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Molecular genetic analysis of the model organism *Caenorhabditis elegans* was used to identify and study mechanisms of action of negative regulators of tyrosine kinase/RAS mediated signal transduction that are candidate tumor suppressors. A homolog of the proto oncogene *cbl,* SLI-1, inhibits Ras activation by the epidermal growth factor receptor homolog LET-23. A missense mutation in the RING finger region of SLI-1 has been identified and provides are reagent to analyze the function of this domain. New screens for additional negative regulators have identified at least four genes that are now being analyzed.

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Introduction

An important short-term goal of breast cancer research is to identify candidate genes for identifying pre cancerous cells and cancerous cells. Ideally, pre cancerous or cancerous cells could be characterized by a set of molecular markers. These markers would best be chosen from the set of genes altered during early stages of precancer formation. It is also crucial to understand the cellular regulatory pathways in which these genes act. *C. elegans* molecular genetics provides a facile model system with which to identify and to examine gene interactions *in vivo* [reviewed by Sternberg and Han, 1998]. For example, it has been clearly shown that the *C. elegans* ras protein LET-60 acts downstream of EGF-receptor homolog LET-23 [Han & Sternberg, 1990; Aroian et al. 1990], that the adaptor protein SEM-5 acts between LET-23 and ras [Clark et al., 1992; Katz, et al., 1996] and that the LIN-45 raf protein acts downstream of LET-60 ras [Han et al., 1993]. The ligand for LET-23 is likely LIN-3, an EGF-like growth factor [Hill and Sternberg, 1992; Katz et al., 1995]. This universal signaling pathway is the target of many mutations contributing to oncogenesis in humans. Overexpression or activation by mutation of LIN-3, LET-23, LET-60 results in excessive vulval differentiation; thus activation of the homologous genes leads to cancer in humans and vulval differentiation in nematodes. Therefore, negative regulators of vulval differentiation defined by loss-of-function mutations that lead to excessive signaling are analogous to tumor suppressor loci. During normal development, the gonadal anchor cell induces precisely 3 of 6 vulval precursor cells (VPCs) to divide and differentiate into vulval tissue. Even if 10% of experimental animals have excessive vulval tissue -- indicative of hyperactivation of the ras pathway -- we can detect it with confidence.

Using the powerful genetics of *C. elegans*, we and others have identified multiple pathways of negative regulation of LET-23 mediated signaling have been identified. We are carrying out genetic screens that can identify additional genes involved in negative regulation, either in known pathways or in new ones. Our genetic studies have identified several negative regulators of LET-23-mediated signaling in *C. elegans* [reviewed by Sternberg, 1994]: SLI-1 [Jongeward et al., 1995; Yoon et al., 1995], UNC-101 [Lee et al., 1994], GAP-1 [von Hajnal et al., 1998] ROK-1, and SLI(sy341) [see details below], and *lin-15* and related genes [Ferguson and Horvitz, 1989]. In general, these negative regulators are redundant, such that elimination of any one has no effect on the normal signal transduction. In the absence of two regulators, excessive vulval differentiation occurs. These genetic properties are similar to the synergistic action of oncogenic mutations, where several mutations are necessary for a phenotypic change. However, such synthetic mutations are difficult to study except in genetically facile organisms such as *C. elegans.*

If we can identify many loci possibly involved in the analogous processes in *C. elegans*, and identify human homologs, this will help human geneticists in several ways. First, this will provide candidate genes for the positional cloning of tumor suppressor loci defined by human cancer genetics. Second, this will provide molecular probes with which to examine tumorous tissue for alterations. The sooner we can identify the many potential tumor suppressor loci, the more effectively that

analysis of the role of tumor suppressor mutation in breast cancer initiation and progression can be assessed. Current technology easily allows parallel processing of samples, and is thus limited by the number of molecular probes. Lastly, we can link together tumor suppressor genes in functional pathways, much as we have been able to do for the LET-23-mediated pathway. If there are multiple pathways of tumor suppressor gene action, then we need to know how to intervene in each one.

One implication of multistep carcinogenesis is that synergism occurs between mutations. The genetics of the negative regulators that we have identified is analogous: mutation of, for example, SLI-1 or ROK-1 alone causes no defect, yet inactivation of both leads to increased signaling. The roles of such apparently redundant genes are difficult to study except in powerful genetic systems; *C. elegans* vulval differentiation provides such a system.

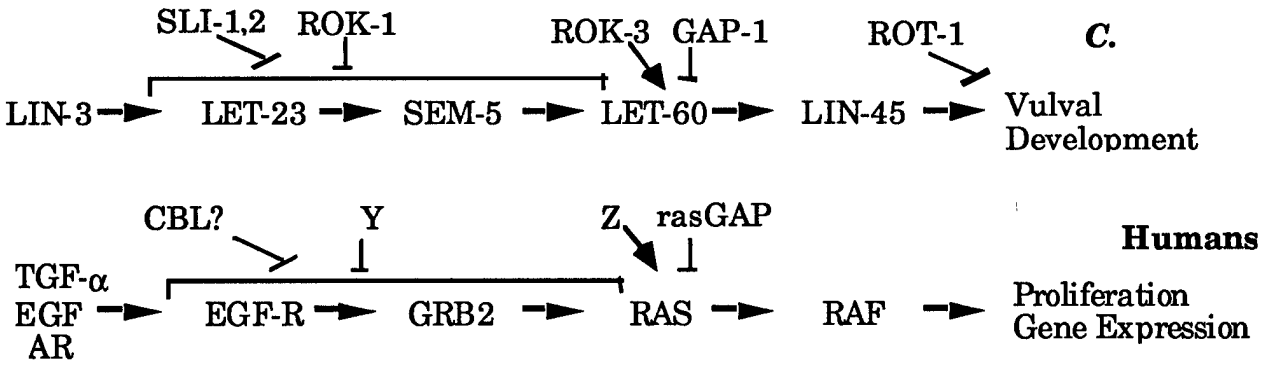

Figure 1. Pathway for the major EGF-R signaling pathway in humans and the analogous LET-23-mediated signaling pathway in *C. elegans*. Arrows indicated positive regulation; bars represent negative regulation. Other components are known. The universality of the side branches is yet to be shown rigorously.

The oncoprotein cbl has continued to be linked to signaling in many human cell types, and shown to be a negative regulator of signaling by the T cell receptor in mice [Murphy et al., 1998]. Our molecular genetic studies of SLI-I were the first indication of its negative role in signaling, and our studies are designed to help understand the function(s) of this family of proteins. The roles of cytoplasmic tyrosine kinases in signaling and its regulation is another major area of research. We have discovered that *rok-1* encodes such a tyrosine kinase, and we have begun to examine its mechanism of action as well. Lastly, regulation at the level of the nuclear targets of tyrosine kinase signaling is yet another area of uncertainty, and analysis of the new gene *sli-(sy341)* [called rot-1 in last year's report] may help clarify this area.

The goal of this project is to identify potential tumor suppressor genes and link them into functional pathways with each other, and with known proto-oncogenes and tumor suppressor genes. *C. elegans* vulval differentiation provides a facile model system with which to study EGF-receptor/*c-neu*-mediated signal transduction and its regulation. The *C. elegans sli-1, sli-2* and *rok-1* genes negatively regulate LET-23-ras signal transduction. We are using *C. elegans* molecular genetics to study and clone these genes in *C. elegans*, and will use molecular biology to clone their human homologs.

Body

The specific goals of the project are as follows.

1. **Analyze SLI-1 function** in *C. elegans* through molecular genetics.
2. **Molecularly clone *sli-2*.**
3. **Molecularly clone *rok-1*.**
4. **Identify and clone additional genes acting in concert with *sli-1*, *sli-2*, and *rok-1***
5. **Examine the functional interactions of *sli-1*, *sli-2*, *rok-1* in regulating other conserved signaling pathways.**
6. **Clone human *sli-2*, *rok-1*, and newly identified genes from human breast tissue libraries** to generate reagents with which to test the hypothesis that these are novel tumor suppressor loci.
7. **Test the functional homology of *c-cbl* and *sli-1*** by introducing the human cDNA into transgenic nematodes defective in *sli-1*.

1. Molecular genetics of *sli-1*, *C. elegans* homolog of cbl and cbl-b.

We identified the *sli-1* locus as a negative regulator of LET-23, the *C. elegans* homolog of EGF-R/c-neu/HER3/HER4, using extragenic suppressor analysis [Jongeward et al., 1995]. We cloned the *sli-1* locus by correlating genetic and physical maps and rescuing a *sli-1* mutant in transgenic nematodes [Yoon et al., 1995]. A 10.5 kilobase genomic fragment has the ability to provide all known functions of *sli-1* in transgenic animals, i.e., it rescues the suppression phenotype of *let-23*.

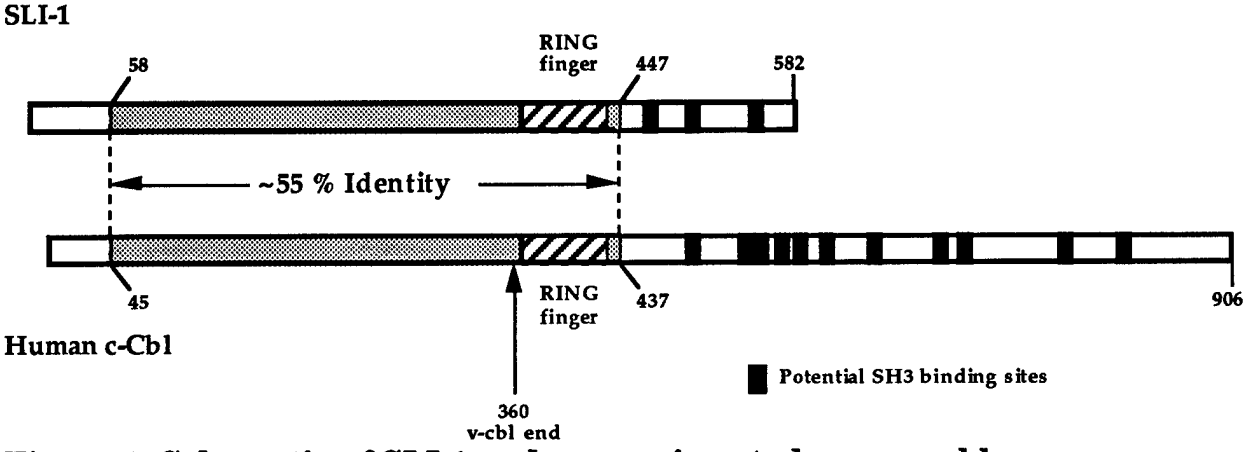

Figure 2. Schematic of SLI-1 and comparison to human c-cbl.

A manuscript describing our structure/function studies, along with genetic data on *let-60 ras* suppression and the SLI-1/SEM-5 two-hybrid has been provisionally accepted by Oncogene pending revisions on the manuscript.

The tyrosine site 2 in the carboxyl terminal tail of LET-23 mediates negative regulation of LET-23 mediated signaling by SLI-1

Since the interaction between SLI-1 and SEM-5 is not required for the negative regulation by SLI-1, and SLI-1 acts upstream of LET-60 but does not work as a positive regulator of GAP-1, we focused our attention on a postulated GNEF and LET-23. So far, no any biochemical experiment has revealed any interaction between SLI-1 homologs and GNEFs, however, many show that SLI-1 homologs (c-Cbl and D-Cbl) associate with EGFR in an activation-dependent manner (Hime *et al.*, 1997; Meisner *et al.*, 1997). We are exploring the possibility that SLI-1 may exert its inhibitory effect by direct or indirect binding to specific pTyr sites in LET-23. The *let-23(sy97)* mutation deletes the last 56 amino acids of the receptor which include the only three tyrosines (Tyr site 6, 7 and 8) in the receptor carboxy terminal tail which, if phosphorylated, would create SH2 binding sites matching the consensus binding site for the SEM-5 SH2 domain. *sli-1(sy143)* strongly suppresses *let-23(sy97)* which suggests that pTyr site 1 through pTyr site 5 could mediate, directly or indirectly, negative regulation of LET-23 mediated signaling by SLI-1 under the above hypothesis. Codons specifying LET-23 carboxy-terminal tyrosine residues were systematically mutagenized and substituted with phenylalanine to prevent SH2 and PTB domains binding (Lesa and Sternberg, 1997). The engineered LET-23 constructs were then introduced into nematodes with a *let-23(null)* background with or without SLI-1 and assayed their activity by scoring transgenic animals for vulval differentiation. Our results suggest that SLI-1 function appears to be dependent upon tyrosines in the LET-23 carboxy-terminal tyrosine residues. Furthermore, a particular tyrosine (Tyr2) in the carboxy terminal tail of LET-23 appears to negatively regulate LET-23 mediated RAS activity and this negative regulation is overcome by mutations in SLI-1. This is consistent with SLI-1 binding to that site or requiring another protein binding to that site.

Identification of a missense point mutation in the RING finger motif of SLI-1

sy115 was isolated as a suppressor of the vulvaless phenotype of viable, hypomorphic allele of *let-23*. It is defined as one allele on *sli-1* locus based on it's mapping to the left end of the X chromosome, left of *dpy-3*. *sy115* mutation was determined by single worm PCR and subsequent sequencing. The mutation *sy115* is a C-> T transition and causes the substitution of serine for proline at codon 404. This is the first point mutation found in the RING finger motif and certainly will help us to understand the roles of RING finger motif in the negative regulation by SLI-1. We will engineer this mutation in mouse cbl and test its interactions with know partners in vitro and in tissue-culture cells.

Two hybrid analysis of SLI-1.

Last year we reported the identification of 4 SLI-1 Interacting Proteins (SIPs). So far, 18 colonies have been analyzed. 15/18 colonies interact specifically with SLI-1 but not SEM-5 or LIN-3. 12/15 encode a 2090 amino acid novel protein (SIP#1) which contains putative EGF-like domains. 1/15 encodes an ATP synthetase (SIP#2). 1/15 encodes a 1302 amino acid ubiquitin-specific protease which is similar

to the *Drosophila* fat facets gene (SIP#3). 1/15 encodes a 349 amino acid novel protein which contains no identifiable motifs (SIP#4).

We tested each by RNA-mediated gene inactivation [RNAi; Fire et al, 1998]. Control RNAi with *sli-1* in a *rok-1* background indicated we could see function by this assay.

clone	chromosome	number obtained	protein product
SIP#1	V	12	2090 aa novel protein
SIP#2		1	ATP synthetase
SIP#3	II	1	1302 aa U6-specific protease
SIP#4	IV	1	349 aa novel

Table 1. Summary of For each clone class, the *C. elegans* chromosome assignment based on comparison to the extensive genomic sequence is indicated, as is the name of the closest relatives based on a BLAST search of non-redundant protein and nucleic acid databases maintained at NCBI. aa, amino acid residues

2. *sli-2.*

sli-2(sy262) was originally isolated as a dominant suppressor of *let-23(sy1)*, a reduction-of-function allele of the C. *elegans* EGF receptor homolog. *sli-2(sy262)* cannot suppress *let-60(n1876)*, a strong reduction-of-function allele of the C. *elegans* Ras homolog (Beitel *et al.*, 1990). This suggests that *sli-2* acts between *let-23* and *let-60*ras. Alone, *sli-2(sy262)* shows no vulval phenotype. However, double mutant combinations of *sli-2(sy262)* with negative regulators of the LET-60 RAS pathway show excessive vulval differentiation. This suggests that *sli-2* may act in a complex, redundant network which regulates RAS pathway signaling. A summary of *sli-2(sy262)* interactions is shown in Table 2.

sli-2 has been mapped to chromosome V, between *unc-46* and *dpy-11*, close to *unc-46*. A duplication spanning this region is capable of partially suppressing the vulvaless phenotype of *let-23(sy1)*, while a deficiency in the region enhances the vulvaless phenotype of *let-23(sy1)* (Table 3). These results, along with *sli-2(sy262)*'s dominant interaction with various *let-23* alleles, suggests that *sli-2(sy262)* may be a gain-of-function mutation, and therefor *sli-2* may act as a positive regulator of the RAS pathway. We continue to inject cosmids in the *unc-46* region to molecularly clone *sli-2*.

Table 2. *sli-2* interactions

Genotype	% animals<3.0 vulval cells differentiating	% animals=3.0 vulval cells differentiating	% animals>3.0 vulval cells differentiating	Avg. # of vulval cells/animal differentiating
sli-2(+)	0	100	0	3.0 (n=many)
sli-2(sy262)	0	100	0	3.0 (n=31)
let-23(sy1); sli-2(+)	95	5	0	0.8 (n=29)
let-23(sy1); sli-2(sy262)	0	25	75	3.9 (n=31)
let-23(sy1); sli-2(+)/sli-2(sy262)	22	35	43	3.1 (n=60)
let-23(sy97); sli-2(+)	100	0	0	0.0 (n=20)
let-23(sy97); sli-2(sy262)	0	100	0	3.0 (n=40)
let-23(sy97); sli-2(+)/sli-2(sy262)	65	35	0	1.7 (n=20)
let-60(n1876); sli-2(+)	100	0	0	0.0 (n=20)
let-60(n1876); sli-2(sy262)	100	0	0	0.0 (n=20)
rok-1(sy247); sli-2(+)	0	100	0	3.0 (n=many)
rok-1(sy247); sli-2(sy262)	0	65	35	3.4 (n=20)
unc-101(sy108); sli-2(+)	0	100	0	3.0 (n=20)
unc-101(sy108); sli-2(sy262)	0	70	30	3.2 (n=20)
sli-2(+); gap-1(n1691)	0	100	0	3.0 (n=20)
sli-2(sy262); gap-1(n1691)	0	70	30	3.3 (n=20)
sli-2(+); sli-1(sy143)	0	100	0	3.0 (n=20)
sli-2(sy262); sli-1(sy143)	2	86	12	3.1 (n=60)
sli-2(+); lin-15A(n767)	0	100	0	3.0 (n=20)
sli-2(sy262); lin-15A(n767)	0	95	5	3.03 (n=20)
sli-2(+); lin-15B(n744)	0	100	0	3.0 (n=20)
sli-2(sy262); lin-15B(n744)	0	90	10	3.06 (n=20)

Table 3. *sli-2* Duplication and deficiency data

Genotype	% animals<3.0 vulval cells differentiating	% animals=3.0 vulval cells differentiating	% animals>3.0 vulval cells differentiating	Avg. # of vulval cells/animal differentiating
let-23(sy1); sli-2(+)	95	5	0	0.8
let-23(sy1); nDf32/sli-2(+)	100	0	0	0.0
let=23(sy1); sli-2(+); yDp1	61	33	6	1.9

3. Genetics and molecular cloning of *rok-1*

We identified the *rok-1* (*r*egulator *o*f *k*inase) locus (*IV*) of the nematode *C. elegans* in a genetic screen for new negative regulators of the vulval induction pathway. We are waiting to obtain a targeted knockout mutation of *rok-1* to confirm the loss-of-function phenotype prior to publication. We have failed to obtain a deletion in a screen of approximately 500,000 EMS-mutagenized gametes using primers for PCR 3.5 kb apart. We will continue to screen until we obtain such a deletion.

Figure ---. Schematic of ROK-1 protein, which has a similar structure to human Ack and is 50% identical in its tyrosine kinase domain.

4. Identification and cloning of additional negative regulators

We have carried out several screens to obtain additional inhibitors of LET-23 - RAS signaling. In one screen, we seek mutations that cause RAS pathway activation (multivulva phenotype) in the absence of *sli-1* function. This screen identified *rok-1*, *rok-2* and several new candidates. In a second screen, we sought mutations that allow vulval formation when the LIN-3 growth factor is present at too low a level. This screen identified *rok-3* an X-linked semi-dominant suppressor of the *lin-3* vulvaless phenotype. In the past year we initiated an additional screen for enhancers of activated LET-23.

lin-3 suppressors.

sli-1(sy341) acts downstream of MAP kinase at the level of transcriptional regulators. The *sli-1(sy341)* mutation confers ligand-independent vulval development: 4 of 8 gonad-ablated animals had some vulval differentiation. *sli-*

1(sy341) maps close to *unc-4* on chromosome II based on three-factor mapping and failure to complement chromosomal deletions. Further deletion mapping will allow its molecular cloning this year. *sli-1(sy341)* appears to act downstream of *lin-25* as indicated by its suppression of *lin-25* null mutation. We mapped *sli-1(sy341)* to a small interval on chromosome II between the right breakpoint of deletion *mnDf29* and the left breakpoint of deletion *mnDf62*. This genetic interval corresponds to a region of 12 cosmids. Test of these cosmids for their ability to rescue the *sli-1(sy341)* phenotype of suppression of a *lin-3* vulvaless mutation is in progress.

Enhancers of *let-23(sa62)*

Previously, we characterized an allele of *let-23*, *sa62*, which displays properties of a constitutively active receptor tyrosine kinase. When present at two copies per cell, 90% of animals exhibit a strong multivulva phenotype which is no longer dependent on the LET-23 ligand, LIN-3. However, when *sa62* is present at only one copy per cell, only 5% of animals are multivulva. In order to identify mutations that can enhance the signaling activity of low levels of an activated EGFR family member, we mutagenized animals [*rol-6(e187)let-23(sa62)/mnC1*] that were heterozygous for *sa62*, and looked for F2 animals possessing 1 copy of *sa62* that now displayed a highly penetrant multivulva phenotype. From 6700 mutagenized gametes, we recovered 10 independent mutations which enhance the penetrance of the multivulva phenotype to varying degrees (see Table 4 for examples).

Table 4. Enhancement of sa62/+; all mutants significantly different that 3.0.

Mutation	Linkage group	Avg. number of vulval Cells induced
parent	not applicable	3.0
46-1	1	4.5
50-5	1	4.3
15-3	1	4.2
29-1	1	3.6
14-1	1	3.4

Thus far, all seven analyzed mutants map to linkage group I, and all 9 tested are semi-dominant. Present work is aimed at positionally cloning these loci (locus), and determining the mechanism for increasing the signaling output of low levels of activated LET-23. None of the mutations appear to constitutively activate the LET-23 pathway on their own, but preliminary data indicate that they are able to restore weak signaling by reduction of function alleles of *let-60* (Ras) and *lin-45* (Raf) to wildtype in the vulva.

5. Gene interactions

Most of the negative regulator mutations confer no phenotype on their own but are multivulva (resulting from pathway activation) in presence of another negative

regulatory mutation. Lack of synergy of null mutations implies a functional interaction. A *sli-1* null mutation synergizes with a *gap-1* null mutation: while none of *sli-1* or *gap-1* single mutants are multivulva, 30% of the *sli-1 gap-1* double mutants are multivulva. We are testing mutations in *gap-2*, a putative rasGAP in our interaction matrix.

6. Human homologs

Searches of human est databases continue.

7. Human cbl in *C. elegans*.

Completed.

Conclusions

•**Mechanistic studies of SLI-1.** A new allele of *sli-1* was sequenced, indicating a role of the ring finger domain. SLI-1 depends on tyrosine site 2 of LET-23 for its inhibitory effect.

•**New regulators.** New potential negative regulators have been identified and analyzed. *rot-1* (now called *sli-(sy341)* acts downstream of MAP kinase, and several transcription factors regulated by MAP kinase. A new screen has identified a novel locus on *C. elegans* chromosome 1.

Progress by task as per original Statement of Work:

A brief description of progress on each task is listed.

Task 1A. Determine whether SLI-1 truncation decreases or increases activity of the protein as assayed in transgenic animals. •[Completed]

Task 1B. Determine role of alternative spliced form of SLI-1. •[Completed].

Task 1C. *sli-1* point mutation sequencing •[on schedule].

Task 1D. *sli-1* antisera. We have obtained antisera that will be used to test for physical interactions of SLI-1 with candidate binding partners obtained from a two-hybrid screen in yeast. [behind schedule].

Task 2A Genetic characterization of *sli-2*. [on schedule]

Task 2B. Molecular cloning of SLI-2 from *C. elegans*. [behind schedule]

Task 3. Genetics and molecular cloning of ROK-1 from *C. elegans*. Waiting on one final experiment [almost completed]

Task 4. Identification by genetic screens of new loci.

a. Screen for new mutations, carry out screens in parallel. [on schedule]

b. Genetic mapping and complementation of new mutations, parallel experiments •[on schedule]

c. Molecular cloning [on schedule]

Task 5. Examination interactions of genes in vivo [ongoing] •[on schedule]

Task 6. Human homologs. •[ongoing]

Task 7. Introduction of c-cbl cDNA into transgenic nematodes. a. **Construct *sli-1/c-cbl* hybrid genes b. Examine phenotypes of transgenic animals.** •A chimera of c-cbl and SLI-1 functions in *C. elegans*; [completed].

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