EVOLUTION

Evolutionary transitions from camouflage to aposematism: Hidden signals play a pivotal role

Karl Loeffler-Henry[1]†, Changku Kang[2,3]*†, Thomas N. Sherratt[1]

The initial evolution of warning signals in unprofitable prey, termed aposematism, is often seen as a paradox because any new conspicuous mutant would be easier to detect than its cryptic conspecifics and not readily recognized by naïve predators as defended. One possibility is that permanent aposematism first evolved through species using hidden warning signals, which are only exposed to would-be predators on encounter. Here, we present a large-scale analysis of evolutionary transitions in amphibian antipredation coloration and demonstrate that the evolutionary transition from camouflage to aposematism is rarely direct but tends to involve an intermediary stage, namely cryptic species that facultatively reveal conspicuous coloration. Accounting for this intermediate step can resolve the paradox and thereby advance our understanding of the evolution of aposematism.

Selection to avoid being killed by predators has contributed to the diversity of animal color patterns (*1*). These color adaptations include crypsis and disruptive coloration to avoid being detected and/or recognized (*2*), conspicuous warning signals in defended species to indicate unprofitability to would-be predators [an association known as aposematism (*3*)], and mimetic signals that share or exploit the aposematic signals of other organisms (*4*). Although our understanding of the genetics, development, perception, and function of these color signals has substantially progressed in recent years (*5*), we still know little about the macroevolutionary patterns of color pattern evolution. Specifically, large-scale macroevolutionary studies on animal color defense are surprisingly scarce, and even these have tended to consider simple binary classifications of species color, notably whether they are cryptic or conspicuous (*6*–*8*). Although this binary classification captures some well-known antipredator strategies of animals (crypsis and potential aposematism or mimicry), this may not be enough to explain how diverse color defense strategies have evolved in nature. For example, some species are cryptic at rest but have bright color signals hidden on body surfaces that are only exposed when signaling to conspecifics, fleeing, or as part of a defensive posture (*9*–*14*). These flexible signaling strategies could represent intermediate stages and therefore might play a pivotal role in evolutionary processes generating different antipredator defenses (*15*).

Amphibians are an excellent group to explore the evolutionary transitions among different antipredator strategies. Their phylogeny is available in nearly all extant taxa (*16*), and their color patterns have been strongly shaped by predators through natural selection (*17*). A previous study examining macroevolutionary patterns of amphibian antipredator adaptations found that rates of speciation, extinction, and transition vary among species with different defensive traits (*6*). Although this large-scale study revealed important evolutionary pathways from camouflage to aposematism, the inference was based largely on a simple two-color state classification scheme (cryptic and conspicuous) of each species' dorsal coloration. However, many amphibian species show a more complex set of color patterns, such as having a cryptic dorsum yet conspicuous patches on normally hidden body parts (*18*–*21*). These hidden color signals are taxonomically widespread in the animal kingdom yet have seldom been considered in macroevolutionary studies (*13*, *14*).

The hidden color signals of amphibians tend to occur in one of two different forms: conspicuous color present on (i) the whole venter (lower surface), such as those in the genus *Bombina*, or (ii) part of the concealed body surface, such as ventral shanks or hindlimbs commonly found in the family Hylidae. These hidden signals are often exposed through behavioral displays [e.g., via an unken reflex toward approaching predators, or foot flagging for intraspecific signaling (*14*, *22*–*24*)]. In addition, amphibians may sometimes use flashing signals during escape: These colors would be visible only when the prey is mobile and may mislead a predator into assuming that the prey's flash color is its resting color and in so doing hinder subsequent search (*25*). Hidden conspicuous color signals may have evolved from typical aposematic signals to offset the costs of conspicuousness while stationary. Alternatively, they may serve as an intermediate state from camouflage to aposematism because this strategy can gain the advantages of both by only signaling when discovered (*15*, *26*, *27*).

Here, using discrete character evolution models, we investigate the role of hidden conspicuous color signals during the evolutionary transitions between camouflage and aposematism. Specifically, we examine the transitions between different antipredator strategies based on a five-category color classification scheme, accounting for crypsis, conspicuousness, two different types of hidden signals [PV; partially conspicuous venter: cryptic dorsum with conspicuous color present as small patches on normally hidden body parts and FV; fully conspicuous venter: cryptic dorsum with conspicuous colors that fully cover the venter (*28*)], and polymorphism (Poly; defined here as a species having both cryptic and conspicuous forms regardless of whether they are regional variants or coexist in the same population; see Fig. 1 for example species and materials and methods for details). We identified two classes of hidden signals because (i) the classes are distinguishable morphologically and (ii) their putative functions differ in that FV coloration is likely to solely function as an aposematic signal to attacking or approaching predators, whereas PV coloration may also serve as a flashing signal or territorial display (*14*). We analyzed two datasets: color (1106 species with color information available) and color+chem (315 species with both color and chemical defense information available).

Results and discussion

Main evolutionary transitions in the color model

We tested nine different models: three models that allowed transitions between almost all states (with two exceptions, see below) at equal or differing rates [All rates different (ARD), Symmetric (SYM), Equal rates (ER) models], and six models that restricted certain transitional pathways (Intermediate, FV intermediate, Stepwise, PV/FV secondary, FV cost-offset PV secondary, Cost-offset) (table S1 and fig. 1A). Transitions between the FV and Poly states and between the PV and Poly states were not allowed (see materials and methods for details). An intermediate model that did not allow the direct transition of species between the cryptic and conspicuous states was the best-supported (lowest Akaike information criterion) model (Fig. 1A and tables S1 and S2 for descriptions and full results), while the estimated transition rates were qualitatively equivalent among the three best-supported models [the Intermediate, ARD, and a modified Intermediate model in which the PV state cannot evolve directly to the conspicuous state but only through the FV state (FV intermediate); fig. S1]. The parameter estimates from fitting the Intermediate model revealed several major patterns of evolutionary pathways

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(Fig. 1B). First, although species in the cryptic state can evolve directly toward all other states with the exception of the conspicuous state (which is precluded in the best-supported model), the cryptic state is stable (i.e., the transition rates from all other states to the cryptic state are at least three times higher than the rates away from it) and the most likely basal ancestral state (support probability = 69%; Fig. 2). Second, the PV state is most strongly associated with the cryptic state: Species with the PV color mainly evolve from cryptic coloration and tend to transit back to it. However, although the transition rate is low, the PV state is also the most likely state leading to the FV state, which could facilitate the subsequent transition toward the conspicuous state. Third, species in the conspicuous state largely evolve from the FV or polymorphic state, and other routes are less likely. However, because species in the polymorphic state evolve almost exclusively from the conspicuous state, the major pathway to the initial evolution of conspicuous coloration is likely through the FV state. Fourth, the polymorphic state appears unstable and transits rapidly to the cryptic or conspicuous state.

Main evolutionary transitions in the color+chem model

In the color+chem model, the evolutionary pathways among the states are substantially more complex than the color-only models (Fig. 1C). As with the color model, the cryptic state was found to be the most likely basal ancestral color state in the subset of species considered in the color+chem model (support probability = 60%; Fig. 3). Although there was slightly stronger support for the hypothesis that the basal defensive state of frogs and salamanders involved chemical defense, the data were more equivocal (Fig. 3; 58% support probability for chemical defense versus 42% for no chemical defense). Chemical defense frequently coincides with conspicuous coloration: Indeed, the majority of species classified as either conspicuous, FV color, or polymorphic have chemical defense (more than 90% in all three groups; table S3). However, chemical defense also occurs in less-conspicuous species as well (65% in species with PV color, 51% in the cryptic species; table S3). This is not surprising, as chemical defense is known to provide survival advantages to both conspicuous and cryptic species through aposematic signaling and taste rejection, respectively (*29*–*31*). On average, the transition rates toward the acquisition of defense are higher than the transition rates away from this defensive state, but the only color state that is more likely to lose than acquire chemical defense is crypsis (Fig. 1C). This is consistent with previous findings that the acquisition of alkaloid sequestration is favored over losing it in poison frogs (*32*). One explanation for the loss of chemical defense in cryptic amphibians is that they experience less risk of detection by predators and therefore less selective pressure for the maintenance of postdetection defense (*33*). Considering that chemical defense may pose a cost to its bearer (*34*, *35*), this may make deterrent toxins less favored in some cryptic lineages. Another nonmutually exclusive reason for the differential in acquisition or loss of chemical defenses in the different types of signaler may be that species with any form of conspicuous signals can potentially benefit from "go slow" signaling that makes predators cautiously sample the prey to determine the presence of chemical defense (*36*). By contrast, predators may sample cryptic prey without caution, possibly making selection for chemical defense to reduce injury after capture weaker in cryptic species.

After accounting for chemical defense, the evolutionary transition from crypsis to aposematism (chemically defended conspicuous state) is not simple but is instead composed

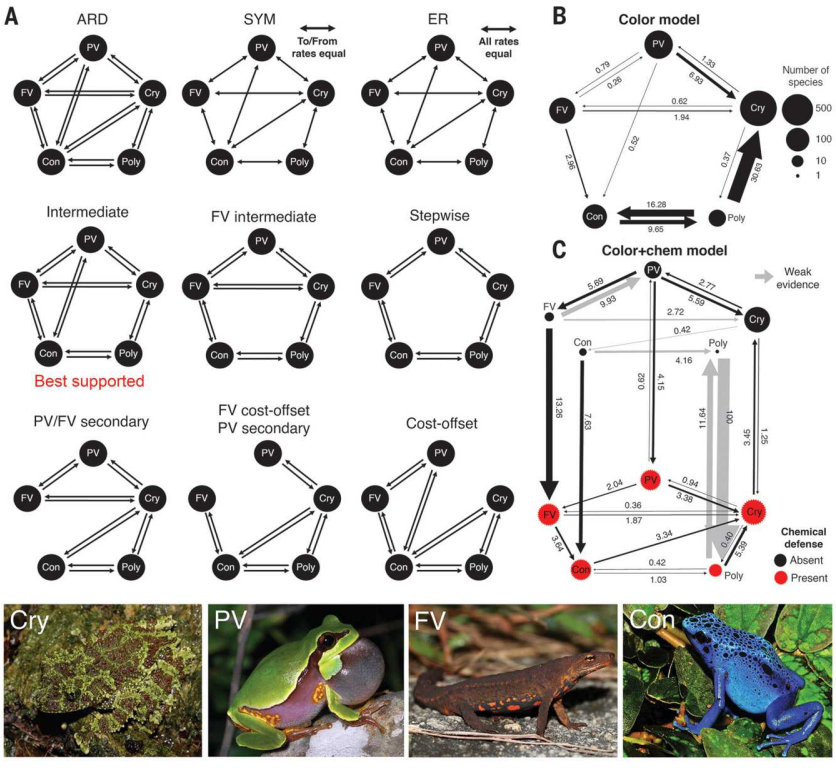

Fig. 1. Diagrammatical representations of the models that we compared and the parameter estimates of the best-supported models. No arrows in the fitted transition models (**A**) indicate that the transition rates were constrained to be zero. See table S1 for the descriptions of the models. Panels (**B**) and (**C**) show the estimated transition rates among different states from the best-supported models using color dataset [(B); species with color information available; *N* = 1106] and color+chem dataset [(C); species with both color and chemical defense information available; *N* = 315 species]. Species that were classified as uncertain color (*N* = 47) were excluded. Arrow thickness reflects the estimated transition rates, which are also given by the values listed next to each arrow (those transitions that are possible but not depicted have estimated rates less than 0.00001). The radius of circles is proportional to the log-transformed number of species in each state. Gray arrows indicate that the strength of evidence is weak because the estimated transition rates were inconsistent between different functions ("fitMk" and "corHMM") (*28*), most likely because they were estimated from very few changes in the tree or they were estimated from only one extant species (undefended Poly). Cry: cryptic; PV (partially conspicuous venter): cryptic dorsum with conspicuous color present as small patches on normally hidden body parts; FV (fully conspicuous venter): cryptic dorsum with conspicuous colors fully covered on the venter; Con: conspicuous; Poly: a species showing multiple distinct morphs that include both cryptic and conspicuous forms. ARD: All rates different model; SYM: symmetric model; ER: equal rates model (see table S1 for details). The photos show sample species from each color category that we used. From left to right, *Theloderma corticale* (Dan Rosenberg), *Hyla andersonii* (Troy Hibbitts), *Paramesotriton hongkongensis* (Dan Rosenberg), *Dendrobates tinctorius* (Michael Gäbler).

of multiple pathways that involve intermediate states (Fig. 1C; see fig. S2 for the visual comparisons of how accounting for chemical defenses modifies the transition rates among the color states from the color model results). Whereas the transition from the undefended conspicuous state to the defended conspicuous state is high, the transition rates toward the undefended conspicuous state from any other states are either zero or very low. This is reasonable because any mutations that make an individual conspicuous without having chemical defense would be detrimental and as such would be selected against unless other defensive strategies, such as Batesian mimicry, are involved (*37*, *38*). Thus, a more stable route to aposematism is via the chemically defended FV state. There are multiple pathways to evolve the chemically defended FV state, but the main routes appear through the defended PV state or undefended FV state, which also mainly evolve from the undefended PV state. These observations collectively suggest that at least the FV state (but often involving the PV state) is likely to be involved in transitions from camouflage to aposematism. Once aposematism has evolved, it goes back to neither the PV nor FV state but instead either evolves back to the cryptic state directly or becomes cryptic/conspicuous-mixed polymorphic.

Hidden signals and their implications for amphibian color evolution

We hypothesized that the PV signals potentially function as a secondary defense (flash displays or postdetection warning) or are used for intraspecific signaling in normally cryptic species (*14*, *25*); thus, the PV signals are mainly associated with cryptic coloration. Our results of both color and color+chem models support this view in that the PV state is primarily associated with the cryptic state with a stronger tendency to go back to the cryptic state (Fig. 1, B and C). Also, the PV state is the most likely state that can lead to the evolution of the FV state, which is a major precursory state toward conspicuous coloration. The presence of PV signals implies that a species has managed to express bright (e.g., carotenoid or pteridine pigments based) colors potentially acquired through diet and/or manufactured de novo and presumably evolved for antipredator or conspecific signaling (*14*, *39*, *40*). This could further facilitate the expression of conspicuous colors on other parts of the body, resulting in the evolution of the FV states.

In both color and color+chem models, species having FV color evolve from either the cryptic or PV color, but not from the conspicuous color (Fig. 1, B and C). Thus, the conjecture that the FV color evolves from the conspicuous color to offset the cost of being continuously conspicuous is not supported (*15*). Instead, the FV state is the most likely intermediate stage that is required for the transition from crypsis to aposematism. About 91% of species with FV color have chemical defense (table S3), suggesting that their ventral warning coloration is likely an honest signal of their defense, rather than a bluff. Theoretically, the FV state can have a selective advantage over the conspicuous state when a species has no chemical defense: Having a conspicuous dorsum without defense should be highly detrimental to individuals, leaving less opportunity to evolve chemical defense subsequently. However, because the FV strategy does not involve the loss of crypsis, this strategy may be able to persist until the evolution of chemical defense follows. Indeed, the results of the color+chem model suggest that the nonchemically defended conspicuous state rarely evolves from any other nonchemically defended states, but the FV state could evolve from the PV state in the absence of chemical defense (Fig. 1C).

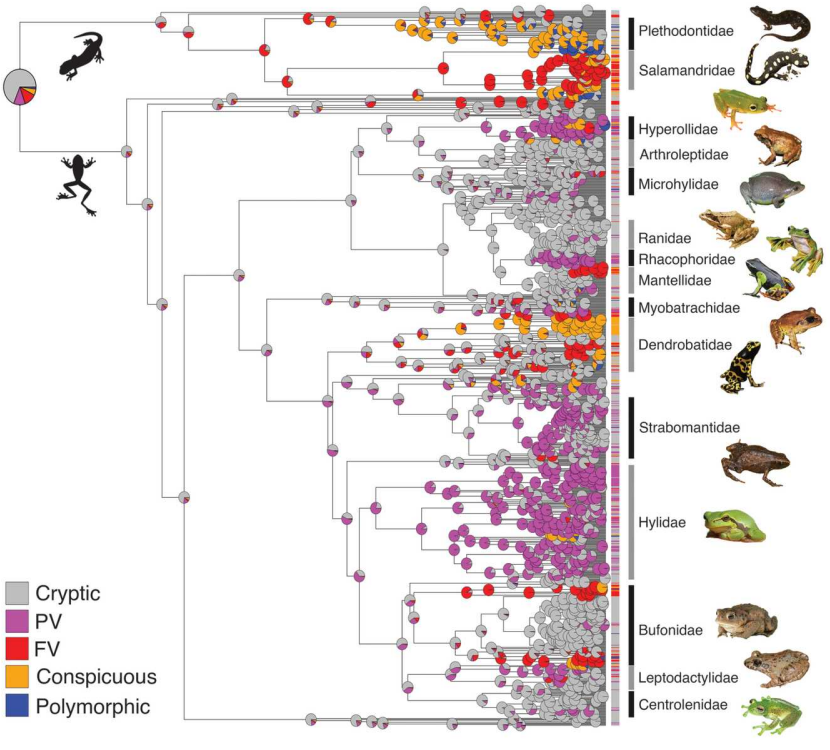

Fig. 2. Ancestral state estimation of each color state (*N* = 1106 species) in frogs and salamanders. Pie charts at each node show the probabilities of ancestral states. The ancestral state of frogs and salamanders is likely to be cryptic coloration. The hidden color signals (PV and FV) are widespread and have evolved multiple times in different lineages. PV: cryptic dorsum with conspicuous color present as small patches on normally hidden body parts; FV: cryptic dorsum with conspicuous colors fully covered on the venter. See table S11 for photo credits.

Transitional patterns of the cryptic/conspicuous-mixed polymorphic state

Only 2.3% of all species in our dataset were considered to exhibit both cryptic and conspicuous morphs, i.e., were classified as polymorphic. Despite its relative rarity, our results suggest that this cryptic/conspicuous-mixed polymorphism has evolved multiple times in different lineages independently (Fig. 2). Most of these polymorphic species have chemical defense (10 out of 11 species) and have evolved mainly from the conspicuous states (Fig. 1, B and C). Both the color and color+chem models suggest that once the cryptic/conspicuous-mixed polymorphic state is reached, it tends to rapidly evolve toward either the cryptic or conspicuous state with a stronger tendency toward the cryptic state (Fig. 1, B and C). The low number of species in the mixed-polymorphic state may reflect this evolutionary instability.

Conclusion

Our study highlights the importance of hidden color signals for the evolutionary processes that generate diverse antipredator coloration in amphibians. Our results suggest that (i) species with hidden color signals, especially those with conspicuous colors that cover the whole venter, represent a key stage in the evolution of aposematic species from cryptic

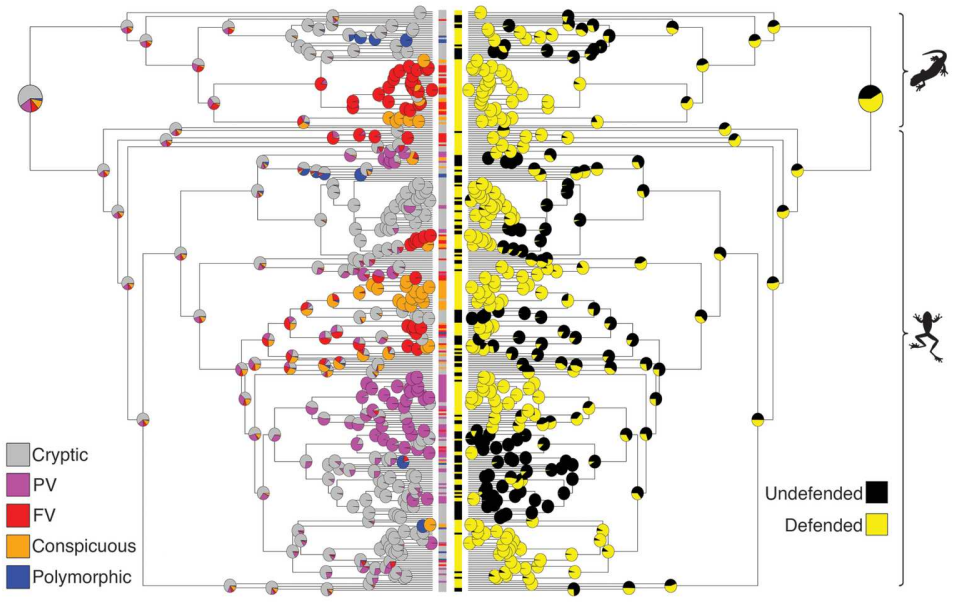

Fig. 3. Ancestral state estimation of each combination of color and chemical defense in frogs and salamanders (*N* = 315 species). Pie charts at each node show the probabilities of ancestral states. The ancestral state of frogs and salamanders is likely to be cryptic coloration, but the evidence of whether the basal state was chemically defended or not was equivocal. Most states have evolved multiple times in different lineages. Transitions from cryptic to aposematic (chemically defended conspicuous) states have usually occurred via intermediate states.

species; (ii) cryptic/conspicuous-mixed polymorphism plays a pivotal role in transitions from aposematism back to crypsis; and (iii) the transition rates toward acquisition of chemical defense are higher than those to its loss in most color states, with the exception of the cryptic state. A number of complementary models confirmed the robustness of these conclusions (see materials and methods for details and figs. S3 to S9 and tables S4 to S10 for the results).

Biologists have long wondered how rare conspicuous mutants of a cryptic defended species can spread in a population when they have a higher predation risk before predators learn to avoid them (*41*, *42*). The fact that aposematic species appear to seldom derive directly from cryptic species confirms the long-standing intuition that the evolution of aposematism from crypsis is challenging. Here we show macroevolutionary evidence for an important yet unrecognized solution in which the problem is effectively side-stepped: Rather than evolve from cryptic species, aposematic mutants appear to derive largely from species with hidden signals. One intuitive way that this might work is if would-be predators that are already exposed to a species with hidden warning signals continue to treat permanently aposematic mutants of this species with caution. The initial evolution of hidden color signals might be facilitated by predatory pressure that promotes the evolution of secondary defenses such as flash or warning displays (*15*, *24*, *25*), or via sexual selection that results in the expression of conspicuous signals in body parts that are only visible during behavioral displays (*14*). These complex transitional routes from crypsis to aposematism would not be revealed if traditional dichotomous classifications of animal antipredator coloration (either cryptic or conspicuous) are applied (see fig. S10 for the results when the binary classification was used). Thus, macroevolutionary studies on animal coloration should take into account these underappreciated hidden signals, which are both common and widespread across the animal kingdom (*13*, *43*, *44*), to advance our understanding of the evolution of antipredator defenses. Indeed, many animal taxa such as snakes, fishes, and a variety of arthropods (see fig. S12 for example groups) include species that are cryptic, are aposematic, and have hidden conspicuous signals. We therefore encourage follow-up studies in other taxa to evaluate the generality of the stepping-stone hypothesis as a route to aposematism.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S12
Tables S1 to S12
References (*46*–*70*)

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NEURODEGENERATION

Mechanism of *STMN2* cryptic splice-polyadenylation and its correction for TDP-43 proteinopathies

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Loss of nuclear TDP-43 is a hallmark of neurodegeneration in TDP-43 proteinopathies, including amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). TDP-43 mislocalization results in cryptic splicing and polyadenylation of pre–messenger RNAs (pre-mRNAs) encoding stathmin-2 (also known as SCG10), a protein that is required for axonal regeneration. We found that TDP-43 binding to a GU-rich region sterically blocked recognition of the cryptic 3′ splice site in *STMN2* pre-mRNA. Targeting dCasRx or antisense oligonucleotides (ASOs) suppressed cryptic splicing, which restored axonal regeneration and stathmin-2–dependent lysosome trafficking in TDP-43–deficient human motor neurons. In mice that were gene-edited to contain human *STMN2* cryptic splice-polyadenylation sequences, ASO injection into cerebral spinal fluid successfully corrected *Stmn2* pre-mRNA misprocessing and restored stathmin-2 expression levels independently of TDP-43 binding.

In the human nervous system, the ability to maintain proper RNA metabolism is thought to decline during aging (*1*). Disruption of RNA metabolism is a common feature of many human neurodegenerative disorders, including the fatal paralytic disease amyotrophic lateral sclerosis (ALS) and the two most common dementias: Alzheimer's disease (AD) and frontotemporal dementia (FTD) (*1*–*5*). Although the exact gene expression profiles and affected neuronal populations vary among the dementias and other neurodegenerative disorders, there is growing evidence supporting common molecular mechanisms (*6*, *7*).

TDP-43 proteinopathy describes a set of neurological disorders that are characterized by mislocalization of the RNA-binding protein TDP-43 [encoded by the *TARDBP* (TAR DNA-binding protein) gene]. TDP-43 relocates from its typically nuclear location and accumulates in the cytoplasm of affected neurons in the form of aggregates. TDP-43 mislocalization and aggregation is found in 97% of ALS patients, about half of FTD patients, and 30 to 50% of AD patients (*8*–*11*). TDP-43 pathology has also been reported in a growing number of brain disorders (*2*), including Huntington's disease (*12*), Perry syndrome (*13*), and chronic traumatic encephalopathy (CTE) (*14*). Recently, a subset of aged Alzheimer's patients has been reclassified as limbic-predominant age-related TDP-43 encephalopathy (LATE disease) (*15*) because their post mortem brain samples show aberrant TDP-43 instead of the expected amyloid beta (Aβ). Although cytoplasmic accumulation of TDP-43 has been reported in ALS and FTD, nuclear clearance of TDP-43 is often observed even without apparent aggregation(s) (*16*, *17*). Loss of nuclear TDP-43 affects expression and processing of multiple mRNA targets across different cell types and tissues (*18*–*24*).

TDP-43 closely regulates maturation of the pre-mRNA encoding stathmin-2 (also known as SCG10, encoded by the *STMN2* gene) (*25*, *26*). Stathmin-2 is a neuronally enriched protein that plays a crucial role in axonal outgrowth during development (*27*) and regeneration (*25*, *26*, *28*). Developmental deletion of mouse *Stmn2* causes motor deficits with denervation of neuromuscular junctions (*29*, *30*). Among the four members of the stathmin gene family, stathmin-2 has the highest expression in mouse and human motor neurons (*25*); *STMN2* is among the top 20 most enriched mRNAs in the ALS-vulnerable motor neurons of the anterior gray column of the spinal cord (*25*, *31*). In post mortem FTD and ALS patient brain and spinal cord tissues, loss of nuclear TDP-43 results in the use of cryptic splice (*25*, *26*, *32*) and polyadenylation sites (*25*) within the first intron of the *STMN2* pre-mRNA (*25*, *26*). This leads to inclusion of a cryptic exon 2a and the production of an mRNA encoding only a truncated open reading frame (17 codons) (*25*) and suppression of the full-length 179–amino acid stathmin-2 protein (*25*, *26*, *32*).

Stathmin-2 is a tubulin-binding protein that is thought to affect microtubule dynamic instability (*33*, *34*), although its mechanism of action in axons and axonal growth cones is unclear (*35*–*38*). Lowering stathmin-2 in induced pluripotent stem cell (iPSC)–derived human motor neurons inhibits the regenerative capacity of injured axons (*25*, *26*). Diminished regeneration capacity of injured motor neurons when TDP-43 function is reduced can be rescued by restoring stathmin-2 expression levels (*25*), which suggests that restoration of stathmin-2 expression in TDP-43 proteinopathies may provide a therapeutic strategy.

In this work, we determined the regulatory elements through which TDP-43 regulates *STMN2* pre-mRNA processing and identified steric binding antisense oligonucleotides (ASOs)

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