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Opinion

Hypermutator fungal pathogens: from threat to meltdown

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Recent research on human and crop fungal pathogens has highlighted a set of unexpected and seemingly unrelated mechanisms fuelling adaptation to drugs and the host immune system. These mechanisms include the loss of RNA interference (RNAi) in human pathogens, the rapid accumulation of point mutations, and the activity of transposable elements. Despite mechanistic differences driving the extreme accumulation of mutations (i.e., hypermutation) in some pathogens, we argue that the origins follow defined principles. The appearance of hypermutation phenotypes puts pathogens on a unique evolutionary trajectory, and mitigation strategies need to be carefully adapted.

Highlights

Hypermutation is a convergent property of multiple human and plant fungal pathogens. The appearance of hypermutation can severely complicate pathogen control, in particular due to rapid gains in fungicide resistance.

At the mechanistic level, hypermutation arises through distinct molecular pathways as diverse as DNA mismatch repair defects to losses of RNA interference (RNAi) mechanisms or the reactivation of transposable elements (TEs).

The emergence of hypermutators is likely shaped by historical gene loss and pathogen lifestyle.

Following hypermutation and fixation of key adaptive mutations in the population, selection is expected to favour mutations that restore mutation rate to a 'ground state', which we argue can arise through three distinct mechanisms.

Recognising hypermutation as a convergent and widespread adaptive trait rather than a rare exception is essential to confronting rapid pathogen evolution in the face of rapidly changing environments.

Hypermutation phenomenon in fungal pathogens

Fungal pathogens of humans and plants pose some of the most serious threats to human health and food security. The rapid and repeated adaptation of fungal pathogens limits the effectiveness of existing treatment strategies and intensifies the need for novel antifungal drugs. This urgency is underscored by the World Health Organization's fungal pathogen priority list (www.who.int/publications/i/item/9789240060241). Research on antifungal resistance has centred on identifying mutations, including single-nucleotide polymorphisms (SNPs) and copy-number variations (CNVs) causing resistance. However, recent studies have drawn attention to the fact that some pathogens appear to have an inherent tendency to accumulate mutations at an extraordinarily high rate. This phenomenon is termed hypermutation and has arisen repeatedly in independent lineages.

The elevated mutation rate enables pathogens to adapt faster to environmental stressors, including antifungal drugs [1]. Different types of hypermutator phenotypes were found in plant pathogens such as *Erysiphe* spp. [2], *Blumeria graminis* [2], and *Zymoseptoria tritici* [3–5], as well as in human and animal pathogens including *Cryptococcus* spp. [6–12], *Candida* spp. [13,14], *Aspergillus fumigatus* [15,16], and *Batrachochytrium salamandrivorans* and *B. dendrobatidis* [17]. Hypermutator phenotypes appear to arise through independent mechanisms and are based on unknown triggers. While hypermutation facilitates rapid adaptation, it also increases the risk of accumulating deleterious mutations – which, in turn, can reduce pathogen fitness. Here, we propose that the seemingly disparate mechanisms underlying fungal pathogen hypermutator emergence converge within a unifying framework. We present a three-stage model which helps to classify the origin of the hypermutator phenotype, to disentangle mechanisms contributing to hypermutation, and provide a framework to assess the trajectory of hypermutators in pathogen species. Our model helps also to evaluate the risks of hypermutator emergence and attenuate resistance evolution in order to aid drug application strategies.

Mechanistic basis of hypermutation in fungi

Hypermutator phenotypes have been found to arise through at least three distinct mechanisms: (i) loss of RNAi, (ii) loss of DNA mismatch repair (MMR) components, and (iii) uncontrolled

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transposable element (TE) activity triggered by stress and/or the breakdown of TE control systems (Figure 1). RNAi is a genome defence mechanism used by fungal hosts to suppress TE activity and maintain genome integrity. In *Cryptococcus neoformans*, RNAi has been shown to play a critical role in TE silencing [10,18–20]. Analysis of *C. neoformans* hypermutator strains by Priest *et al*. [10] identified two clinical hypermutator isolates of *C. neoformans* that harboured extensive accumulations of the *C. neoformans* LINE-1 element (Cnl1) at subtelomeric regions. This accumulation was unexpected given the presence of a functional RNAi pathway in *C. neoformans*, and that Cnl1 amplification is not present in the commonly used *C. neoformans* strain H99 [10]. Further analysis revealed that the TE proliferation was driven by a nonsense mutation (C>T) in *ZNF3*, which encodes an RNAi component that localises to P-bodies [10]. The resulting

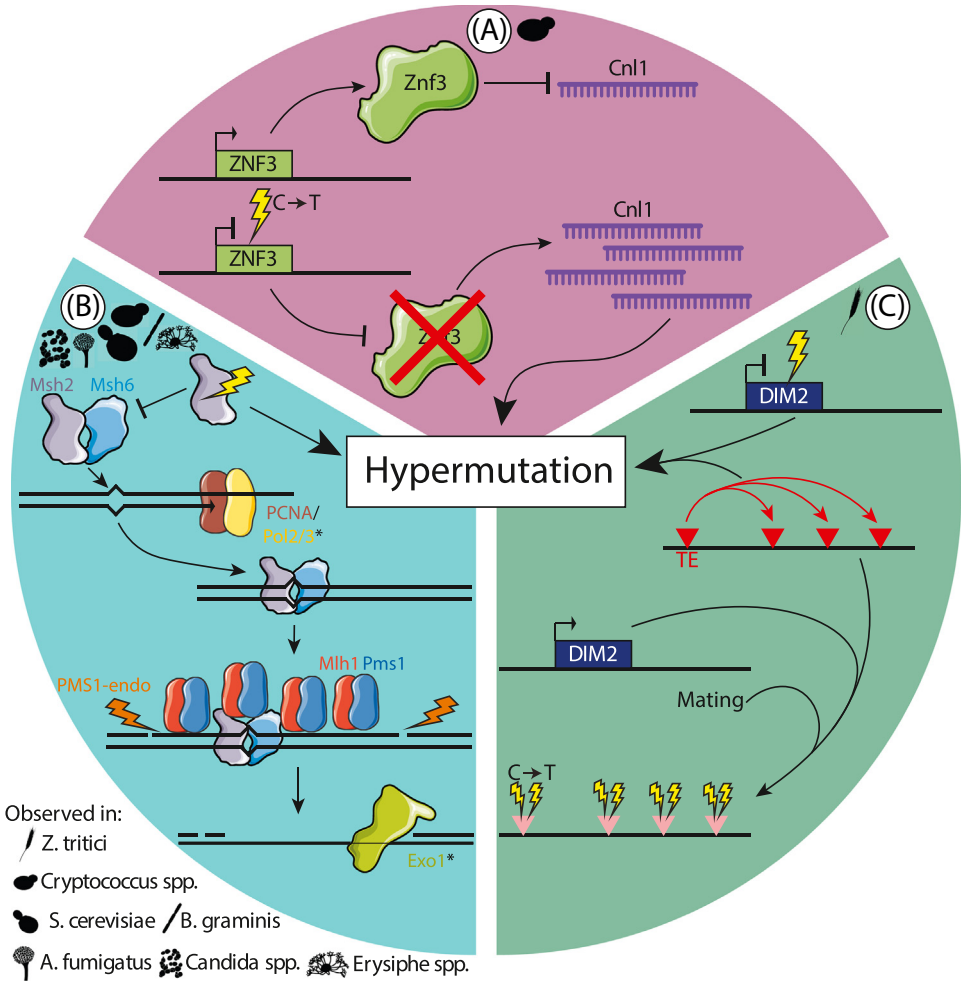

Figure 1. Three distinct mechanisms converge to result in hypermutator phenotypes. (A) Loss of function of *ZNF3* leads to loss of RNA interference (RNAi) and expansion of Cnl1 retrotransposons in *Cryptococcus neoformans*. (B) Loss of *Msh2* impairs the DNA mismatch repair (MMR) pathway, leading to rapid accumulation of mutations. (C) Loss of *DIM2* impairs the repeat-induced point mutation (RIP) mechanism, enabling transposable elements (TEs) to maintain function and generate novel insertions. Asterisks (*) indicate optional components in the DNA MMR repair pathway. Species outside of the main circle indicate known species with a history of hypermutator emergence. *A. fumigatus*, *Aspergillus fumigatus*; *B. graminis*, *Blumeria graminis*; *S. cerevisiae*, *Saccharomyces cerevisiae*; *Z. tritici*, *Zymoseptoria tritici*. Abbreviation: PCNA, proliferating cell nuclear antigen. Non-original artwork was sourced from BioIcons (https://bioicons.com). Protein icons by Servier (https://smart.servier.com/) are licensed under CC-BY 3.0 Unported https://creativecommons.org/licenses/by/3.0/.

truncated Znf3 protein renders the RNAi pathway non-functional, allowing Cnl1 elements to transpose unchecked (Figure 1A).

DNA repair is essential for controlling genome-wide mutation rates. Unsurprisingly, loss of MMR components has been repeatedly observed in fungal hypermutator lineages. Among these, *MSH2* is the most commonly disrupted gene and has been implicated in the emergence of hypermutator phenotypes in drug-resistance contexts. For example, *MSH2* loss has been linked to hypermutation in *Cryptococcus deuterogattii* lineages resistant to 5-fluorocytosine, FK506, and rapamycin [11,12]; in azole-resistant *C. neoformans*, mutations have been found not only in *MSH2*, but also in *MSH5*, *RAD5, MLH1*, and *PMS1* [6,21]. Loss-of-function MMR mutations have also been identified in multidrug-resistant isolates of *Nakaseomyces glabrata* (formerly *Candida glabrata*) [13–15] and in *Candida albicans* strains with disruptions in *MSH2* and *PMS1* [22].

In *A. fumigatus,* multi-azole-resistant hypermutator lineages carry a G233A variant in *msh6*, which shows strong genetic linkage with the canonical resistance allele TR_{34}/L98H in *cyp51A* encoding the molecular target of azole fungicides [15]. Similar patterns are observed in obligate plant pathogens: *Erysiphe necator*, *Erysiphe pisi*, *Erysiphe pulchra*, and *Blumeria graminis* each lack 5-21 MMR genes. This loss is associated with elevated frequencies of mononucleotide runs, longer microsatellites, elevated AlT mutational bias, decreased GC content, and a hypermutator phenotype [2]. At the mechanistic level, MMR deficiency results in hypermutation by preventing the correction of replication-induced base mispairing [23] (Figure 1B). In *Saccharomyces cerevisiae*, the MMR system operates via two pathways: one coupled with the replication machinery using proliferating cell nuclear antigen (PCNA) as a mispair sensor, while the other relies on Msh2-Msh6 recognition independently of PCNA [23]. In both cases, Msh2–Msh6 recruits multiple Mlh1–Pms1 complexes to the mismatch site, leading to Pms1 endonuclease activity and excision and repair, sometimes via recruitment of Exo1. Thus, loss of *MSH2* or *MSH6* disrupts mismatch recognition and blocks downstream recruitment of repair factors, ultimately giving rise to the hypermutator phenotype (Figure 1B). Beyond MMR, hypermutation has also been observed arising from perturbations in DNA double-strand break repair pathways (i.e., homologous recombination (HR) and non-homologous end joining (NHEJ)). Disruptions in *RAD51* (HR), *KU70*, or *KU80* (NHEJ) induce a hypermutator phenotype in *C. neoformans* due to loss of regulation of the DNA damage response via the PI3K pathway [24].

TEs are ubiquitous selfish components of eukaryotic genomes. The majority of TE activity is thought to be deleterious due to potential impacts on host genes and genome integrity [25]. Consequently, hosts have evolved mechanisms to defend against TE activity, including the piwi-interacting RNA (piRNA) pathway [26], RNAi [10], DNA methylation and histone modification [27–29], and repeat-induced point mutation (RIP). The latter is a fungal-specific hypermutation mechanism on its own but with a narrow focus on introducing C-to-T transitions at a high rate in any duplicated sequence, and occurs only during sexual reproduction [30]. Generally, TE activity in pathogen genomes is expected to be low due to strong selection to control TE proliferation, especially since TE insertions can be fatal if they interrupt essential host genes. However, environmental stresses, such as fungicide exposure, and loss of control mechanisms can lead to the derepression of TEs, which in turn increases the likelihood that TEs can insert into new loci. This activity can generate significant genome variation as observed in the major wheat pathogen *Z. tritici*, where a burst of TE activity in a North American population led to ~15 new TE insertions per generation over a period of a couple of decades [5]. This expansion is likely driven by activation of TEs in response to environmental stress combined with relaxed purifying selection [31]. In addition to environmental triggers, this expansion is also driven by the loss of TE control mediated by the RIP defence mechanism [32,33], which prevents recognition and removal of novel TE

insertions following sexual reproduction (Figure 1C). Infrequent sexual reproduction, as observed in some fungal pathogens, could add complexity to the impact of RIP. Prolonged clonal reproduction could allow for TE activity even in RIP-proficient lineages. Loss of TE control via environmental stress and/or loss of TE control machinery therefore leads to novel TE insertions and, by this, genomic novelty.

The origins of hypermutator phenotypes

Given that hypermutation is a product of convergent evolution, rather than being driven by a single mechanism, it is important to inspect general conditions facilitating the rise of these phenotypes. We propose that hypermutators are likely to originate under two different scenarios: as a result of a historical contingency, or as a consequence of pathogen lifestyle (Figure 2).

Historical contingency

Hypermutators often emerge after the loss of machinery involved in maintenance of genome integrity or repair. Therefore, the likelihood of hypermutator emergence may well be connected to the propensity of a clade to lose the machinery. A patchy distribution of host gene maintenance could therefore be used as a predictor of hypermutation emergence (Figure 2A). Patchy distributions have been observed in several lineages, with evidence to support the role of historical contingency in hypermutator emergence through all three proposed hypermutator mechanisms.

Phillips *et al*. showed that MMR genes are generally well conserved among Ascomycota (median of 49/52 MMR genes found among 1107 species [2]). However, there is historical contingency for MMR gene loss in the four closely related obligate plant pathogens *E. pulchra, E. pisi, E. necator*, and *B. graminis*, and the loss of MMR components has been linked to hypermutator emergence [2]. Further evidence was found in three distantly related lineages in the subphylum Saccharomycotina with reduced sets of DNA repair genes and accelerated evolutionary rates [34].

Beyond DNA repair, other mechanisms also show patchy distributions of their underlying genes, which could pre-empt hypermutator emergence. In *Z. tritici*, the methyltransferase involved in RIP TE control, *dim2*, exhibits recent loss and creates within-species variability [32]. Lineages without a functional *dim2* show reduced C-to-T transitions and less pronounced TE silencing [32], promoting hypermutator emergence via increased TE activity. Further, Huang *et al*. showed

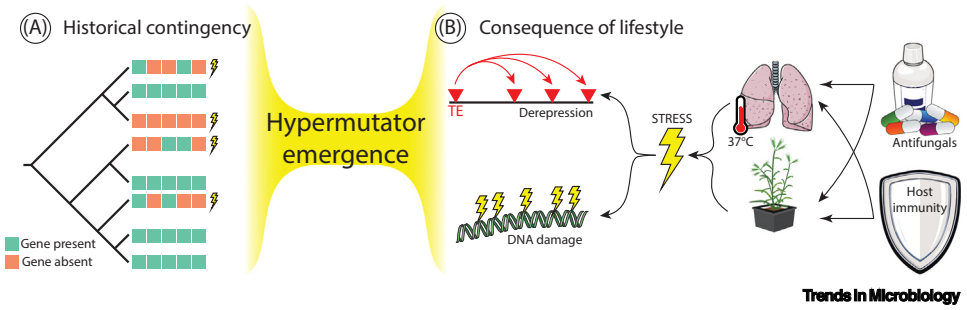

Figure 2. Hypermutators can emerge through two main pathways. (A) Lineages with patchy distributions of gene presence related to mismatch repair (MMR), transposable element (TE) control, and epigenetic modification may be predisposed to hypermutator emergence through stochastic and tolerable loss of such genes. (B) Pathogenic lifestyles are stressful, and fungal pathogens are exposed to environmental challenges including the host immune response, antifungal drugs, and suboptimal conditions such as those at higher temperatures in human infection. Stress responses can lead to DNA damage and TE derepression, in turn enabling hypermutation. Non-original artwork was sourced from BioIcons (https://bioicons.com). Lung and drug icons by Servier (https://smart.servier.com/) are licensed under CC-BY 3.0 Unported https://creativecommons.org/licenses/by/3.0/. Brachypodium_flowering_plant icon by Frédéric Bouché (https://figshare.com/authors/Plant_Illustrations/3773596) is licensed under CC-BY 4.0 Unported https://creativecommons.org/licenses/by/4.0/.

that RNAi loss is common among the natural diversity of *C. neoformans* [7]. However, a high TE burden is required for hypermutator phenotypes to emerge, even in the absence of RNAi. [7]. Thus, the emergence of hypermutators in this context appears to follow a two-step evolutionary process: (i) a high TE load acts as a latent risk factor, and (ii) loss of RNAi-mediated TE suppression permits TE mobilization, accelerating genome-wide mutation rates through uncontrolled transposition. Taken together, there is ample evidence for historical contingency in losses of MMR, RNAi, and TE control machinery. Lineages sharing such properties seem well disposed for hypermutator emergence in pathogens and beyond.

Consequences of lifestyle

Pathogens face a variety of environmental stressors, including changing environments, fungicide exposure, and host immune responses. Stress-induced mutation resulting from a pathogenic lifestyle, either directly or indirectly, can lead to DNA damage and, possibly, hypermutator emergence (Figure 2B). In *C. deneoformans*, growth at 37°C is stressful and results in hypermutation by means of TE mobilization [8]. Importantly, inactivation of RNAi was not sufficient to recapitulate TE movement observed at 37°C at a lower temperature, highlighting the role of stress-dependent TE mobilization in hypermutator emergence. Beyond stress-induced TE activation, reactive oxygen species (ROS) generated as a by-product of mitochondrial activity [35], metabolic flux [36], host immune responses [37,38], and fungicide exposure [39–42] can threaten genome integrity and inflict DNA damage. Such lifestyle-dependent stress conditions can, hence, induce loss-of-function mutations in key genes underpinning mutation rates (i.e., MMR, RNAi, and TE control genes). Such a phenomenon does not seem limited to human pathogens, as TE activity also increases in response to nutrient starvation and host infection stress in the wheat pathogen *Z. tritici* [43], whilst heat shock and copper stress lead to TE activation in the cereal pathogen *Magnaporthe oryzae* to generate high levels of genetic diversity [44].

Evolutionary trajectories for hypermutators

Producing progeny with highly variable fitness is advantageous as a response to severe environmental challenges, such as drug exposure for pathogens. Hypermutator lineages are uniquely capable of producing a broad range of fitness outcomes, with most being deleterious, but few will likely carry significant advantages. In other words, hypermutator emergence enables accelerated exploration of genomic variation on which selection can act. [6,8,9,11,14,15,34,45]. Furthermore, hypermutators may persist through multiple generations by hitchhiking with beneficial alleles. However, once key adaptive mutations are fixed in the population and the population is well adapted to its environment, selection may favour mutations that restore the mutation rate to a 'ground state' baseline [46]. Thus, we propose that there are three distinct fates for hypermutators: evolutionary dead end, constitutive hypermutation, or functional recovery.

The evolutionary dead end is represented by the continued accumulation of deleterious mutations leading to gradual fitness reductions with selection ultimately purging hypermutators from populations [47] (Figure 3A). The speed at which hypermutators reach this dead end will depend on the hypermutation rate, the severity of deleterious mutations and the ability to purge deleterious mutations from the genome. However, new hypermutators may continue to arise from non-hypermutator background in response to novel environmental stresses, leading to a resurgence in hypermutator proportions within populations.

A constitutive hypermutation scenario was established, for example, in the stable hypermutator lineages of the Saccharomycotina subphylum [34] (Figure 3B). Loss of DNA repair gene repertoires lead to substantial mutation burdens. However, the evolutionary dead end was likely avoided through complementation of lost functions either through neofunctionalisation [48,49]

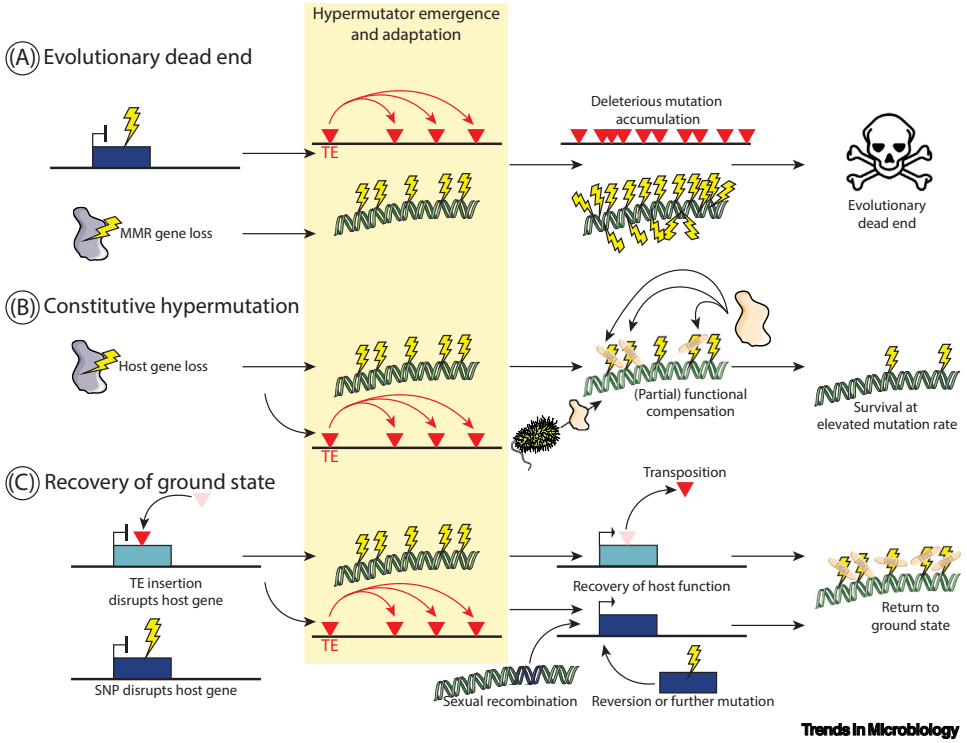

Figure 3. Hypermutators are proposed to embark on one of three evolutionary trajectories. (A) Unstoppable accumulation of deleterious mutations after the population is well adapted to the new environment will lead to the demise of hypermutator individuals. (B) Lost host functions can be partially, or completely, compensated through acquisition of machinery through means such as horizontal gene transfer, enabling the lineage to persist with an elevated mutation rate. (C) Depending on the mutation underlying the hypermutator phenotype, host function has the potential to be restored. For example, transposable element (TE)-disrupted genes can be reinstated if the TE transposes out of the gene locus, resulting in a return to the ground state mutation rate, whilst interrupted genes can be recovered via sexual recombination or mutation to reinstate function. Abbreviations: MMR, mismatch repair; SNP, single-nucleotide polymorphism. Non-original artwork was sourced from BioIcons (https://bioicons.com). Protein and bacterium icons by Servier (https://smart.servier.com/) are licensed under CC-BY 3.0 Unported https://creativecommons.org/licenses/by/3.0/.

or, as observed in the Wickerhamiella/Starmerella (W/S) clade of Saccharomycotina, horizontal gene acquisition [34]. In this case, a bacterial photolyase gene rescues UV-induced DNA damage repair through nonvertical evolution, highlighting the potential for horizontal gene transfer (HGT) as a route for functional compensation [34]. In bacterial hypermutators, MMR genes are also found to frequently undergo HGT [50]. Frequent HGT is observed among fungi [51–59], in addition to horizontal transfer of whole accessory chromosomes [60,61], and Starship-mediated gene mobilization and exchange [3,16,62–64]. Consequently, the likelihood of partial functional compensation is likely higher than previously appreciated and could, as observed in the W/S clade, enable lineage survival at elevated mutation rates, resulting in constitutive hypermutator populations.

Functional recovery may be possible if hypermutator lineages are selected for re-acquiring machinery to control mutation rates (Figure 3C). This is most likely to arise in lineages where TE activity is responsible for hypermutator emergence. Recovery may occur if the TE responsible for inducing hypermutation by disruption of a control machinery gene transposes out of the locus to reinstate host gene function. Such occurrences would mirror the well-characterized process in maize, originally discovered by Barbara McClintock [65,66]. In this case, transposition would represent a stress-responsive mechanism which can enable hypermutation, before

recovering normal host function once the population is well adapted and would support McClintock's original hypothesis of TEs being stress-responsive components of host genomes. In addition, pathogen lineages capable of sexual reproduction could rescue functional RNAi and MMR machineries through sexual reproduction with lineages carrying intact machineries, and hence return to a 'ground state' mutation rate. Further, very recently lost control mechanisms could be restored through back or compensatory mutations.

Concluding remarks

Hypermutation is increasingly observed, and it poses a major challenge to sustainable control of fungi affecting human and plant health. Understanding the mechanisms and triggers of hypermutation is therefore essential for assessing the risk of hypermutator emergence and informing strategies to minimise their occurrence (see Outstanding questions). Our proposed three-stage framework helps to assess the likelihood of hypermutator emergence and may contribute to mitigate resistance evolution. Although hypermutator phenotypes arise via diverse mechanisms, their recurrent convergence suggests a shared adaptive strategy among fungal pathogens. The emergence of hypermutators is likely shaped by historical gene loss and pathogen lifestyle, with high-risk lineages often lacking key DNA repair, genome integrity, and TE control genes, compounded by stress-intensive environments that accelerate mutation accumulation. In contrast to theoretical expectations, hypermutators do not always meet an evolutionary dead end. Consequently, vigilance is warranted given the potential pathways of escape that enable hypermutator persistence, limiting our ability to effectively manage pathogens. Recognising hypermutation as a convergent and widespread adaptive trait rather than an exception is essential to confronting challenges of rapid resistance emergence. Integrating molecular insights, evolutionary context, and monitoring efforts, will be key to anticipating and mitigating the emergence of hypermutators.

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Declaration of interests

No interests are declared.

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Outstanding questions

How can hypermutator emergence be detected in pathogens? Sequencing efforts will help assess loss of mutation control machinery genes (e.g., linked to DNA repair, RNA interference, or TE control), whilst assessing mutation spectra in a population can also be used to detect hypermutation manifesting by shifts to high TE activity compared to a reference population.

How can hypermutator emergence be detected in newly sequenced species? A phylogenetic approach may aid to assess whether closely related lineages show characteristics associated with hypermutator emergence, but the power of this approach depends on broader characterization of hypermutator dynamics across the fungal tree of life.

At what rate do hypermutators occur outside of pathogen lifestyles? Assessing hypermutator appearance systematically across the diversity of the fungal kingdom can be used to interrogate lifestyle or environmental niche associations. This effort will move research towards searching for causal factors beyond the attention paid to human and crop disease agents.

Once hypermutators are identified, how can their risk level be assessed? Evaluating the potential threat of a hypermutator lineage will likely depend on the mechanisms underlying its emergence, its capacity for long-term persistence, and its ability to evade existing management strategies. Understanding these factors will be essential for prioritising surveillance and mitigation efforts.

How can drug treatments be adapted to hypermutator pathogens? Hypermutation will facilitate the gain of multidrug resistance, and hence drug combinations will need to be even more carefully considered. Alternative control strategies such as microbial competition may be options in agricultural settings, whilst early detection and treatment will be key to forestalling the accumulation of resistance gains.

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