The Role of *sptf*-3 in *C. elegans* Growth and Development

Honors Research Thesis

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Abstract

The Sp family of transcription factors has important functions during development in animals. In the nematode *Caenorhabditis elegans* there are three Sp-related transcription factors (Sptf) which perform similarly important roles in development. The purpose of this project is to learn more about SPTF-3 in *C. elegans*. Worms with a mutation in *sptf-3* exhibit embryonic lethality, reduced fertility, and a bivulva phenotype that may result from abnormal vulval development. The first goal of this project was to identify the role of SPTF-3 in the differentiation of egg-laying system cells. Transgenic expression of genes in wild-type worms was compared to expression of the same genes in *sptf-3* mutants. In both uterine and vulval tissues, improper expression of genes occurs in *sptf-3* mutant background. These alterations suggest that inappropriate differentiation of cells is occurring in worms with the mutant protein. The second goal of this project is to identify the source of the morphological defects of *sptf-3* mutants. Tissue-specific RNAi was used to reduce the expression of SPTF-3 under three conditions: all cell types, germline cells only, and somatic cells only. A proportion of embryonic lethality resulted in the conditions where protein expression was reduced in all cell types and in germline cells only. This suggests that knockdown of *sptf-3* in the germline causes observed somatic abnormalities in affected worms. The third goal of this project is to gain insight into the in-vivo function of the SPTF-3 protein. This is being done by introducing the mutant form of the protein into wild-type worms, to determine whether the mutant protein can compete with the wild-type protein in-vivo. The results of this study could provide more information about SPTF proteins in *C. elegans* as well as Sp transcription factors as a whole.

Introduction

Appropriate activity and function of regulatory transcription factors are important for healthy development in animals. The specificity protein (Sp) family plays a range of roles in regulation of gene expression during animal development. Members of this family of transcription factors are characterized by a conserved pattern of three zinc finger DNA binding domains at the C-terminus of each protein (reviewed by Zhao and Meng, 2005). These domains bind to regulatory sequences on target genes, often the commonly conserved GC-box and GT-box promoter elements, in order to modulate transcription.

Within this nine-member group, some proteins have other structural regions located on their N-terminus for activation or repression of gene expression (reviewed by Zhao and Meng, 2005). These structural differences give them further distinguishing characteristics and they also
account for tendencies of certain Sp1-related proteins to preferentially bind to some regulatory sequences over others. For example, Sp1 is normally an activator of gene expression, while others, such as Sp3 and Sp4, have the ability to function through either activating or inhibitory elements depending on the specific target gene (reviewed by Bouwman and Philipsen, 2002). Despite these distinct differences between the proteins, there is evidence to suggest overlapping function among some members of the family, especially between Sp1 and Sp3 (reviewed by Zhao and Meng, 2005).

A related family of proteins is the Krüppel-like transcription factors, whose name comes from their similarity to the *Drosophila* protein Krüppel. These proteins possess C-terminal zinc finger DNA binding domains that are similar to those present in Sp1-related proteins. The difference between the Sp1-related factors and the Krüppel-like factors lies in the presence of a specific Sp binding region found only on the N-terminus of seven of the nine the Sp1-related proteins (reviewed by Black et al., 2001).

Sp1-related proteins are widely expressed in animals, with some expressing ubiquitously (Sp1, Sp3) and others expressing only in specific tissues or at discrete developmental stages (Sp4) (reviewed by Zhao and Meng, 2005 and Bouwman et al., 2002). One important role for these proteins, specifically in vertebrates, is activity during embryonic development (reviewed by Zhao and Meng, 2005). For example, the loss of expression of an Sp protein in vertebrates can cause a range of developmental abnormalities. Specifically, knockout mice exhibit defects that may include embryonic lethality, early death, slowed growth, or abnormal reproduction (reviewed by Safe and Abdelrahim, 2005).

In humans, it is particularly important to learn more about the function of these transcription factors because of the protein family’s emerging role in tumor growth. For
example, levels of Sp1 protein have been shown to be elevated in pancreatic, breast, and thyroid cancers, with studies suggesting that, in these tissues, concurrently upregulated genes that are playing a role in tumor growth are under the control of Sp1. Less is known about the role of other Sp family members in cancer progression, but Sp3 and Sp4 may also be involved in development of pancreatic tumors specifically (reviewed by Safe and Abdelrahim, 2005).

In the nematode *Caenorhabditis elegans*, there are three Sp1-related transcription factors, denoted *sptf*. The purpose of this project is to investigate the role and activity of one of these three sp-related transcription factors. Worms with a point mutation in the gene *sptf-3*, which disrupts the DNA-binding region of the protein, exhibit a varied phenotype composed of many defects which make them distinct from their wild-type counterparts. These defects include embryonic lethality, reduced fertility, a reduced lifespan, and abnormal egg-laying system phenotypes (protruding vulva and bivulva) that are characteristic of abnormal vulval development. Studying the function of this protein in a model system such as *C. elegans* could produce information about how this important family of transcription factors might be functioning in other animals and even humans. This functional information could provide valuable insight into their activity in healthy tissues as well as tumors.

**Materials and Methods**

**Lifespan Assay**

In order to compare the lifespan of *sptf-3* mutant worms with the lifespan of wild-type worms, N2 (wild-type) and *sptf-3* mutant hermaphrodites were placed individually on *E. coli* OP50 plates during the mid-L4 larval stage of development. These were then scored daily for survival throughout their entire life span. During the first 4-5 days, they were moved daily to
new plates to separate them from offspring and prevent crowding on the plate. After their fertile stage ended and they ceased laying eggs, the worms’ survival was assayed daily but they were not moved to fresh plates.

One feature of the variable sptf-3 phenotype that affects many but not all mutant worms is inability to successfully lay eggs. This results in the eventual death of the parents when their offspring hatch within them. To account for differences between the cause of death of the N2 worms (normally old age) and sptf-3 worms, all worms were scored for number of days of survival as well as ultimate cause of death. This information provided two categories in which to place the subset of sptf-3 mutant worms: those that died of old age and those that died prematurely as a result of general egg-laying system defects. 19 N2 worms and 39 sptf-3 worms were included in this analysis.

**Embryonic Lethality Assay**

Mid-L4 worms were placed on individual plates and allowed to lay eggs overnight. After 24 hours, the parent worms were removed from the plates and their eggs were counted. Two days after this initial count, all healthy eggs would be expected to have hatched, so the number of hatched offspring was counted to determine the proportion of surviving worms. 3 N2 worms, laying a total of 151 eggs, and 12 sptf-3 worms, laying a total of 88 eggs, were scored.

**Role of sptf-3 on gene expression in egg-laying system tissues**

Because of the egg-laying system defects that occur in sptf-3 mutant worms, cell markers for both uterine and vulval tissues were studied in the background of the sptf-3 mutation to determine whether the presence of the mutant protein could interfere with normal cell marker
expression patterns. In the uterine tissue of wild-type worms, the *ida-1* green fluorescent protein (*ida-1::GFP*) transgene normally expresses in the uv1 cells of the worm (Zahn et al., 2001). In the first set of experiments, N2 and *sptf-3* worms both carrying the *ida-1::gfp* transgene were scored for uv1 expression during the mid-L4 stage (N2 mid-L4 n=27, *sptf-3* mid-L4 n=45). In order to observe expression at a second, later developmental time point, worms in both genotype categories were also scored for uv1 expression during the adult stage. Adult worms were scored after being picked during the mid-L4 stage one day earlier (N2 adults n=29, *sptf-3* adults n=30). GFP expression patterns were recorded according to the total number of uv1 cells expressing on one side of the worm only, such that the three possible expression categories consisted of zero, one, or two expressing uv1 cells.

The vulB and vulC cell marker *ceh-2* was used as a tool to observe potential effects of *sptf-3* on gene expression in vulval cells. During the mid-L4 stage, expression is expected in each of the two vulB1 and each of the two vulB2 cells, with a total of two cells expressing anterior and two expressing posterior to the vulva. Towards the end of and in the stages subsequent to mid-L4, expression is replaced by both left and right vulC cells, for a total of two cells expressing anterior and two expressing posterior to the vulva (Inoue et al., 2005) (see fig. 1 for specific locations of vulval cells during stages). Worms with the *sptf-3* mutation frequently exhibit abnormalities in the shape and number of cells present in the vulva, making it difficult to confirm the identity of expressing cells. In addition, bivulva animals sometimes exhibit expression in the ectopically-induced vulva. Therefore, instead of scoring expression patterns based upon the identity of the cells expressing the *ceh-2::GFP* transgene, the overall number of cells expressing anterior and posterior to the vulva was instead recorded. The stages scored were the same as those used during observation of uv1 cell expression; mid-L4 worms as well as adult
worms were assayed. N2 wild-type worms mid-L4 n=29, sptf-3 mutant worms mid-L4 n=42; N2 worms at adult stage n=28, sptf-3 worms at adult stage n=44.

**RNAi to reduce sptf-3 in wild-type worms**

RNA interference was used to determine the effect of a reduction in sptf-3 on the survival of wild-type worms. sptf-3 cDNA was cloned into the RNAi feeding vector pPD129.36 and transformed into *E. coli* HT115 RNAi feeding cells. Three different genotypes of parent worms were used in an effort to further pinpoint the tissue location of sptf-3 function: N2 worms are wild-type and are able to undergo RNAi in all tissue types. Worms with a mutation in *rrf-1* are deficient in RNAi processing in the somatic cells, and because of this undergo RNAi only in the germline (Sijen et al., 2001). Conversely, worms with a mutation in *ppw-1* are deficient in RNAi processing in the germline, and thus RNAi only occurs in the somatic cells (Tijsterman et al., 2002). Mid-L4 worms were placed on individual plates of HT115 containing the transformed plasmid one day after the bacteria was spotted onto the plates. After feeding overnight the parents were removed and the eggs on the plate were counted. Hatched offspring were subsequently counted three days after initial egg count in order to determine the proportion of surviving worms, and also to observe whether additional morphological defects occurred as a result of RNA interference treatment. Results include 3 separate trials of RNAi with a total of 6 RNAi-treated parent worms in each genotype condition. N2 wild-type worms, total eggs: empty vector condition n=306, sptf-3 cDNA vector n=333; *rrf-1* worms, total eggs: empty vector n=356, sptf-3 cDNA vector n=289; *ppw-1* worms, total eggs: empty vector n=336, sptf-3 cDNA vector n=316.
Heat-shock induction of wild-type SPTF-3 protein

To determine whether transgenic introduction of SPTF-3 has the ability to rescue the phenotypes that are present in sptf-3 mutant worms, we introduced the wild-type protein using full-length sptf-3 cDNA in the pPD49.83 heat-shock vector. In order to induce protein expression in a population of worms at a uniform developmental stage, adult worms were bleached and their eggs were allowed to hatch overnight at room temperature. The hatched larval (L1) offspring were then plated onto E. coli OP50 plates and heat-shocked in a water bath for one hour at 34°C. After three days worms were scored during the adult stage for frequency of visible egg-laying system defects, namely the protruding vulva and/or bivulva phenotypes commonly observed in sptf-3 mutants. GFP cDNA in the same pPD49.83 vector was used as a control. A second control condition consisted of plates that were prepared in the same way as the experimental plates but not subjected to the heat-shock treatment. Total number of worms scored: control in sptf-3; unc-119 no h.s. n=104, h.s. n=109; wild-type sptf-3 in sptf-3; unc-119 mutants no h.s. n=58, h.s. n=59.

Results

Worms with a mutation in sptf-3 exhibit reduced lifespan and reduced embryonic survival

A lifespan assay was carried out to investigate the effect of the sptf-3 mutant phenotype on the overall health of homozygous affected worms. The variability of the phenotypic characteristics of sptf-3 mutant worms makes them difficult to group into discrete categories. One subset of defects associated with the sptf-3 mutation is frequent inability of the worms to lay eggs, seemingly due to developmental abnormalities of the egg-laying system. Because of this, offspring commonly hatch inside the worm, causing the premature death of the parent. Since N2
worms to not suffer from this trait, their death normally results from old age. As a whole, the overall lifespan of sptf-3 worms is reduced compared to that of wild-type worms [see fig. 2, curves N2 and sptf-3(all)]. Because some mutant worms are egg-laying defective, their premature death gives a skewed view of how long the worms would otherwise live [see fig 2, curve sptf-3(b)]. Excluding this group, however, the remaining sptf-3 worms that die of old age still live fewer days than wild-type worms [see fig 2, curve sptf-3(a)].

In addition to the egg-laying defects and the reduced lifespan observed in sptf-3 mutant worms, these worms also fail to thrive during the embryo stage. In an effort to learn more about the effect of the mutation in sptf-3 on embryonic health in the worms, the eggs of sptf-3 worms were assayed to investigate the potential occurrence of embryonic lethality. Specifically, just over 80% of sptf-3 eggs hatch, compared to a 100% hatch rate in wild-type worms (see fig. 3).

**Mutant sptf-3 results in disruption of normal gene expression in uterine and vulval tissues**

In order to determine the influence of sptf-3 on expression of genes involved in egg-laying system tissues, we studied the expression patterns of two known cell markers in sptf-3 mutant worms to observe whether expected expression patterns were altered in uterine or vulval tissue.

First, expression of uv1 cell marker *ida-1* was compared between wild-type and mutant worms. In wild-type worms, expression is expected in all four of the uv1 cells during the adult stage, as well as several neurons throughout the worm (Zahn et al., 2001). When comparing expression between the two genotypes, expression patterns are similar and somewhat unstable during the mid-L4 stage, when presumably the uv1 cells have not achieved complete differentiation (see Fig. 4). However, during the young adult stage, when expression in wild-type
worms occurs in uv1 cells with great regularity, expression occurs unpredictably in spf-3 mutant worms. In fact, expression patterns in this set of worms resembles patterns observed in the L4 stage of wild-type animals rather than the stable and predictable expression found in their adult wild-type counterparts.

Expression of ceh-2, a vulval cell marker, was used to visualize the effect of mutant spf-3 on gene expression in vulval cells. The expected expression for this marker is in vulB1 and vulB2 cells in mid-L4 worms and in vulC cells in the late L4 and adult stage (Inoue et al., 2002). While the expression pattern exhibits some variability in wild-type worms, expression was even more widespread and unpredictable in spf-3 mutant worms, suggesting expression maintenance abnormalities that could be similar to those observed in the ida-1 uv1 cell expression patterns (see Fig. 5).

**Tissue-specific RNAi suggests a maternal role for spf-3**

Tissue-specific RNAi knockdown of spf-3 under three different genetic conditions gives information about one potential method of function for spf-3. Embryonic lethality, one feature of the spf-3 mutation, results in offspring of treated N2 parents as well as offspring of treated rrf-1 parents, or those which have undergone RNAi only in the germline. The embryonic lethality that results in offspring of worms with germline knockdown of spf-3 suggests a maternal function for the protein.

**Introduction of wild-type spf-3 in mutant background is sufficient to rescue visible egg-laying system deficiencies**
In order to determine whether introduction of wild-type SPTF-3 protein could rescue the morphological defects observed in \textit{sptf-3} mutants, wild-type \textit{sptf-3} cDNA was introduced as a transgene under the control of heat-shock promoter pPD49.83. Frequency of normal egg-laying system development was scored based on absence of protruding vulva and bivulva phenotypes and compared to non-heat-shocked control worms. Heat-shocking mutant larval worms at the L1 stage to produce SPTF-3 results in adult worms that develop normally nearly 70\% of the time, indicating that introduction of wild-type SPTF-3 in is sufficient to significantly rescue vulval morphological defects in adult mutant worms compared to worms that have not been treated with the heat-shock.

**Discussion**

**Observations of defects associated with \textit{sptf-3} mutants**

The variable nature of the phenotype of \textit{sptf-3} mutants makes them difficult to characterize and generalize. While the mutant worms clearly fail to thrive at the level of wild-type worms, the reasons for this can differ, even from worm to worm. The choice for multiple categories in determination of lifespan is one consequence of this variability. While the \textit{sptf-3} worms in general exhibit a reduced lifespan compared to N2 worms, those most severely affected by the mutation live by far the shortest length of time. These groupings are further complicated by the fact that some worms are sterile or have a reduced brood size, which could mask functional egg-laying deficiencies that would otherwise present themselves in fertile worms with the mutation. However, it is not clear whether this observed reduction in lifespan even in those worms that are able to functionally lay eggs is a direct result of the presence of the
mutant protein or an indirect culmination of the multiple phenotypic defects combining to halt normal development.

The embryonic lethality associated with sptf-3 mutants suggests that a fully functional protein is important for embryonic development. This is consistent with the importance of Sp1-related proteins in vertebrate embryonic development (discussed above and reviewed by Zhao and Meng, 2005). Interestingly, the presence of mutant SPTF-3 seems to be the most detrimental to the worm either early in development (eggs and young larvae) or late in development (adults). If they survive to reach the L3 and L4 larval stages, sptf-3 mutant worms appear relatively healthy until they reach the adult stage and begin to lay eggs, at which point their abnormal development begins to be more obvious and damaging to their ability to thrive.

Along with the larger-scale morphological defects in sptf-3 worms are deficiencies at the tissue and cell level. The experiments with uterine and vulval cell markers suggest that the presence of the mutant SPTF-3 protein has a detrimental effect on general expression of cell markers in these tissues, as observed in the unpredictable and even ectopic GFP expression found in sptf-3 worms. Considering the widespread nature of the regulatory sequences to which Sp1-related proteins tend to bind, it is not surprising that an error in the DNA binding domain of sptf-3 has an unfavorable effect on maintaining general gene expression, even for genes that are not necessarily known direct targets of sptf-3.

**Implications regarding introduction of wild-type SPTF-3 via heat-shock transgene**

Introduction of the SPTF-3 protein via heat-shock at the L1 stage, an early larval stage, had the greatest ability to rescue the developmental abnormalities observed in the adult stage. This is notable due to the wide range of developmental time points that occur between the stage
of heat-shock and the stage of observed rescue, and is also interesting considering that the effect of the endogenous mutant protein, as previously mentioned, is most detrimental during the very early and very late developmental stages.

It is not clear whether this rescue effect indicates that the induced protein lasts from the larval stage through the adult stage to bring about rescue, or whether the presence of the protein early in larval development is sufficient to carry out early developmental regulatory decisions that allow for normal development many hours later during the adult stage. Perhaps there is a time point at which it is too late for induced wild-type SPTF-3 to be able to repair abnormal regulatory decisions resulting from the mutant protein.

**Conclusions**

Overall, *sptf-3* worms exhibit a wide range of defects that indicate the importance of the protein for regulatory decisions. The embryonic lethality defect can be replicated through RNAi against *sptf-3* in wild-type and *rrf-1* worms, with results suggesting that the protein itself seems to function in a maternal manner to aid in embryonic development. Also, bivulva and protruding vulva phenotypes can be rescued through introduction of the wild-type protein via a heat-shock promoter transgene as long as the rescue protein is introduced early enough in development.

Abnormal gene expression in uterine and vulval tissues suggests that the presence of the mutant protein has a detrimental effect on transcriptional regulation, even in genes that are not necessarily known downstream targets of SPTF-3. This results in improper expression of known cell markers in these two tissue types. Evidence in other animals suggests that some Sp1-related factors may be able to compensate for a loss in function due to overlapping function of other family members (discussed above), but it is not yet known whether other Sp1-related factors in
C. elegans have this same ability. The results presented provide information about this important family of transcription factors in C. elegans that functionally supports their relationship to Sp1-related transcription factors in other animals with regards to regulation of gene expression and role in embryonic development, and also introduces a potential maternal role for the function of this transcription factor specifically.

References


Tables and Figures

**Fig. 1** – Location of vulval cells in *C. elegans* (Inoue et al., 2002). Note the locations of vulB1, vulB2, and vulC cells, which express the *ceh-2::*GFP reporter transgene.

**Fig. 2** – Comparison of the life spans of N2 and *sptf-3* worms. N2 wild-type worms n=19, *sptf-3* mutant worms n=39; N2 category includes all wild-type worms. *sptf-3*(all) category includes all 39 *sptf-3* worms plotted together on same curve, regardless of cause of death; *sptf-3*(b) denotes worms from *sptf-3* group that died as a direct result of abnormal egg-laying system development, not old age as in N2 worms (n=33); *sptf-3*(a) denotes worms from *sptf-3* group that died of old age, not as a consequence of abnormal morphology (n=6).

**Fig. 3** – Assay of *sptf-3* egg survival. Bar graphs show proportion of embryonic survival for wild-type and *sptf-3* mutant genotypes. N2 wild-type worms n=151 eggs from a total of 3 N2 worms. *sptf-3* n=88 eggs from a total of 12 *sptf-3* worms. Eggs were counted ~24 hours after mid-L4 worms were individually placed on plates. Survival was assayed by counting number of hatched offspring two days after eggs were counted and comparing to total number of eggs laid. Two-sample Z test, p<0.0002.
**Fig. 4a** – Analysis of GFP expression of uv1 cell marker *ida-1* in wild-type and *sptf-3* worms. N2 worms mid-L4 n=27, *sptf-3* mutant worms mid-L4 n=45; N2 adult worms n=29, *sptf-3* adult worms n=30. Developmental stage for adult worms was unified by observing expression in worms one day after the mid-L4 stage.

**Fig. 4b** – Potential uv1 expression patterns using *ida-1::GFP* transgene. The typical *ida-1* expression pattern consists of expression in several neurons as well as all four uv1 cells in the adult stage. Other patterns observed in mutant worms include expression in only one of the two uv1 cells or expression in neither uv1 cell. Expression was scored in the uv1 cells on one side of the worm only; since there are two uv1 cells on each side of the worm, proportions are based on a total of two cells only. T-test, p<0.0001.

**Fig. 5a** – Analysis of GFP expression of vulval cell marker *ceh-2* in wild-type and *sptf-3* worms. N2 wild-type worms mid-L4 n=29, *sptf-3* mutant worms mid-L4 n=42; N2 worms at young adult stage n=28, *sptf-3* worms at young adult stage n=44. As in uterine cell expression analysis, developmental stage for adult worms was unified by observing worms one day after the mid-L4 stage. Expression was scored by counting the number of cells expressing anterior and posterior to the vulva and plotting them accordingly, rather than identifying each cell individually.

**Fig. 5b** – Variable expression patterns of *ceh-2* in *sptf-3* in L4 as well as adult stages includes abnormal expression both anterior to and posterior to the vulva.
**Fig. 6** – Offspring survival of parents treated with RNAi. Data from 3 trials of RNAi with a total of 6 affected parent worms in each condition. Parent worms were placed on individual plates of feeding RNAi bacteria during L4 stage; after one day they were removed and eggs were counted. Surviving offspring were counted three days after initial egg count for survival data. N2 wild-type worms total eggs: empty vector condition n=306, *sptf-3* cDNA vector n=333. Two-sample Z test p<0.0001. *rrf-1* worms total eggs: empty vector n=356, *sptf-3* cDNA vector n=289. Two-sample Z test, p<0.0001. *ppw-1* worms total eggs: empty vector n=336, *sptf-3* cDNA vector n=316.

**Fig. 7** – Rescue of normal egg-laying system development after introduction of wild-type SPTF-3. Worms were heat-shocked during the first larval stage (L1) for one hour at 34°C. Total number of worms scored: ctl. in sptf-3;unc-119 no h.s. n=104, h.s. n=109; w.t. sptf-3 in sptf-3;unc-119 mutants no h.s. n=58, h.s. n=59.
Fig. 1 – Uterine and vulval cells in the egg-laying system of *C. elegans*
Fig. 2 – Decrease in lifespan of *sptf-3* worms compared to wild-type

**Lifespan of *sptf-3* mutant worms vs. wild-type**

![Graph showing lifespan comparison between N2, sptf-3 (all), sptf-3 (a), and sptf-3 (b) over 22 days.](image)

Fig. 3 – Deficiencies in embryonic survival in *sptf-3* mutant worms

**Survival of *sptf-3* mutant eggs**

![Bar graph showing survival comparison between N2 and sptf-3 genotypes.](image)
Fig. 4 – Uterine cell expression disruption in *sptf-3* background

**ida-1 expression in uv1 cells**

![Bar chart showing ida-1 expression in uv1 cells across different stages and genotypes](chart)

**Typical uv1 expression in young adult stage**

- Expression in both uv1 cells
- Expression in one uv1 cell
- No uv1 cell expression

**Atypical uv1 expression in young adult stage**
Fig. 5 – Vulval cell expression disruption in sptf-3 background

(a) Mid-L4 ceh-2 expression

(b) CEH-2 in sptf-3 worms – L4 stage

CEH-2 in sptf-3 worms – young adult stage
Fig. 6 – Embryonic survival after RNAi treatment

![Survival after RNAi treatment graph]

Fig. 7 – Rescue of egg-laying system development after introduction of wild-type SPTF-3 into sptf-3 mutants

![Introduction of wild-type SPTF-3 into sptf-3 mutants graph]
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