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An Animal Model to Discern Torsin Function: Suppression of Protein Aggregation in *C. elegans*

Guy A. Caldwell, Songsong Cao, Christopher C. Gelwix, Elaina G. Sexton, and Kim A. Caldwell

The University of Alabama, Department of Biological Sciences, Tuscaloosa, Alabama

Dystonia is a movement disorder characterized by sustained muscle contractions that frequently cause twisting or repetitive movements or abnormal postures (1). Oppenheim's dystonia, the most severe early-onset form of this disorder, is transmitted in an autosomal-dominant manner with reduced penetrance (30% to 40%) and has been linked to specific deletions in the human *DYT1 (TOR1A)* gene that encodes a protein of unknown function termed torsinA (2–4). These breakthroughs have established the groundwork for subsequent investigation of the cellular mechanisms responsible for dystonia using the comparative genomics of model organisms.

The nematode, *Caenorhabditis elegans*, is a genetically tractable organism for which a wealth of molecular markers and defined cell lineage is available. This microscopic worm grows from embryo to adult in 3 days and has a transparent anatomy, facilitating examination of developmental and cellular change over time in an intact animal. Whereas the human brain contains over 100 billion neurons, *C. elegans* has exactly 302. Moreover, the complete neuronal connectivity of this animal has been determined and diagrammed by electron microscopy (5). Despite its simplicity, this nematode shares many of the hallmarks of human neuronal function including ion channels, neurotransmitters (dopamine, serotonin, acetylcholine) and axon pathfinding cues, among other molecules (6). The *C. elegans* genome contains many predicted proteins that exhibit a high degree of similarity with gene products implicated in human diseases and this model system has been exploited to gain insights into sensory and neurologic mechanisms underlying a variety of disorders (7,8).

The genomic sequence of *C. elegans* predicts three gene products that share significant amino acid similarity to torsins (9). The identification of a nematode torsin-like protein was first reported in the original paper on positional cloning of the human *DYT1* gene (2). This protein has been shown to be encoded by the *ooc-5* gene (9). The *C. elegans* OOC-5 protein has not been shown to function neuronally but is involved in the establishment of embryonic asymmetry and oogenesis in *C. elegans* (9,10). Two additional torsin-related genes have been since identified within the completed genome sequence of *C. elegans*, and we named these *tor-1* and *tor-2*.

Although strides toward discerning sites of torsin expression and localization have been

made, a precise cellular activity for members of this protein family has not been determined (11–13). Torsins share amino acid sequence similarity with the diverse AAA+ family of adenosine triphosphatase (ATPase) proteins that includes heat shock proteins, proteosome subunits, proteases, and dynein (14). A recurrent theme in neurologic disease is the evidence of aberrant protein folding (15–17). Overexpression of molecular chaperones has been shown to suppress formation of protein inclusions in cells and decrease neurotoxicity (18,19). In this regard, mutations in neuroprotective molecules that function in a capacity to monitor protein folding may also be responsible for the symptomatic features of dystonia. We hypothesize that torsins function in a capacity similar to molecular chaperones in facilitating the proper cellular management of misfolded proteins. To experimentally test this hypothesis, we utilized an *in vivo* assay for examining states of intracellular protein aggregation in living nematodes (20). These experiments serve to simultaneously define a cellular activity for torsin proteins while establishing *C. elegans* as a model system for the analysis of torsin function.

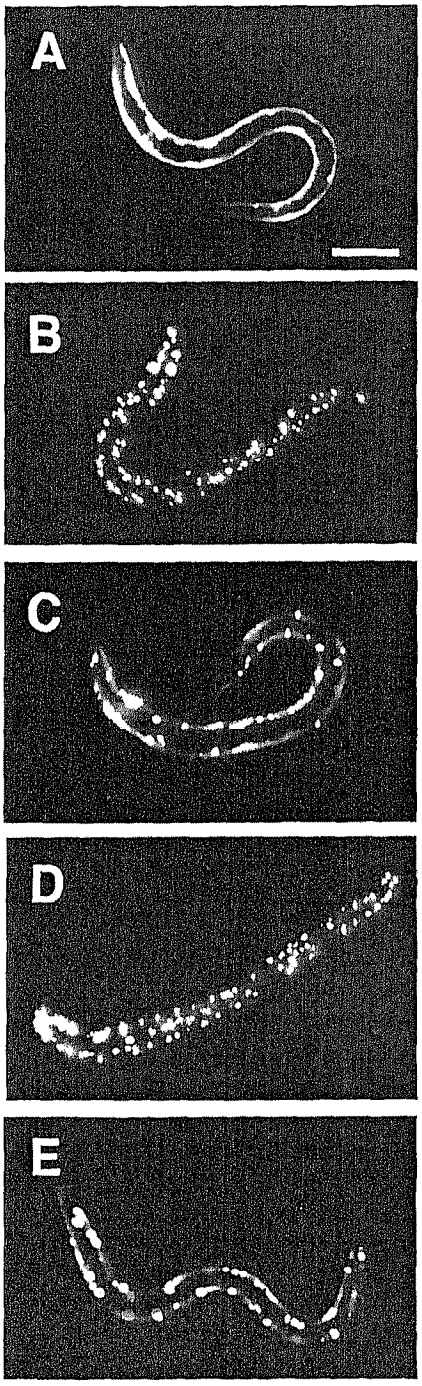
RESULTS AND DISCUSSION

Suppression of Protein Aggregation by Torsins

While all human and nematode torsin-like gene products contain adenosine triphosphate (ATP)-binding and other sequence motifs common to AAA^+ proteins (9,14), a functional role for torsins in mediating protein folding has not been previously demonstrated. We isolated a complementary DNA (cDNA) corresponding to the worm *tor-2* gene product, the nematode torsin homologue that shares highest global sequence identity to human torsinA. The 1.3-kilobase (kb) *tor-2* cDNA encodes a protein of 412 amino acids that is detected in *C. elegans* extracts by TOR-2–specific peptide antisera (not shown). This cDNA corresponds precisely to *C. elegans* open reading frame Y37A1B.13 in GenBank; all predicted splice junctions and amino acids have been verified by DNA sequencing.

Differential states of protein solubility were generated *in vivo* by ectopic expression of gene fusions between variable lengths of CAG codons (encoding polyglutamine repeats) fused to the green fluorescent protein (GFP) in *C. elegans* using the *unc-54* body wall muscle promoter (20). Direct comparison between transgenic animals expressing either a fusion of 19 glutamines to GFP (Q19::GFP) or 82 glutamines fused to GFP (Q82::GFP) demonstrated that fluorescent protein aggregation could be induced by polyglutamine expansion and readily visualized in these transparent animals. The evenly distributed and diffuse GFP localization associated with Q19::GFP (Fig. 11-1A) was dramatically transformed into a pattern of distinct fluorescent cellular aggregates in Q82::GFP animals (Fig. 11-1B). In contrast, coexpression of wild-type TOR-2 protein dramatically reduced GFP-containing aggregates in animals containing Q82::GFP (Fig. 11-1C), even partially restoring diffuse body wall muscle GFP fluorescence. Torsin suppression of polyglutamine-induced protein aggregation persisted over time as these animals aged post-adulthood. Coexpression of TOR-2 with Q19::GFP did not alter the normal cytoplasmic distribution of GFP in these animals (not shown).

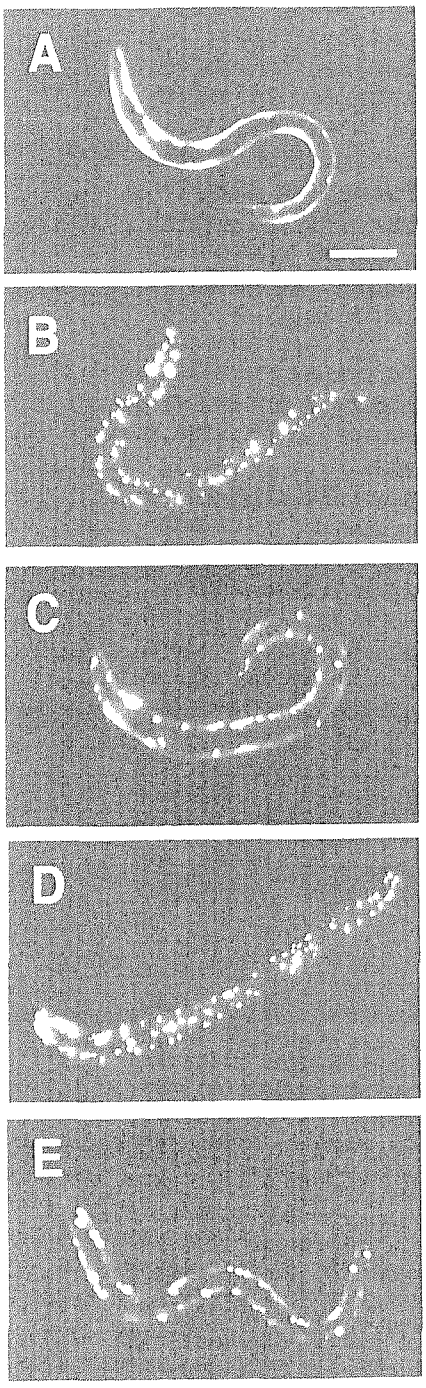
Specific amino acid deletions in torsinA have been linked to Oppenheim's dystonia, implicating the carboxy-terminus of this protein as being essential for torsin function (2–4). Although the glutamic acid residue deleted in patients with early-onset torsion dystonia (ΔE302/303) is not strictly conserved across species, it is found within a stretch of overall high-sequence identity at the C-terminus (14). We used site-directed mutagenesis to examine the consequences of altering this portion of the worm TOR-2 protein. To putatively mimic the effects of aberrant torsin activity associated with dystonia, we generated a mutant *tor-2* gene that encoded a protein lacking a serine at position 368 of TOR-2 [TOR-2(Δ368)]. Coexpression of the TOR-2(Δ368)



mutation with Q82::GFP protein was incapable of ameliorating protein aggregation and did not restore the diffuse body-wall fluorescence to these animals (Fig. 11-1D). Interestingly, coexpression of TOR-2(Δ368) with Q19::GFP did not alter the general cytoplasmic expression of GFP from what is found in Q19::GFP animals alone (*data not shown*). Immunoblotting of *C. elegans* extracts indicated that the various lines of transgenic animals, including TOR-2(Δ368), all contained equivalent levels of TOR-2 protein (not shown). Therefore, these data are indicative of a loss of torsin activity that is associated with the TOR-2(Δ368) mutation rather than a change in protein stability.

To test if the sequence homology shared between torsin proteins extended to functional homology, we obtained a human *DYT1* cDNA encoding torsinA and placed it under control of the *C. elegans unc-54* promoter. As was previously determined for nematode TOR-2, the human gene product was capable of suppressing protein aggregate formation and restoring diffuse body wall fluorescence in transgenic animals when coexpressed with Q82::GFP protein (Fig. 11-1E). This indicates that conservation of torsin protein activity is maintained across species boundaries and among members of this medically significant protein family.

FIG. 11-1. *C. elegans* lines containing transgenes expressing polyglutamine-GFP fusions and torsin proteins under the control of the *unc-54* body wall muscle specific promoter. **A:** Transgenic nematodes expressing a Q19::GFP protein fusion exhibit a normal diffuse green fluorescent protein (GFP) expression pattern, in comparison to animals expressing a Q82::GFP fusion in either the absence of TOR-2 **(B)** in the presence of TOR-2 **(C)**, or the presence of TOR-2(Δ368) mutant protein **(D)**. Suppression of polyglutamine-induced protein aggregation is evident in the presence of wild-type TOR-2 protein **(C)**, whereas the TOR-2(Δ368) mutant has lost this activity **(D)**. Likewise, coexpression of human torsinA in the presence of Q82::GFP also results in a clear reduction in protein aggregation **(E)**. The anterior of all animals is on the left of each panel. Scale bar represents 50 μM.



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Overexpression of human torsinA ΔE302/303 has been shown to alter the subcellular distribution of this protein and result in the formation of membranous whorls in culture (21,22). In contrast, O'Farrell et al. (23) have shown in transfected human cell cultures that expression of an altered torsinA protein, lacking the 18 base pair (bp) (ΔF323-Y328) clinically linked to early-onset dystonia, does not change the localization of torsinA or result in the formation of membranous inclusions. Our data indicate that deletion of one specific C-terminal residue in the nematode TOR-2 protein (serine 368) results in a loss of this protein's ability to suppress protein aggregation without an apparent change in either TOR-2 localization or cellular morphology (see below). Further experimentation will be needed to clarify the significance, or lack thereof, of aberrant membrane inclusions on torsin activity and dystonia. Systematic structure-function analyses of torsin activity using this *C. elegans* assay system will allow for changes in cytoplasmic architecture to be directly coordinated with protein function.

TOR-2 Localizes to the Sites of Protein Aggregation

We examined the localization of wild-type and mutant TOR-2 in animals containing Q82::GFP aggregates by immunofluorescence microscopy using a TOR-2–specific affinity-purified antibody. Figure 11-2(A,B) depicts TOR-2 localization to the sites of polyglutamine-induced protein aggregation in a tight ring-like pattern completely surrounding the fluorescent protein aggregates. Immunolocalization of TOR-2 in animals overexpressing TOR-2(Δ368) did not lead to a discernible change in the cellular distribution of this protein (Fig. 11-2C,D). Thus, although this mutant torsin appeared to be localized to protein aggregates, it was incapable of functionally altering their solubility.

The distinctive localization of *C. elegans* TOR-2 to sites of protein aggregation is overtly reminiscent of cellular bodies called aggresomes (24). These inclusions, which also contain ubiquitinated proteins, proteosome subunits, and chaperones, are formed in response to excess misfolded proteins. Likewise, human torsinA has been shown to be in abundance at sites of α-synuclein aggregation termed Lewy bodies, a clinical characteristic in the brains of patients with Parkinson's disease (25). Interestingly, we have also shown that the sites of TOR-2/polyglutamine association are also sites of concentrated ubiquitin staining (Fig. 11-2E,F). Coupled with a report that heat shock proteins can suppress neurotoxicity associated with misfolding of α-synuclein in *Drosophila* (19), these combined data represent an exciting insight into the relationship between Parkinson's disease and dystonia at a molecular level.

Toxic misfolded protein intermediates are being heavily scrutinized as potential causative agents in a variety of neurologic disorders (15–17). The failure of intracellular quality control mechanisms can result in aberrant protein aggregation and associated disease states. Studies in yeast have identified numerous proteins involved in the cellular response to misfolded proteins; however, metazoan counterparts to these mechanisms still remain poorly defined (26). Our combined data implicating torsins in protein folding and co-localizing with ubiquitin at sites of protein aggregation are suggestive of a possible role for this exclusively metazoan family of proteins in the ATP-dependent retrotranslocation of misfolded proteins at the endoplasmic reticulum. Deficits in this mechanism may have significant consequences on neuronal activity, and dystonia may be a clinical manifestation of subtle changes in the ability of torsins to properly manage malformed protein structures in response to cellular stress.

Continued investigation of torsin function in *C. elegans* and other animal models will provide further insights into the molecular mechanism underlying dystonia. For example, the effect of structural alterations to torsin proteins may be evaluated for their functional consequences on chaperone-like activity and

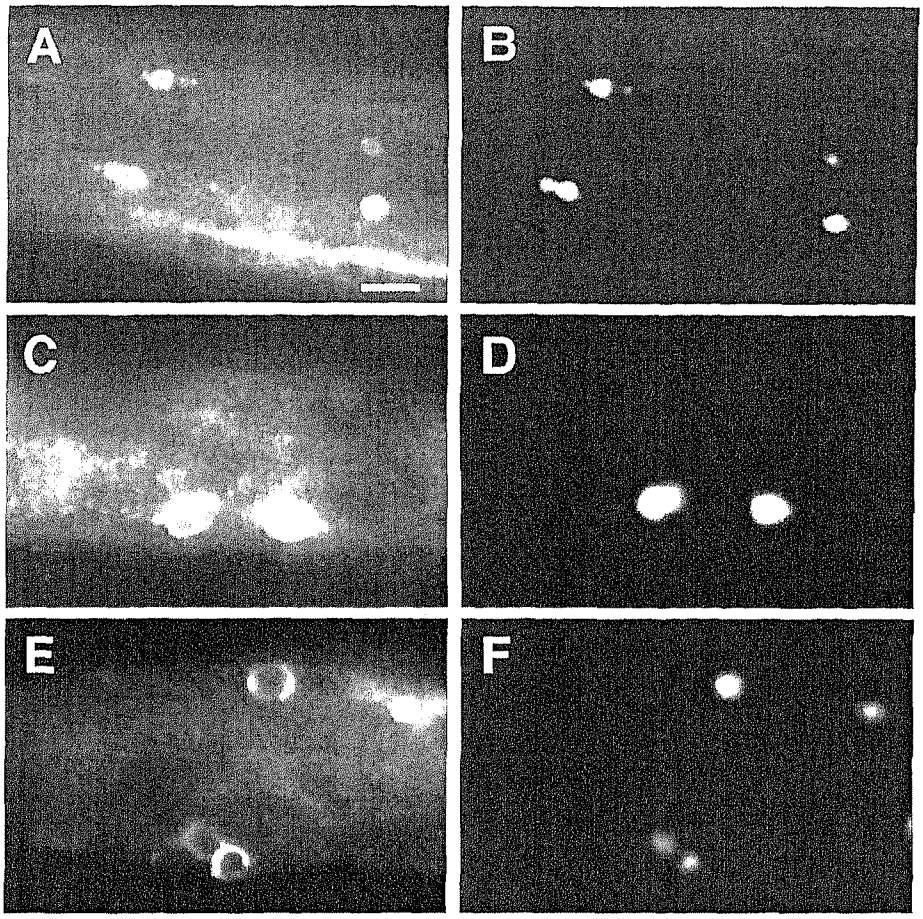
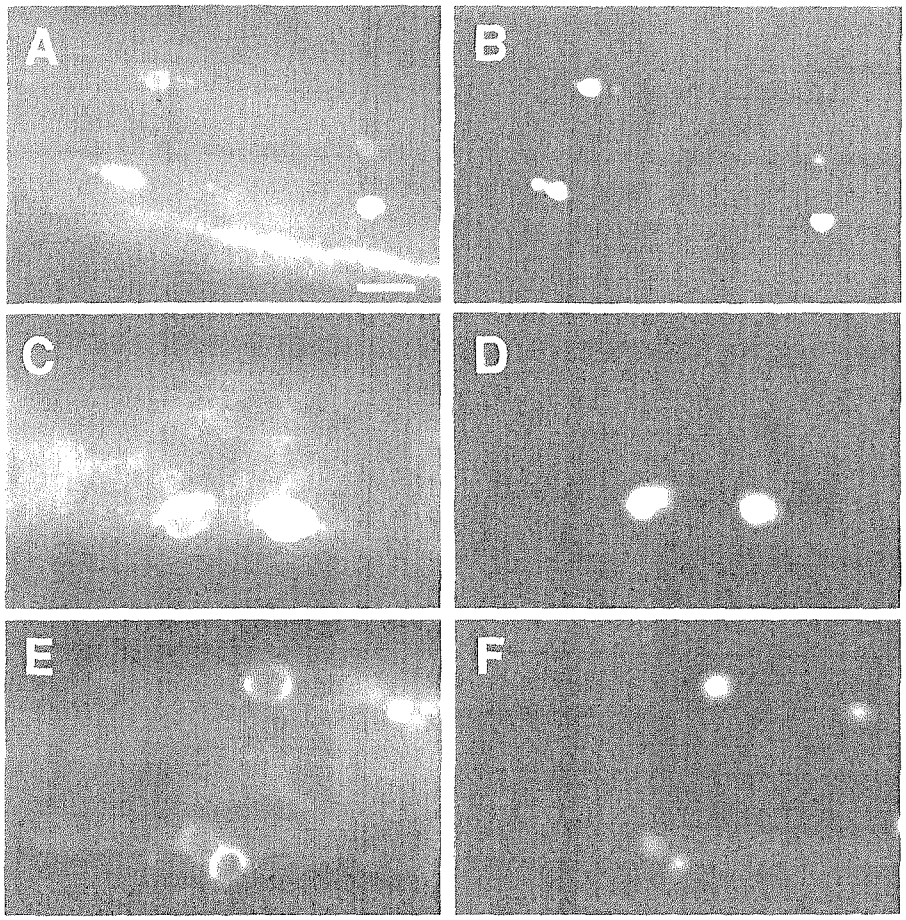


FIG. 11-2. Localization of TOR-2 or ubiquitin in transgenic *C. elegans* lines by immunofluorescence microscopy. Using an affinity purified TOR-2 specific antibody, both wild-type TOR-2 **(A)** or mutant TOR-2(Δ368) protein **(C)** are found to be highly localized to sites of Q82::GFP protein aggregation in body wall muscles **(B,D)**. Transgenic animals stained with an affinity purified antiubiquitin antibody exhibit a very similar pattern of localization **(E)** around fluorescent Q82::GFP protein aggregates **(F)**. The scale bar represents 5 μM.

then subsequently transferred to neuronal cells (i.e., dopaminergic) for examination of putative dominant negative phenotypes. As *C. elegans* is amenable to both traditional genetic and large-scale genomic screening methods such as RNAi (double-stranded RNA-mediated interference), the strengths of this system may be further exploited to facilitate functional characterization of genes influencing torsin activity. Screens for putative suppressors and enhancers of torsin activity using isogenic lines of mutagenized animals containing torsin and polyglutamine::GFP transgenes will yield effectors of torsin function (Fig. 11-3). Such studies may concurrently interface with ongoing human genetic mapping efforts to more rapidly discern genetic loci corresponding to other dystonias.



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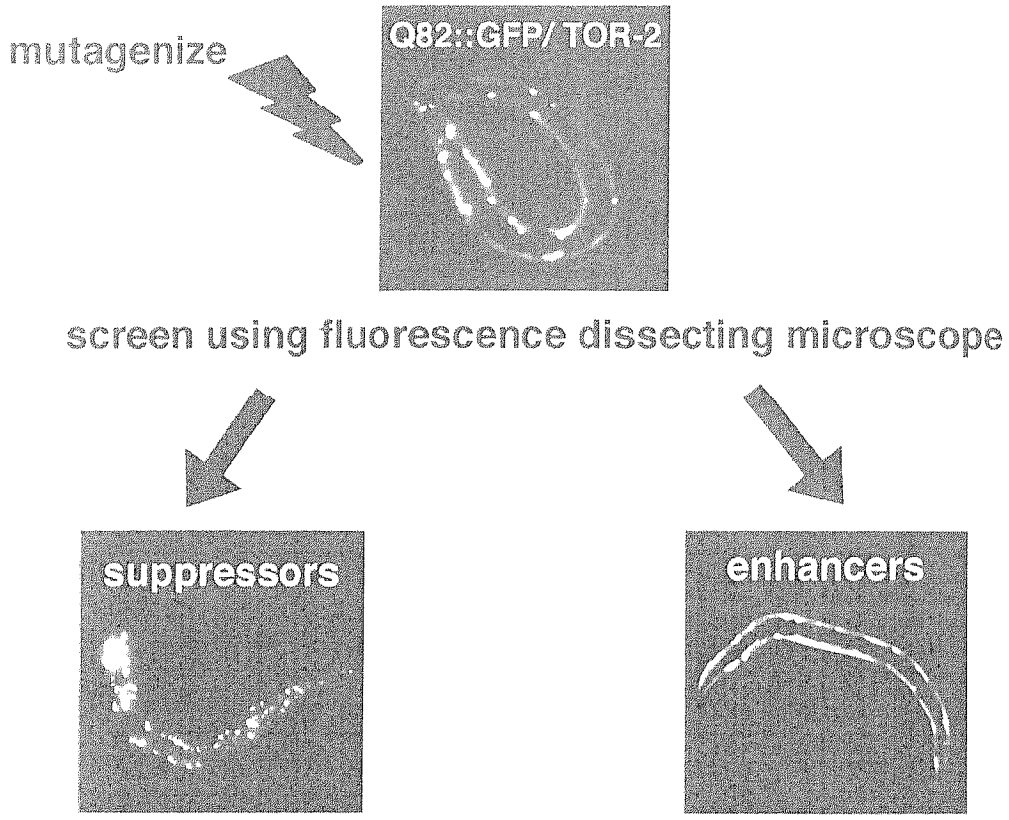

FIG. 11-3. Use of *C. elegans* to identify putative effectors of torsin activity by genetic screening. An isogenic line of transgenic *C. elegans* expressing a torsin protein that exhibits partial suppression of protein aggregate formation may be mutagenized. Progeny from these hermaphrodites can be rapidly screened under a fluorescence dissecting microscope for candidate genes that enhance or suppress protein aggregation when mutated.

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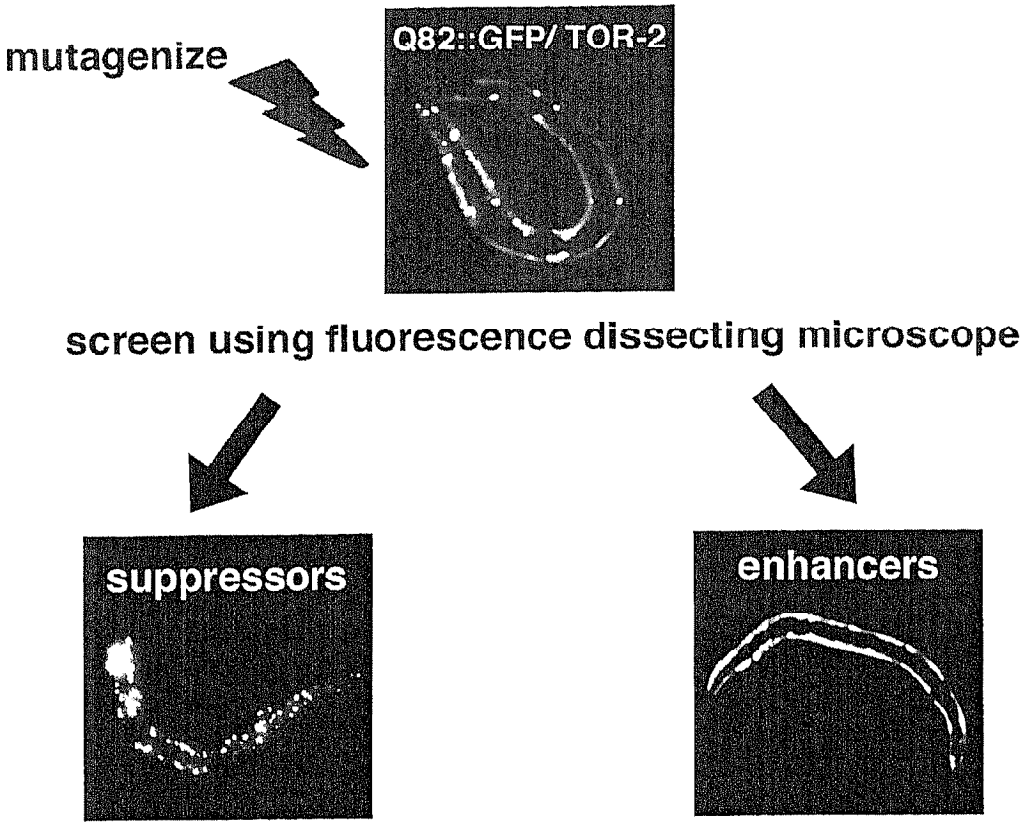

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