Suppression mechanisms

themes from variations

Suppressor analysis is a commonly used strategy to identify functional relationships between genes that might not have been revealed through other genetic or biochemical means. Many mechanisms that explain the phenomenon of genetic suppression have been described, but the wide variety of possible mechanisms can present a challenge to defining the relationship between a suppressor and the original gene. This article provides a broad framework for classifying suppression mechanisms and describes a series of genetic tests that can be applied to determine the most likely mechanism of suppression.

The analysis of any biological process by classical genetic methods ultimately requires multiple types of mutant selections to identify all the genes involved in that process. One strategy commonly used to identify functionally related genes is to begin with a strain that already contains a mutation affecting the pathway of interest, selecting for mutations that modify its phenotype. Modifiers that result in a more severe phenotype are termed enhancers, while mutations that restore a more wild-type phenotype despite the continued presence of the original mutation are termed suppressors. Historically, suppressors have proven extremely valuable for determining the relationship between two gene products *in vivo*, even in the absence of cloning or sequence information. In this current era of whole genome sequencing projects, where the DNA sequence provides little or no clues about the function of ~60% of the open reading frames[1,2], suppressors continue to be useful for determining the roles of these pioneer proteins *in vivo*. The goal of this article is to provide a condensed framework for understanding the molecular basis for suppression, with an emphasis on suppressors that are most informative for analyzing genetic pathways. Early studies of suppressors and enhancers have been described previously (reviewed in Refs 3–8).

Suppressors have been used increasingly to analyze genetic pathways since they were first described by Sturtevant in 1920 (Ref. 9). There are two main reasons for the increased use of suppressors. First, many genes are resistant to identification by more direct genetic selections. A pre-existing mutation often serves to sensitize that pathway, allowing the identification of additional components through suppressor selections. Second, and perhaps more importantly, suppression of a pre-existing phenotype establishes a genetic relationship between the two genes that might not have been established by other methods. Correctly interpreting the molecular relationship between the two gene products, however, requires knowledge of the possible mechanisms of suppression. A wide variety of potential mechanisms have been discovered; they can be grouped into six classes (Fig. 1) and can be used logically to determine the most likely suppression mechanism (Fig. 2). Each class of suppressors provides different types of information and not all classes are useful for defining genetic pathways. The criteria described here provide a basis for distinguishing the classes, allowing the investigator to: (1) pursue the class that best suits their particular interests; (2) identify systematically the relationship between the two gene products; and (3) design rationally future suppressor hunts to increase the likelihood of obtaining the desired classes of mutations. Some practical aspects should be considered when designing suppressor hunts (Box 1).

Intragenic suppression

The simplest suppression mechanism to conceptualize is intragenic suppression, where a phenotype caused by a primary mutation is ameliorated by a second mutation in the same gene. The suppressing mutation might be a true revertant, restoring the original DNA sequence; it might be an alteration of the same codon, resulting in a less detrimental amino acid at that position; or it might affect a different codon, causing an amino acid change at another position that now restores the function of that protein closer to wild-type activity. Intragenic suppressors are distinguished from extragenic suppressors by linkage analysis; by definition intragenic suppressors must be very tightly linked to the original mutation, whereas extragenic suppressors are unlikely to be tightly linked. Although intragenic suppressors provide valuable information about the structure–function relationships within a protein, they do not identify any new proteins that are functionally related to the original mutant, and therefore are generally not the goal when undertaking a suppressor hunt. In some organisms specific selections can be designed either to enrich for, or to screen away intragenic suppressors, depending upon the interests of the investigator[10–12].

Informational suppressors

A major unexpected class of suppressors identified by early suppressor hunts consisted of mutations in components of the translational machinery that suppress nonsense or frameshift mutations. These suppressors were termed informational suppressors because they altered the passage of information from DNA to protein, in apparent violation of the genetic code. For this reason, informational suppressors were pursued with great interest and have proven to be extremely valuable tools for studying phenomena such as codon–anti-codon recognition, the triplet nature of the genetic code and translational accuracy[7]. Because

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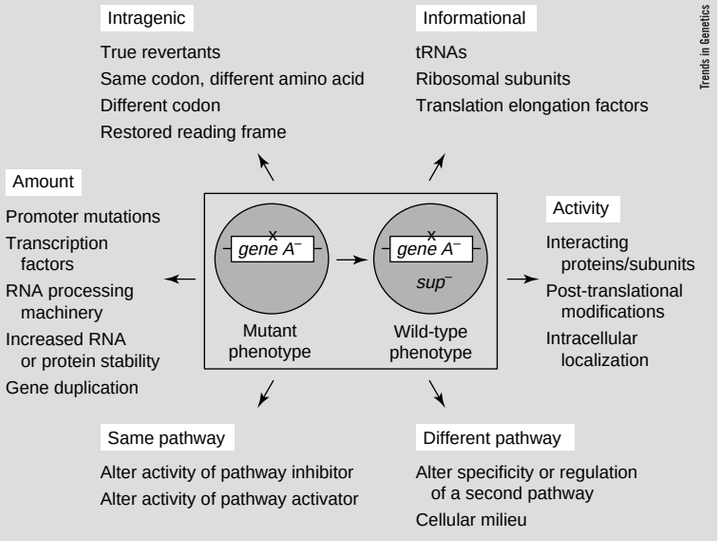
FIGURE 1. Classes of suppression mechanisms



In a typical suppressor hunt, selection for phenotypic revertants of a *gene A* mutation usually results in a cell that still contains the original mutation, but has a more wild-type phenotype due to a suppressor mutation. The six classes of suppressors described in the text are shown in white boxes, with examples of specific mechanisms listed below each class.

informational suppressors are specific for a type of mutation, and not for a particular gene product, their distinguishing genetic characteristic is often considered to be their allele-specific and gene non-specific patterns of suppression. This criterion is not absolute, however, because allele-specific, gene non-specific suppressors that are not classically defined informational suppressors have been described subsequently[13–15]. Mutations in *SPT* genes or in *su(Hw)*, for example, suppress certain transposable element insertion mutations in *Saccharomyces cerevisiae* and *Drosophila melanogaster*, respectively. Because transposon insertions are specific types of mutations that can occur in essentially all genes, *spt* and *su(Hw)* mutations have genetic characteristics of informational suppressors, even though they affect transcription and not the information content of the open reading frame.

Although informational suppressors have provided valuable information about the mechanism and fidelity of translation, they are not particularly useful when the goal of a suppressor hunt is to study other cellular processes. Frameshift or nonsense mutations therefore should not be used as starting points for a suppressor hunt when other mechanistic classes are desired, because of the relatively high frequency of obtaining informational suppressors in those genetic backgrounds. Intentionally beginning a selection with nonsense or frameshift mutations, in contrast, is an effective strategy to enrich specifically for informational suppressors when that is the desired goal[16,17].

Altering the amount of the mutant protein

A primary mutation can reduce the overall activity of the encoded protein either by reducing its specific activity, or by reducing the actual amount of the protein. For either case, one expected class of suppressors consists of mutations that increase the amount of the original protein. The suppressor might affect gene expression at many levels: *cis*-acting promoter mutations[18], mutations in the transcription machinery, alterations of mRNA processing, translation, RNA (Ref. 14) or protein stability[19], or duplication of the mutant gene[20]; all, conceivably, could increase the level of the mutant protein. This class of suppressors, therefore, identifies either specific regulators of the gene of interest or general components of gene expression pathways. For example, mutations in the Pup3p subunit of the 20S proteasome suppress temperature-sensitive mutations (Ts⁻) in the Rpo26p subunit of RNA polymerase II in *S. cerevisiae* by increasing the stability of the mutant Rpo26p (Ref. 19). Suppression is specific for Rpo26p and not other Ts⁻ Pol II subunits, suggesting that Rpo26p is particularly sensitive to proteolysis. The *smg* genes, which were identified in *Caenorhabditis elegans* as suppressors of mutations in a variety of seemingly unrelated pathways, constitute an example of suppression via general gene expression pathways[14]. The *smg* genes are involved in nonsense-mediated mRNA decay; suppression is most likely due to increased stability of the mRNAs encoded by the original genes. Suppression by this class, therefore, often provides insight into the regulation of the primary gene of interest, although little is learned about its function. Distinguishing this class of suppressors requires comparing RNA and protein levels of the original gene in wild-type and suppressed cells.

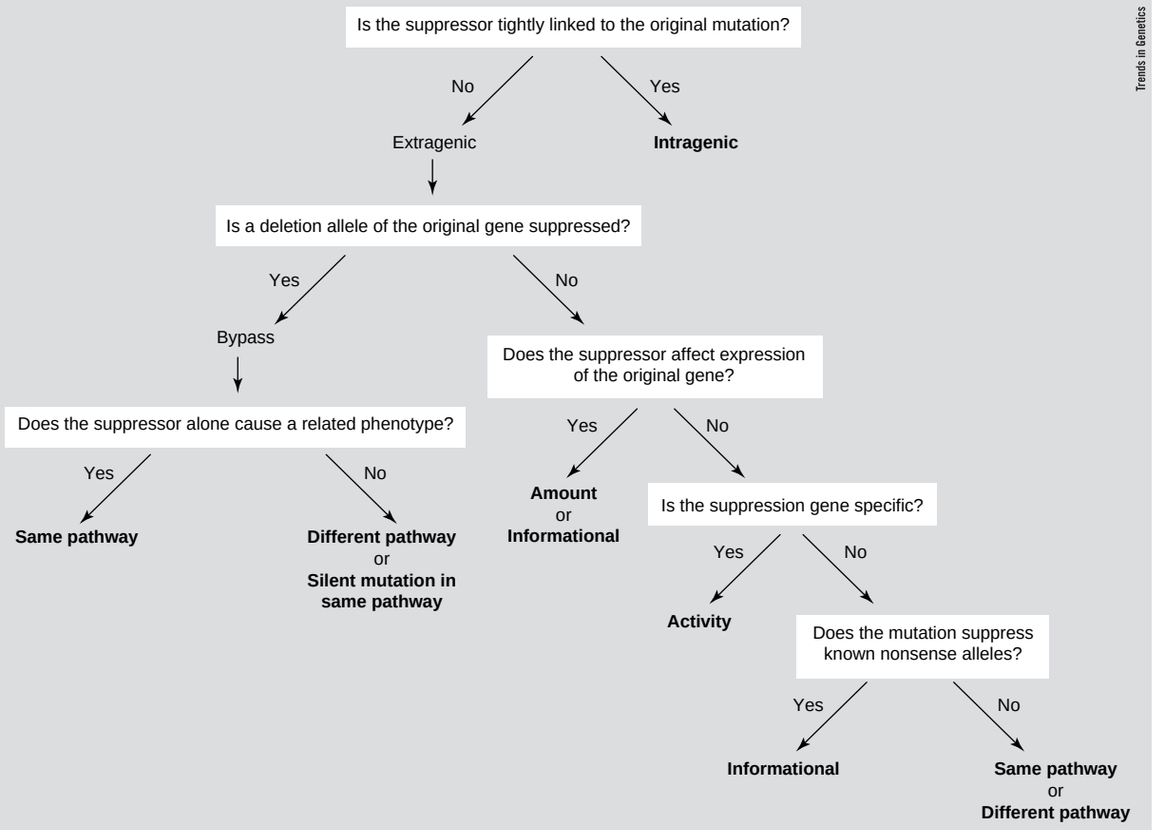
Altering the activity of the mutant protein

Another mechanism for increasing the overall activity of a defective protein is to increase its specific activity. This can occur by at least three mechanisms. As discussed above, certain intragenic suppressors can be considered a subset of this class; in addition, the specific activity of a mutant protein can be increased by altering direct interactions with regulatory subunits, or by affecting post-translational modifications.

Mutations that identify direct interactions between two proteins are among the most commonly desired class of suppressors. In the most simplistic scenario, an amino acid change at the interface between two proteins disrupts their interaction and a compensatory amino acid substitution in the second protein restores that interaction. Suppression by this 'lock-and-key' mechanism demands extreme allele-specificity, because only mutations at amino acid positions that are in direct contact should be suppressed. Although suppression by this mechanism has been documented, it is likely to be rare. An alternative to the lock-and-key model was proposed recently, based on a thorough study of mutations in an *S. cerevisiae* actin-binding protein (Sac6p) that suppress temperature-sensitive mutations in actin[21]. Purified mutant Sac6p proteins bind tighter than wild-type Sac6p to mutant actin, consistent with a restored Sac6p–actin interaction, but unexpectedly they also bind tighter to wild-type actin. The authors propose that suppression results, not from restoring the original disrupted interaction, but from the creation of new contacts between the two proteins. This conclusion is confirmed by structural studies, which demonstrate that the affected Sac6p residues are not normally in direct contact with actin[22]. Based on the larger target size available for the creation of new contacts, it is likely that this mechanism occurs more frequently than the commonly envisioned lock-and-key mechanism.

The *act1* and *sac6* mutations also provide an excellent example of mutual suppression, another genetic phenomenon frequently associated with interacting proteins. The

FIGURE 2. Logic for determining the most likely suppression mechanism



Questions that need to be answered to determine the mechanism of suppression are shown in white boxes, and the classes of suppressors described in Fig. 1 are in bold. These questions are not always addressed in the order shown, owing to the number and types of alleles that are available, the availability of clones for the original gene, and the genetic limitations of the organism being studied; however, the flowchart provides a typical order in which these questions are ideally applied.

sac6 mutations that suppress temperature-sensitive *act1* mutations are themselves temperature-sensitive in an *ACT1*$^{+}$ background[23]; in other words, the *act1* mutations reciprocally suppress the *sac6* Ts^{-} phenotype. Other examples of mutual suppression of the same phenotype have been observed[11,24,25] and in each case the relevant proteins are components of the same multi-subunit complex. This characteristic might, therefore, be diagnostic of proteins that are components of the same complex.

Many proteins are regulated by post-translational modifications that either stimulate or inhibit activity of the modified protein. Suppressors can be used to identify the proteins responsible for these regulatory events; mutations that alter the modification of a mutant protein might restore activity sufficiently to result in a wild-type phenotype. In *Schizosaccharomyces pombe*, for example, overexpression of *mik1* suppresses the semi-dominant *cdc2-3w*-dependent mitotic catastrophe phenotype[26]; mitotic catastrophe refers to a synthetic lethal phenotype caused by premature entry into mitosis that occurs in a *cdc2-3w wee1-50* double mutant. The *mik1* gene encodes a protein kinase that directly phosphorylates Cdc2 on Tyr15, inhibiting Cdc2 activity. Phosphorylation is a common regulatory modification *in vivo*, but other types of modifications have also been identified by suppressors. *RAM1*, for example, was identified as a suppressor of the dominant *RAS2*^{val19} mutation in *S. cerevisiae*[27]; *RAM1* is required for the C-terminal farnesylation and membrane association of RAS and other membrane proteins. In both of these examples, suppression results from altering the modification state of an overactive protein, but suppression of recessive loss-of-function mutations can also occur by this mechanism.

The defining genetic characteristics of this class of suppressors that function by modifying the activity of a mutant protein are that the suppressor is incapable of suppressing a deletion allele of the original gene and that suppression should be gene-specific. Allele-specific suppression is often regarded as a hallmark indicative of a direct interaction; however, allele-specificity should not be overinterpreted, as suppression by other mechanisms could also be allele-specific. When a gene has multiple functions, for example, mutations that differentially affect those functions will be suppressed in an allele-specific manner if a suppressor only affects one of those functions. Allele-specific suppression could also be exhibited by weak suppressors; weak alleles of the original gene will be suppressed, while more severe alleles will not, regardless of the mechanism. Ultimately, comparison of any known biochemical activities of the original mutant protein in suppressed and unsuppressed strains is the only unequivocal way to distinguish this class.

BOX 1. Advice for successful suppressor hunts

Know your starting allele

This cannot be overemphasized. The results of a mutant hunt are absolutely dependent upon the nature of the starting allele: a completely different spectrum of suppressors is expected depending upon whether the selection begins with a loss-of-function, gain-of-function, null, missense or nonsense allele, or a mutation outside the coding region (promoter mutant, splice site mutant, 3′UTR mutation and others). If possible, determine the sequence of the starting mutation. If the mutation is not determined, test beforehand whether it is nonsense suppressible, or use antibodies to determine whether the full length protein is produced. Suppressors are more interpretable when the biochemical defect of the original protein is known.

Consider targeting the selection

A broad selection with few underlying assumptions is usually preferable, but at times a directed selection is more appropriate. If dominant, intragenic, informational or bypass suppressors are desired, set up the selection to get them directly.

Suppressors versus enhancers

Suppressors are very powerful genetic tools for analyzing biological pathways, but there are equally powerful tools at your disposal. Before undertaking a potentially time-consuming suppressor hunt, evaluate whether an enhancer or synthetic lethal selection is more likely to identify the gene products in which you are most interested.

Test the phenotype of the suppressor by itself

Suppressors that cause a phenotype related to the original mutation, whether the same phenotype or the opposite, are much more likely to be relevant to the original pathway. Generally, these candidates should be pursued first.

Starting strain considerations

Time invested to set up strains in a way that will speed up subsequent linkage, dominance and complementation tests, is time well spent. With yeast, for example, begin with strains of opposite mating types containing appropriate nutritional auxotrophies to allow for diploid selection and subsequent cloning attempts with plasmid libraries.

Consider suppression by overexpression

In organisms where it is feasible, selecting for plasmids that suppress due to overexpression of a wild-type gene product is much less time-consuming than genomic suppressor hunts, thus allowing attempts for suppression of multiple alleles without the need to clone the suppressing gene. Because suppression is due to overexpression of a wild-type gene product, some mechanistic classes are eliminated, allowing more reliable assumptions about the relationship between the suppressor gene and the original mutation.

Altering the activity of the mutant pathway

In a multi-step pathway, a mutation that alters one step of the pathway can often be suppressed by mutations in genes that affect other steps within that same pathway. This class of suppressors is often extremely informative, because in addition to identifying other components of the pathway of interest, the suppressors can also facilitate ordering of the pathway[28,29]. The strongest evidence that a suppressor functions within the same pathway is when the suppressor alone confers a phenotype that is related to that of the original mutation. As described above, suppressors often confer the same phenotype as the original mutation when both proteins are components of the same multi-protein complex, but suppressors that affect other steps in the same pathway often confer an opposite phenotype from the original mutation. For example, deletions that inactivate the Ste4 Gβ subunit inhibit the mating-type signaling pathway in *S. cerevisiae*; this defect can be suppressed by dominant mutations in *STE11*, which encodes a protein kinase involved in the same pathway[30]. The dominant *STE11-4* mutation alone partially activates the signaling pathway in the absence of mating pheromone, in contrast to the original *ste4Δ* mutation, which causes an inability to signal. The appearance of a related phenotype is so indicative of a role in the same pathway that it is commonly used as a secondary screen to identify candidates that suppress by this mechanism[31–33]. Suppressors of *S. cerevisiae* temperature-sensitive *cdc* mutants were readily obtained, for example, but screening through the suppressors for mutants that simultaneously conferred a cold-sensitive Cdc$^-$ phenotype allowed rapid identification of the most promising suppressors for subsequent studies. The absence of a related phenotype should not be interpreted as evidence against a role in the same pathway, however, as silent suppressors (those that cause no phenotype by themselves) are not uncommon.

A suppressor that affects other steps in the same pathway can function either upstream or downstream of the original gene; deducing the order of the two gene products depends upon the type of pathway involved and the nature of the original mutation[29]. In a switch or signaling pathway, where the presence or absence of a signal determines whether the pathway is ‘on’ or ‘off’, suppression of a partial loss-of-function allele does not allow reliable ordering of the pathway; suppression might result from mutations in either downstream or upstream components (Fig. 3). Suppression of a null allele, however, is expected to be due to downstream mutations that activate the pathway independent of the original (suppressed) gene product. By contrast, in biosynthetic pathways such as phage assembly or the cell cycle, where the accumulation of intermediate products or the occurrence of successive dependent steps is being detected, the suppressing mutation is usually upstream of the original mutation. When two mutations by themselves cause opposite phenotypes and one mutation suppresses the other, this phenomenon is termed suppression by epistasis and the mutation whose phenotype is observed in the double mutant is said to be epistatic to the suppressed mutation. In the example cited above, a *ste4Δ STE11-4* double mutant signals constitutively; *STE11-4* is therefore epistatic to *ste4Δ*. This simple epistasis test provided early evidence that the Ste11p kinase functions downstream of the Ste4 Gβ subunit.

Altering a different pathway

A mutation that inactivates one pathway can often be suppressed by altering a second pathway. The suppressor might

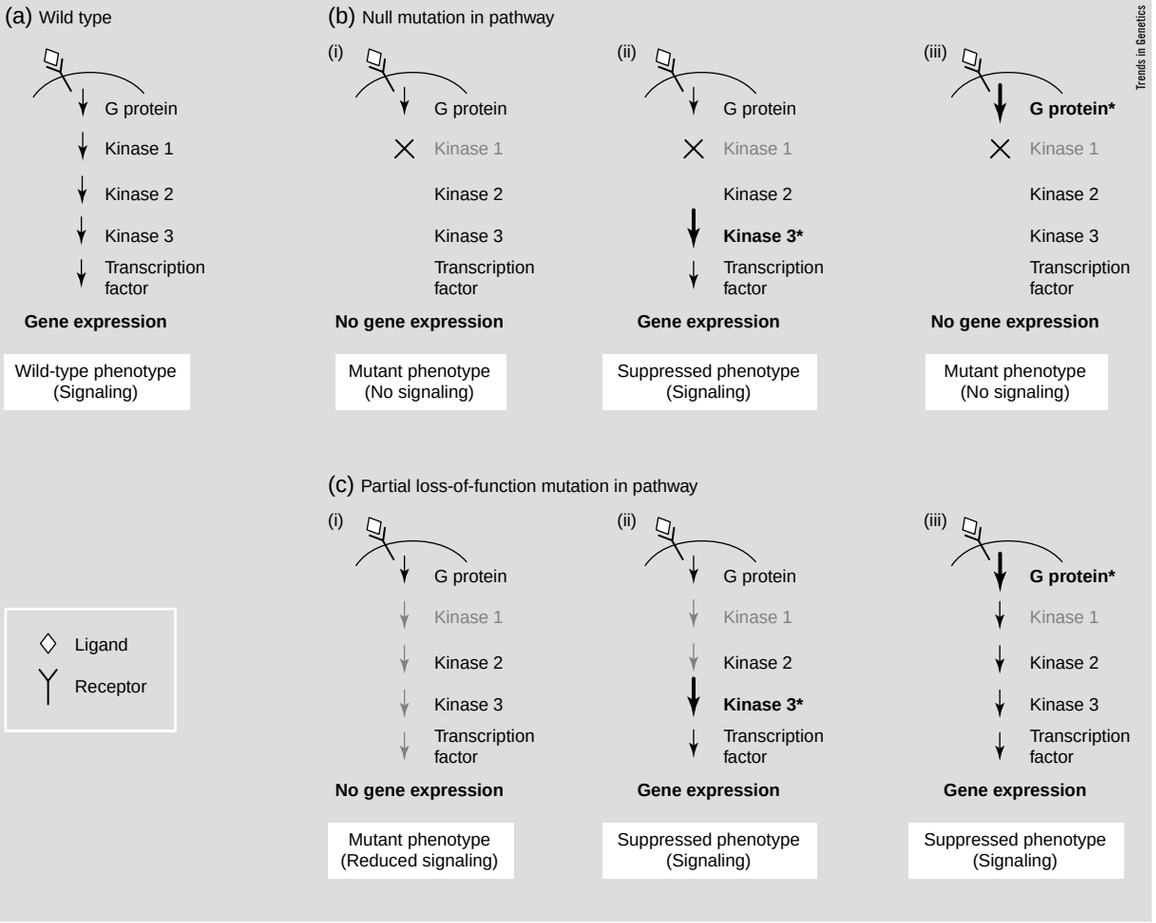
affect the regulation of a pathway that has a related or overlapping function, or the suppressor could alter the specificity of a functionally unrelated pathway. A classic example of the latter involves sugar transport in bacteria; mutations of the *Escherichia coli* maltose permease can be suppressed by altered specificity mutations in the lactose permease that now allows maltose transport, even though the wild-type *lac* permease usually has no role in the transport of maltose[34].

Suppressors that alter the 'cellular milieu' should also be considered as affecting a different pathway. These mutations alter the overall physiology of the cell (e.g. by changing the intracellular pH or Ca^{2+} concentration) such that the function of the original mutant protein is restored in that altered environment[35]. It is not clear how frequently this indirect mechanism actually occurs. Determining whether a suppressor affects a different pathway from the original mutation is greatly aided by identifying the phenotype of the suppressor in the absence of the original mutation. If the suppressor alone causes a phenotype that is completely unrelated to the original mutation, it is likely to be due to an alteration in a different pathway and caution should be taken when interpreting the relationship between the two gene products.

Closing perspectives

Most of the examples cited above involve suppression of recessive loss-of-function mutations by genomic suppressors. Suppressors of dominant gain-of-function alleles have also been obtained with great success and suppression often occurs via analogous, although opposite, mechanisms. For example, suppression of a dominant gain-of-function mutation can occur by reducing the amount of the original protein[12], reducing the activity of the dominant protein[27], or reducing the activity of the pathway[36]. Another strategy commonly used in some organisms is to select for genes whose overexpression suppresses a mutant phenotype[37]. Typically, a mutant strain is transformed with a plasmid library that overexpresses wild-type genes, either from an inducible promoter or simply due to increased plasmid copy number, and suppressors are selected. Suppression by overexpression also occurs through mechanisms analogous to those used by the six main classes of suppressors (Fig. 1).

FIGURE 3. Suppression in a typical signaling pathway



(a) A typical receptor-mediated signal transduction pathway is shown, involving a ligand, a cell surface receptor, a G protein, a kinase cascade and a transcription factor that activates or represses gene expression in response to the signal. (b) A null mutation in the gene encoding kinase 1 blocks the pathway, resulting in a mutant phenotype (i). The null mutation can be suppressed by a mutation in a gene encoding a component (denoted by an asterisk), such as kinase 3 (ii), which activates the downstream portion of the pathway independent of kinase 1 activity. Mutations in upstream components, such as the G protein (iii), restore signaling through only part of the pathway, owing to the kinase 1 defect. (c) When the pathway is inactivated by a partial loss-of-function mutation in kinase 1 (i), mutations in either downstream (ii) or upstream (iii) components can activate the pathway enough to suppress the mutant phenotype. Pathways can, therefore, only be ordered reliably when the starting mutation is a null allele.

To determine correctly the mechanism that results in suppression, it is important that suppressors are analyzed similarly to mutations obtained by more straightforward selections. In particular, dominance tests can be used to infer whether suppression is most likely to be due to a gain or loss of function, and null alleles should be created or identified to determine the phenotype caused by complete loss of suppressor gene function. Deletion alleles of the suppressor gene are particularly important for distinguishing between the classes described above; if a deletion suppresses, for example, suppression clearly cannot be due to restoring a physical interaction between the two gene products. Combining analysis of the suppressor alone with characterization of the suppression phenotype (described in Fig. 2) usually will allow the construction of a reasonable model for understanding the molecular relationship between a suppressor and the original gene product.

The current availability of complete genome sequences does not diminish the necessity for undertaking and understanding classic suppressor analysis. Approximately 40–60% of open reading frames (ORFs) in *S. cerevisiae* and *C. elegans* provide little clue about their function from the sequence. Even those ORFs that do allow inference of the encoded biochemical activity still need to be functionally connected to specific pathways *in vivo*; those connections are often provided by suppressor analysis.

Suppression studies performed in simpler organisms often can provide insights into gene function in more complex organisms where suppressor hunts are not feasible. First, pathways deciphered by traditional suppressor analysis in simpler organisms often are conserved across large evolutionary distances, allowing extrapolation to more complex organisms. Second, suppression can also be used in more imaginative ways, taking practical advantage of that evolutionary conservation to cross species lines. Human cyclin D was originally cloned, for instance, by selecting for human cDNAs that, when overexpressed, suppressed a yeast cyclin-deficient mutant[38]. More recently, overexpression of human BAX was found to cause apoptosis in yeast; selection for human cDNA clones that suppressed the BAX-induced apoptosis identified a novel gene called BI1 (Ref. 39). BI1 overexpression also suppressed BAX-induced apoptosis in human cells, indicating that it was not an artifact of the selection in yeast. The use of analogous innovative suppressor hunts, combined with more traditional selections, will continue to be important for deciphering biological pathways in the future.

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