phenotypes (99). It has been suggested that in the short term, genetic robustness hampers evolvability because it reduces the intensity of selection, but in the long term, it allows the populations to accumulate a larger diversity of genotypes and thus promotes evolutionary innovation (35). Loss-of-function mutations can convert genotypes from neutral to adaptive (99). Such a scenario is often the case with genetic robustness via paralogous genes or gene network buffering, both of which appear to have positive effects in the majority of biological contexts. For example, studies on paralogous genes in multiple model organisms have shown that paralogs are significantly less likely to be essential than singleton genes because of their functional redundancy (9, 48, 65, 148). These observations led scientists to further

evaluate genotype–phenotype relationships through the use of synthetic lethality screens. Synthetic lethality is the phenomenon whereby while the disruption of any of two, or multiple, genes individually has no effect on an organism's fitness, the simultaneous disruption of these genes is lethal. Additionally, measurements of essentiality in human genes provide similar insights (146) and have shown that close paralogs are less likely to be involved in human disease: Genes with close homologs (at least 90% sequence identity) are three times less likely to harbor pathogenic mutations compared with genes with diverged homologs (53).

Nevertheless, TA brings an additional degree of complexity to the concept that genetic robustness has primarily positive effects on mutants' fitness and survival. The reported impact of TA on mutant phenotypes ranges from beneficial [in zebrafish *egfl7* (124) and *capn3a* (92) mutants as well as in nematode (131), zebrafish (140), and mouse cell line (33) *actin* mutants] to apparently neutral [in zebrafish *vegfaa* mutants (33)] and even possibly detrimental [in mouse *Lgr6* mutants (56) and zebrafish *marcksb* mutants (154)] (for a review, see Kontarakis & Stainier 71). Combined with the fact that only PTC-causing mutations trigger TA, it is possible that different mutations—those that trigger TA and those that do not—can lead to the same phenotypic outcome (e.g., zebrafish *vegfaa* mutants). This paradox brings a new level of complexity as far as how genetic robustness impacts the genotype–phenotype relationship. Moreover, TA also impacts haplosufficiency: It was shown that in heterozygous cells and zebrafish, TA leads to the upregulation of the wild-type allele (33), which may translate into the normalization of protein levels. Hence, these observations challenge the current model of haplosufficiency whereby half of the protein activity, as encoded by a single functional allele, is sufficient to maintain a wild-type phenotype; in fact, in cases when TA is at play, the single functional allele will be upregulated. It will also be interesting to revisit some of the classical models of robustness in which a compensation response was attributed to the increased expression of a paralog due to protein loss and determine whether overlooked mutant mRNA degradation and TA may be at play.

Further studies are clearly needed to provide an evolutionary perspective on TA and how this process arose and presumably spread. Currently, TA could be viewed as a byproduct of two essential processes: (*a*) mRNA quality surveillance and (*b*) gene regulation by small RNAs (sRNAs). These two independent processes, each selected via its positive effect in transmitting genetic information (6, 14), seem to contribute equally to the emergence of TA by (*a*) generating mRNA fragments that can be further processed into sRNAs and (*b*) modulating the expression of the adapting genes. A phenomenon called RNA activation, whereby 21-bp RNA duplexes increase the transcription from target promoters by modifying the repressive histone methylation (83), can provide a hypothetical mechanistic basis for how these sRNAs act during TA.

Is TA an elaborate mode of genetic robustness that increases genetic variation in only a select set of genes? Or is it a specialized mechanism of gene regulation, related to genome duplication events that later converged into robustness-conferring pathways? So far, only a few cases of TA have been reported, and the majority of mutants with small indels, as reported in the literature, exhibit phenotypes, raising the questions of how many gene families are actually capable of displaying functional compensation via TA and how many TA models have been missed as negative data seldom get reported? Of course, the phenotype of these alleles, if they display TA, will need to be compared to alleles that do not display TA. In addition to the evolutionary perspective, there are many unknowns regarding how TA occurs mechanistically: What is the nature of the mutant mRNA degradation products and of their potential derivatives? Which proteins are involved at every step of TA? How much sequence similarity is required for TA, and does the important similarity lie in select regions of the adapting genes? How does TA affect the transcriptome of mutant cells? What are the direct targets of TA, and can TA also lead to reduced transcription of certain genes? Can TA mechanisms be exploited to treat human genetic disorders?

COMBINING GENE PERTURBATION STRATEGIES TO GET A BETTER UNDERSTANDING OF GENE FUNCTION

Clearly, different gene perturbation strategies can lead to distinct phenotypic outcomes, e.g., due to off-target effects, reagent toxicity, activation of protein feedback loops, generation of hypomorphic alleles, or induction of mutant mRNA degradation and subsequent transcriptomic alterations via TA (**Figure 1**). Thus, it is extremely important to consider all possible outcomes and carefully select a gene disruption method, preferably even combining a few intersectional methods to cross-validate the observations.

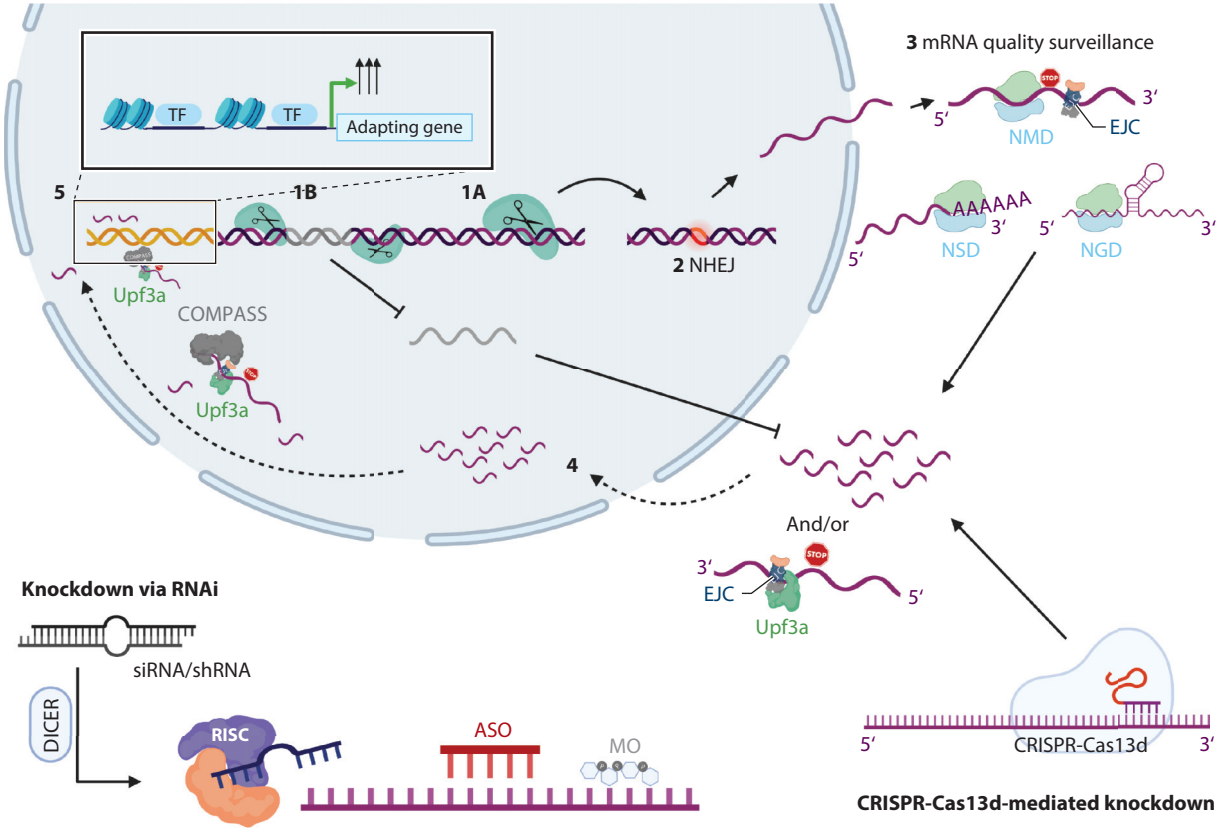

Figure 1

Gene perturbation techniques that lead to or avoid TA. Inducing double-strand breaks with sequence-specific nucleases (*Step 1A*) triggers DNA repair mechanisms, e.g., NHEJ (*Step 2*), that can lead to indels, with or without frameshifts. Aberrant mRNAs are detected by the mRNA quality control pathways (NMD, NGD, or NSD) (*Step 3*), resulting in transcript degradation. Current models of TA propose that mutant mRNA degradation fragments and/or PTC-bearing mRNAs shuttle back into the nucleus (*Step 4*), where, in association with the WDR5-COMPASS complex, they act at the TSS of the adapting gene(s) and modulate their expression (*Step 5*). Gene knockdown via RNAi (siRNAs or shRNAs), ASOs, or MOs has not been reported to lead to TA, whereas mRNA cleavage using CRISPR-Cas13d can lead to TA-triggering mRNA degradation. Gene knockout by generating a large deletion (promoter or full locus deletion) (*Step 1B*) abolishes mRNA expression and thus prevents TA. Abbreviations: ASO, antisense oligonucleotide; EJC, exon junction complex; MO, morpholino; mRNA, messenger RNA; NGD, no-go decay; NHEJ, non-homologous end joining; NMD, nonsense-mediated mRNA decay; NSD, nonstop decay; PTC, premature termination codon; RISC, RNA-induced silencing complex; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; TA, transcriptional adaptation; TF, transcription factor; TSS, transcription start site. Figure adapted from images created with BioRender.com.

Despite the reasonable concerns about the specificity and reproducibility of RNAi and antisense technologies, they remain valuable tools for gene function analysis. Knockdown reagents allow a relatively quick assessment of gene function, which can be a good indicator of whether to proceed with knockout generation. Following recently updated guidelines (138), knockdown studies can complement mutant allele investigation: Such studies can be especially beneficial when investigating essential genes as they allow the easy generation of a knockdown equivalent of an allelic series. Moreover, other knockdown approaches can provide further versatility in gene function research: Such approaches include targeting transcription [e.g., using a dead version of Cas9 fused to a transcriptional repressor (also known as CRISPR interference)], or targeting proteins via small-molecule inhibitors of protein function, degradation tags called degrons, or protein ubiquitination-promoting proteolysis-targeting chimeras (PROTACs) (52).

Knockout generation has several potential pitfalls. Since it is still unclear how prevalent TA is and exactly which genes trigger TA when mutated, nonsense alleles should be used with caution. Deletion of single or multiple exons is another common approach; however, such modifications can also lead to frameshifts and generate PTCs. Selecting small in-frame deletions or insertions when targeting functional domains or evolutionarily conserved regions is a plausible approach. However, one needs to keep in mind that, as mentioned above, in-frame deletions or insertions can lead to NGD-prone alleles, whereas removal of the endogenous stop codon will subject mRNAs to NSD, and mRNA degradation can trigger TA (33). After obtaining the sequence of the generated mutant alleles, it is therefore recommended to use tools such as MutaRNA (101) to visualize and estimate the impact of each mutation on the mRNA's secondary structure and, most importantly, to subsequently determine mutant mRNA levels.

Findings by El-Brolosy et al. (33) and Ma et al. (92) suggest that generating RNA-less alleles, e.g., promoterless or full locus deletion alleles, avoids TA by eliminating the possibility of PTC-containing mRNAs and/or mutant mRNA degradation. Promoter deletions with very high reduction, or even complete loss, of gene expression have been reported in several clinical cases (40, 137); however, due to widespread intergenic, as well as intragenic, *cis*-regulatory elements and some genes having multiple promoters (23), often with poorly defined genomic coordinates, generating a promoterless allele can be a challenging task. In addition, the expression of different isoforms in various tissues should be considered when creating such promoterless alleles, and thus targeting a larger genomic region might be the best approach. Generating large deletions, including the removal of an entire gene locus, is becoming more routine in a number of model organisms (8, 12). Such large deletions come with certain caveats, including the potential removal of noncoding RNAs, regulatory elements of nearby genes, or genes encoded by the antisense strand, again complicating the interpretation of the resulting mutant phenotypes. One should also be aware that excised regions are prone to reintegrating in close proximity to the edited locus (13). Despite these caveats, RNA-less alleles appear to be the most efficient way to avoid TA, and their generation may benefit from recent findings suggesting the superiority over Cas9 of the type I-C CRISPR system from *Pseudomonas aeruginosa* Cascade–Cas3 (*Pae*Cas3c) in performing large deletions (22). If an interesting phenotype is observed in a full locus deletion allele, more restricted lesions (e.g., small in-frame deletions) will need to be generated and investigated in detail, while keeping in mind their potential susceptibility to NGD.

Should the researchers who use mRNA knockdown strategies expect TA in their experiments? Based on current data, mRNA degradation by itself is sufficient to trigger TA. Although Ma et al. (92) did not observe TA by targeting *capn3a* mRNA with *Leptotrichia wadei* Cas13a (*Lwa*Cas13a), El-Brolosy et al. (33) reported that decay-prone uncapped RNAs induce TA in zebrafish and in mouse cells in culture. In addition, a recent study that explored the efficiency of different types of Cas13-mediated gene knockdown reported that CRISPR-*Rfx*Cas13d (CasRx) effectively and

precisely targets and degrades mRNA transcripts in zebrafish embryos and leads to TA (73). However, these effects were not observed with *Psp*Cas13b, *Pgu*Cas13b, or *Lwa*Cas13a (73), the latter in agreement with the results of Ma et al. (92). Thus, one should keep in mind the possibility that gene knockdown approaches that lead to target mRNA degradation induce TA (**Figure 1**).

CONCLUDING REMARKS

The ever-expanding set of genome editing tools has not only accelerated the study of gene function and interactions but also further revealed how the intricate processes of mRNA quality control can regulate gene expression. Investigating the molecular mechanisms of gene knockdown and knockout techniques is essential to advance clinical genetics and introduce gene perturbation approaches into medical practice. Therapeutic gene silencing reached a new milestone in 2018, when the first RNAi-based drug, patisiran, was approved by the US Food and Drug Administration for the treatment of hereditary transthyretin amyloidosis with polyneuropathy (132). Likewise, in 2017, a Hunter syndrome patient received an in vivo ZFN-mediated gene editing treatment as part of the CHAMPIONS phase 1/2 clinical trial (39).

Moreover, the discordant phenotypic outcomes of various gene perturbation strategies have helped uncover a new mode of genetic robustness and led us to rethink genotype–phenotype relationships. Hopefully, this review has made a strong case that, despite their increasing precision and ease of use, different gene perturbation approaches can yield complex and ambiguous outcomes. The recent discovery that the transcription of decay-prone mutant mRNAs can influence various genomic loci and upregulate genes that sometimes compensate for the loss of the mutant gene underscores the fact that there are still many unknowns regarding how cells adapt and respond to various gene perturbations. The design of loss-of-function experiments requires a careful comparison between different experimental approaches, and often the combination of knockdown and knockout strategies is essential to accurately ascribe gene function. TA is a newly identified phenomenon, and it remains unclear how many genes or gene families can exhibit functional compensation via TA. Fortunately, it is becoming easier to generate promoter or full locus deletions, and since completely abolishing mRNA expression is an efficient way to avoid genetic compensation via TA, such alleles are a sensible way to start genetic analysis of gene function.

Finally, the increasing ability to reveal the complex interactions between multiple genes, and thus more accurately evaluate genotype–phenotype relationships, is expected to provide the field with larger data sets of the effects of various mutations. These data sets will address effects not only on single genes or small gene networks but also at the whole-genome level, revealing a precise interactome in which TA and genetic robustness act. The awesome power of gene perturbation techniques might make us feel like we are in the driver's seat, but nature has many tricks up its sleeve. It is important to keep an open mind, and given that a mechanism as prevalent as TA has arguably been hiding in plain sight for many years in the era of genome editing, it is safe to assume that there is still plenty to discover in functional genomics.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank Z. Kontarakis, C.M. Dooley, and F. Gunawan for discussions and comments on the manuscript; research in the D.Y.R. Stainier laboratory is supported by the Max Planck Society,

the European Union (European Research Council AdG project: ZMOD 694455), the Deutsche Forschungsgemeinschaft (DFG), and the Leducq Foundation. G. Jakutis is supported by a predoctoral fellowship from Boehringer Ingelheim Fonds.

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