PID-1 is a novel factor that operates during 21U-RNA biogenesis in Caenorhabditis elegans

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The Piwi–piRNA pathway represents a small RNA-based mechanism responsible for the recognition and silencing of invading DNA. Biogenesis of piRNAs (21U-RNAs) is poorly understood. In Caenorhabditis elegans, the piRNA-binding Argonaute protein PRG-1 is the only known player acting downstream from precursor transcription. From a screen aimed at the isolation of piRNA-induced silencing-defective (Pid) mutations, we identified, among known Piwi pathway components, PID-1 as a novel player. PID-1 is a mostly cytoplasmic, germline-specific factor essential for 21U-RNA biogenesis, affecting an early step in the processing or transport of 21U precursor transcripts. We also show that maternal 21U-RNAs are essential to initiate silencing.

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One of the challenges faced by germ cells is the faithful inheritance of their genomes over generations. An important aspect of this process is to ensure that potentially harmful DNA sequences are not expressed and/or do not replicate. Such sequences include transposons and retroviruses and, when left unchecked, create havoc, leading to fertility problems. One of the mechanisms controlling this line of defense is the Piwi–piRNA system (Ghildiyal and Zamore 2009; Malone and Hannon 2009; Luteijn and Ketting 2013).

The Piwi–piRNA pathway is a small RNA-based mechanism. At its core, Argonaute proteins from the Piwi subfamily use small RNA cofactors called piRNAs to recognize and silence transcripts derived from repetitive elements. Silencing often involves the amplification of a small RNA population and can occur at both the post-transcriptional and transcriptional levels. Post-transcriptional silencing involves cleavage of targeted mRNA molecules by the RNase H activity of Piwi proteins, while transcriptional silencing likely involves the recognition of a nascent transcript and is accompanied by the acquisition of repressive chromatin marks at the targeted locus (Ashe et al. 2012; Buckley et al. 2012; Luteijn et al. 2012; Shirayama et al. 2012; Sienski et al. 2012; Huang et al. 2013; Le Thomas et al. 2013; Rozhkov et al. 2013). This heterochromatin-like state can spread and lead to the silencing of genes that are located close to a Piwi target (Sienski et al. 2012). Despite the fact that a number of proteins have been linked to these activities (Ghildiyal and Zamore 2009; Malone and Hannon 2009; Luteijn and Ketting 2013), the exact mechanisms of this transcriptional silencing mechanism remain unclear.

Although the core principles of Piwi-driven mechanisms appear to be strongly conserved, the precise mechanisms through which Piwi proteins act and piRNAs are produced are remarkably flexible between species. In the nematode Caenorhabditis elegans, the Piwi protein PRG-1 is guided to its targets by piRNAs (named 21U-RNAs in C. elegans) (Ruby et al. 2006; Batista et al. 2008; Das et al. 2008; Wang and Reinke 2008) in a process that tolerates significant mismatches (Bagijn et al. 2012; Lee et al. 2012). Combined with the fact that ~30,000 21U-RNAs have been identified (Gu et al. 2012), 21U-RNAs can identify almost any foreign DNA sequence. Following target recognition by PRG-1, a second class of small RNAs (22G-RNAs) is produced, using the target transcript as a template (Das et al. 2008). In order to produce 22G-RNAs, presumably one of the RNA-dependent RNA polymerase (RdRP) enzymes of C. elegans is required as well as some genes known as mutator genes, like mut-7 and rde-3 (Bagijn et al. 2012). The 22G-RNAs are bound by a different set of Argonaute proteins, including the nuclear protein HRDE-1/WAGO-9 (Ashe et al. 2012; Buckley et al. 2012; Shirayama et al. 2012). The final outcome is reduced transcription and trimethylation of Lys9 of histone H3 [H3K9me3] of PRG-1 targeted genes (Ashe et al. 2012; Luteijn et al. 2012; Shirayama et al. 2012). Intriguingly, this transcriptional silencing response can acquire a PRG-1-independent, self-maintaining state. Under these conditions, HRDE-1, RDE-3, and MUT-7 are still required (Ashe et al. 2012; Luteijn et al. 2012; Shirayama et al. 2012).

The least-understood step in this PRG-1-mediated mechanism is how 21U-RNAs are made. In fact, we only know that 21U-RNA genes are marked by the presence of an upstream consensus motif (Ruby et al. 2006) of one transcription factor that plays a role in their transcription (Cecere et al. 2012) and that they are made as 5'-capped precursors that are at least 26 nucleotides (nt) in length.

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(Gu et al. 2012). From these precursors, the two most 5’ bases are removed, likely followed by binding to PRG-1 and 3’ end trimming [Kawaoka et al. 2011]. We do not know any of the factors acting during precursor processing. Here, we describe a forward mutagenesis screen aimed at the isolation of mutants that are piRNA-induced silencing-defective [Pid]. This screen led to the identification of alleles of known PRG-1 pathway components but also revealed a novel protein, PID-1, that plays an important role in 21U-RNA biogenesis downstream from their transcription. We also describe genetic experiments revealing intriguing maternal and paternal effects of the PRG-1 pathway.

Results and Discussion

Isolation of mutants in the 21U pathway

In order to identify novel genes acting in the PRG-1-mediated silencing pathway, we made use of a strain expressing a GFP-Histone-2B fusion gene that carries a specific 21UR1-RNA recognition site in its 3’ untranslated region [UTR] [21U sensor] [Bagijn et al. 2012]. We previously described how loss of HENN-1, the enzyme catalyzing the 3’ end methylation of 21U-RNAs, leads to partial desilencing of this transgene [Kamminga et al. 2012]. We also demonstrated that this partially activated state is subject to spontaneous desilencing, after which this sensor construct is no longer responsive to removal of PRG-1 [Luteijn et al. 2012]. In order to identify genes that act at any stage of the piwi pathway, we mutagenized a population of animals carrying a partially activated 21U sensor in a henn-1 mutant background with EMS and scored for full activation of the 21U sensor [Supplemental Fig. S1A]. Genome-wide resequencing of retrieved mutant strains identified premature stop mutations in mut-7 and tde-3 [Supplemental Fig. S1B]. We also identified an interesting allele [xf25] of hrde-1 carrying a nonsynonymous mutation affecting the last amino acid of HRDE-1 (A1032T). Given the important role for the most C-terminal residue of Argonaute proteins in making the binding pocket for the 5’ phosphate of the small RNA cofactor [Patel et al. 2006], we reasoned that this mutation might disrupt HRDE-1 activity. Indeed, a deletion allele of hrde-1, tm1200, was unable to complement xf25 [data not shown], showing that HRDE-1[A1032T] is nonfunctional. In summary, our screen effectively identifies genuine components of the C. elegans piwi pathway.

PID-1, a novel, general 21U-RNA biogenesis factor

Most of the alleles identified in our screen displayed normal levels of 21U-RNAs, as assessed by Northern blotting [Supplemental Fig. S1C], except for xf14. The locus affected by xf14 was named pid-1. To probe the generality of the apparent 21U accumulation defect, we analyzed small RNAs from both wild-type and pid-1(xf14) young adult animals that also carry the 21U sensor through deep sequencing [Supplemental Table S1]. Both libraries have a very similar size and display similar microRNA [miRNA] profiles [Supplemental Table S1, Supplemental Fig. S2A], indicating that the libraries can be well compared.

As expected, in wild-type animals, we could detect small RNAs mapping to the 21U sensor [Supplemental Fig. S2B]. These small RNAs display all characteristics of 22G-RNAs [Supplemental Fig. S3]. While in pid-1(xf14) mutants these small RNAs still display all 22G characteristics, their levels are strongly reduced [Supplemental Fig. S2B].

We next checked mature 21U-RNA levels, normalizing to total miRNA counts. We found that, overall, 21U-RNA levels were 10-fold reduced in pid-1(xf14) mutants [Fig. 1A] and that most 21U-RNA loci responded similarly to pid-1(xf14) [Supplemental Fig. S2A]. The loss of 21U-RNAs is slightly less than observed in prg-1(pk2298) mutants [Fig. 1A], suggesting that 21U-RNA biogenesis can proceed partially in the absence of PID-1. The 5’ nucleotide composition of the remaining 21U-RNAs in the pid-1 mutants was still heavily biased toward uracil [Supplemental Fig. S2C].

Finally, we asked whether PID-1 equally affects both type 1 and type 2 21U-RNAs. Most 21U-encoding loci are characterized by a specific sequence motif upstream of the transcription start site [Ruby et al. 2006], and a later study showed that this motif may be a transcription factor-binding site [Cecere et al. 2012]. However, it was shown recently that many loci exist that do not have this sequence motif and still produce 21U-RNAs. These two different classes have been referred to as type 1 and type 2 21U-RNAs, respectively [Gu et al. 2012]. We found that pid-1(xf14) leads to a strong decrease in type 1 as well as type 2 21U-RNAs [Fig. 1B], indicating that PID-1 does not affect recognition of the upstream 21U sequence motif. We conclude that PID-1 is a factor involved in global 21U-RNA biogenesis.

PID-1 affects PRG-1 levels and inhibits RNAi

The only gene thus far known to be required for 21U-RNA accumulation is prg-1. Hence, PID-1 could be required for transcription of prg-1. In order to test this, we performed RT-qPCR on wild-type and pid-1 mutant animals [Supplemental Fig. S4A]. This revealed that transcript levels of

Figure 1. pid-1 affects 21U-RNAs and PRG-1. (A) Bar diagram displaying normalized mature 21U read counts in strains with the indicated genotypes. Normalization was done to total miRNA read counts. Mature 21U reads were defined as those reads starting at the annotated mature 5’ end. (B) Bar diagram displaying type 1 and type 2 21U-RNA abundance in the indicated strains. Read counts reflect 21U counts obtained from NaIO 4-oxidized small RNA populations normalized to total mapped non-RNA reads. (C) Western blotting analysis of extracts from wild-type, prg-1, and pid-1 mutant animals [young adults]. Tubulin was used as a loading control.


prg-1 are unaffected by pid-1. In contrast, PRG-1 protein levels were strongly reduced upon mutation of pid-1
(Fig. 1C), indicating a post-transcriptional effect of pid-1 on PRG-1. Although we formally cannot exclude a role for
PID-1 in PRG-1 translation, we interpret this result as instability of PRG-1 in the absence of 21U-RNAs, much like
Drosophila Piwi is unstable without piRNAs, and Argonaute proteins are destabilized by loss of miRNAs
(Olivieri et al. 2010; Smibert et al. 2013).

Since many PRG-1 pathway components affect RNAi
triggered by exogenous dsRNA, we also probed the RNAi
sensitivity of pid-1 mutants. Using pos-1 RNAi by feed-
ing, we found that pid-1 mutants, like prg-1 mutants, are
slightly hypersensitive [Supplemental Fig. S4B]. Most
likely, this reflects competition for components shared
by the exo-RNAi machinery and the prg-1 pathway, such as
MUT-7 and RDE-3.

Identification of pid-1 as F18A1.8

To identify the pid-1 gene, we first crossed the pid-1(xf14)
allele into a henn-1 wild-type background. This showed
that the loss of 21U-RNAs in pid-1(xf14) animals is henn-
1-independent [Supplemental Fig. S5A]. Genetic mapping
revealed that pid-1 is genetically closely linked to the
21U sensor transgene on chromosome II (data not shown),
and genome-wide resequencing revealed three nonsilent
mutations in the area where pid-1 was mapped; these three
mutations affect F18A1.8, ZK1320.5, and F37H8.3.
We then selected recombinants that separated the 21U
sensor from these three mutations. Only recombinants
that picked up a wild-type copy of F18A1.8 produced 21U-
RNAs [Supplemental Fig. S5A], suggesting that F18A1.8
is pid-1. To test this, we generated two additional alleles
(xf35 and xf36) of F18A1.8 using CRISPR technology and
probed the effect of these alleles on the activity of the
21U sensor and on 21U-RNA biogenesis. First, xf35 and
xf36 do not complement pid-1(xf14) [Supplemental Fig.
S5B, data not shown]. Second, pid-1(xf35) was unable to
silence a 21U cherry sensor transgene that has been crossed in from
a mutant animal carrying a mCherry-21U sensor transgene that has been crossed in from
a

Figure 2. Identity of pid-1. (A) A typical pid-1(xf35) mutant animal carrying a mCherry-21U sensor transgene that has been crossed in from
a mutant-7(pk264) mutant background. The depicted animal is homo-
ygous wild type for mutant-7. (B) Schematic of the PID-1 protein.

at position 61 (R61C) [Fig. 2B]. This mutation somehow
affects PID-1 stability, since Western blotting reveals that
PID-1 expression is strongly reduced in pid-1(xf14) mutant
extracts, while the xf35 and xf36 alleles do not produce
detectable PID-1 protein at all [Fig. 3A]. Next, we tested
whether PID-1, like PRG-1, is expressed specifically in the
germline by performing Western blot analysis on mutants
with a temperature-sensitive allele of glp-4, resulting in
a lack of germline tissue in animals grown at 25°C. This
revealed that PID-1 is expressed predominantly in the
germline. This is consistent with published data showing
that pid-1 transcripts are selectively enriched in the
primordial germ cells of C. elegans [Spencer et al. 2011].

No obvious domain structure is detected through
regular BLAST analysis. However, at the N terminus,
a bipartite nuclear localization signal may be present
[KRFSHITLASTPFKKR], and the protein may contain
a nuclear export signal [LSAGLSL] as well [Fig. 2B],
suggesting that PID-1 may be cycling between the nucleus
and cytoplasm. Subcellular fractionation experiments sug-
gest that the majority of PID-1 is cytoplasmic (Fig. 3C).
However, given that the PID-1 signal is retained better in
the nuclear fraction than the non-PID-1-specific signals, it
is possible that a small part of the PID-1 pool may be
associated with or be inside the nucleus [Fig. 3C].

PID-1 does not affect 21U-RNA methylation

To further probe at which level PID-1 affects 21U-RNA
biosynthesis, we NaIO₄-oxidized small RNA. This pro-
cedure enriches for small RNA species that carry a 2'-
O-methyl modification at their 3' end, such as mature 21U-
RNAs in C. elegans [Ruby et al. 2006], and it is believed
that this methylation step finalizes 21U-RNA biogenesis.
To assess the effect of the oxidation, we compared the
ratio of 21U with miRNA reads in the tobacco alkaline
phosphatase (TAP)-treated and the oxidized RNA-derived
libraries. Using this analysis, we were unable to detect
a significant difference between the wild type and pid-1
mutants [Supplemental Table S1], indicating that the
remaining 21U-RNAs in pid-1(xf14) mutants are still 2'-
O-methylated. Furthermore, the global requirement of
pid-1(xf14) for 21U-RNA accumulation is also observed
in the libraries made from oxidized RNA [Supplemental
Fig. S6A]. We also checked whether the step right before methylation
is affected by PID-1. This step involves the trimming
of the 21U 3' end, presumably by an exonuclease. To assess
this, we profiled the length distribution of those 21U reads
from the TAP-treated libraries whose 5' ends match pre-
cursor transcripts. It has been suggested before that these
21U precursors mostly start 2 nt upstream of the 5' end of
the mature 21U-RNA, are 5'-capped, and are at least 26 nt in
length (Gu et al. 2012). These species should be repre-
sented in our TAP-treated libraries, as TAP has decapping
activity. Hence, we analyzed all reads that come from

PID-1 acts during 21U precursor processing

We also probed our libraries for potential 21U-RNA pre-
cursor transcripts. It has been suggested before that these
precursors mostly start 2 nt upstream of the 5' end of
the mature 21U-RNA, are 5'-capped, and are at least 26 nt in
length. These species should be represented in our TAP-treated libraries, as TAP has decapping
validate nuclear fractions (nuc).
validate cytoplasmic fractions (cyt), and Histone H3 was used to
signals from the custom-raised antiserum. (C) Western blot detecting PID-1 and PRG-1 protein in
mutant animals grown at permissive (with germline) and restrictive
(asterisks mark nonspecific signals from the custom-raised antise-
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Figure 3. PID-1 is a mostly cytoplasmic, germline-expressed pro-
tein. [A] Western blot for PID-1 in the indicated backgrounds. The
asterisks mark nonspecific signals from the custom-raised antiserum. [B] Western blot detecting PID-1 and PRG-1 protein in glp-4(bn2ts)
mutant animals grown at permissive (with germline) and restrictive
(asterisks mark nonspecific signals from the custom-raised antiserum). [C] Western blot analysis of a subcellular fractionation experiment. TBA-1 was used to validate cytoplasmic fractions (cyt), and Histone H3 was used to validate nuclear fractions (nuc).

Figure 4. PID-1 acts during 21U precursor processing. [A] Relative coverage of individual bases of 21U loci by obtained cDNA reads from prg-1(pk2298), wild-type libraries made from TAP-treated small RNA. The annotated 5’ ends of the analyzed loci are at position 0. The dotted lines reflect the standard deviation from the two wild-type samples and the three pid-1 alleles. [B] A length distribution plot for 21U-RNA precursor reads obtained from the CIP-TAP-treated libraries.

unique 21U loci (Supplemental Table S2) and start within
5 nt of the annotated mature 5’ end. In order to prevent
strong biases by heavily expressed individual 21U loci, we
restricted our analysis to those 21U loci that individually produce <1% of the 21U reads. We then plotted read
coverage over these 21U loci, starting at position ~5 and extending until position ~40, with the start of the annotated mature 21U-RNA at position 0. First, such analysis in two replicate samples of wild-type animals reveals that this approach yields very reproducible patterns (Supple-
mental Fig. S6C). We then performed the same analysis on
prg-1(pk2298) small RNA-derived cDNA libraries. This revealed that in the absence of PRG-1, ~80% of the reads derived from 21U loci correspond to precursor transcripts, starting mostly at position ~2 and extending to ~35 nt in length (Fig. 4A). We then analyzed the effects of the three pid-1 alleles (Fig. 4A; Supplemental Fig. S6C), revealing a clear accumulation of 21U-RNA precursors in animals lacking PID-1. Interestingly, the effect of pid-1 is somewhat less than that of prg-1. Given the fact that pid-1 mutants still produce low levels of 21U-RNAs while prg-1 mutants almost lack them completely, the simplest interpretation of these data is that in pid-1 mutants, 21U precursors can still be partially processed into mature 21U-
RNAs. Finally, we profiled the length and locus coverage of 21U precursor transcripts in the diverse wild-type and
mutant backgrounds using a protocol that selects for capped transcripts (Gu et al. 2012). This experiment did not reveal significant differences in the 21U precursor structure between any of the tested strains, suggesting that PID-1 does not affect the structure of 21U precursor transcripts (Fig. 4B).

Genetic requirements for pid-1 and prg-1 during
transgene (re)silencing

Finally, in order to better understand the global role of
21U-RNAs during the process of transgene silencing, we
assessed how active transgenes from 21U-defective back-
grounds react to the re-establishment of the 21U pathway.
For that, we crossed pid-1(xf14);21U sensor males into either wild-type or pid-1 mutant hermaphrodites. As expected, offspring of these males mated with pid-1-defective hermaphrodites show strong transgene activ-
ity. Interestingly, however, when mated to wild-type hermaphrodites, these males sire offspring with active transgenes, albeit the fluorescence intensity is notably lower (Table 1). We observed similar transgene activity in offspring from prg-1(pk2298);21U sensor males mated with wild-type hermaphrodites. Full silencing of the trans-
gene is only observed in the next generation (Table 1). This shows that while 21U-RNAs start to silence a novel target within one generation, full silencing can be achieved only upon joint passage through the germline. Possibly, 22G-
RNA levels need to accumulate in order to establish full silencing.

Next, we crossed an active 21U sensor from mut-7(pk204)
male into both wild-type and pid-1 mutant hermaphro-
dites and analyzed transgene expression in the F1. This revealed a very strong maternal effect of 21U-RNAs: The wild-type hermaphrodites produced fully silenced off-
spring, while the progeny of the pid-1 mutant mothers could not initiate detectable silencing even though they were heterozygous wild type for pid-1. This result highlights at least two properties of the 21U pathway. First, the maternally provided 21U-RNA pool is essential to estab-
lish resilencing of a mut-7(pk204)-activated transgene, suggesting that the initiation of silencing by 21U-RNAs happens during embryonic development using maternally provided 21U-RNAs. Second, a mut-7-activated transgene coming in via the sperm is easier to resilience by 21U-
RNAs than a pid-1 or a prg-1-activated transgene. We conclude that some silencing information must be present in mut-7-derived sperm but is lacking in prg-1-defective sperm and that this information can be efficiently used by the maternal 21U-RNA pool.
Materials and methods

C. elegans strains

C. elegans was cultured on NGM plates, with Escherichia coli OP50 as a food source. Alleles that were used in this study are listed in the Supplemental Material.

Table 1. Parental effects in the 21U pathway

| Genotype          | Mother          | Father          | Fluorescence |
|-------------------|-----------------|-----------------|--------------|
| pid-1(xf14)       | pid-1(xf14)     | pid-1(xf14); sensor | ++           |
| prg-1(pk2298)     | prg-1(pk2298)   | prg-1(pk2298); sensor | ++           |
| pid-1(xf14)+/F1   | +/-             | pid-1(xf14); sensor | +            |
| pid-1(xf14)+/F2   |                 | Selling of above F1 | -            |
| prg-1(pk2298)+/F1 | +/-             | prg-1(pk2298); sensor | +            |
| prg-1(pk2298)+/F2 |                 | Selling of above F1 | -            |
| mut-7(pk204)+/F1  | +/-             | mut-7(pk204); sensor | -            |
| mut-7(pk204)+/+pid-1(xf14)+/F1 | pid-1(xf14) |                                        |

All analyzed animals were offspring from a 21U sensor-carrying male crossed with a nontransgenic hermaphrodite. Genotypes of the parents are indicated. Fluorescence intensity was scored under a dissection microscope. [++] Detectable at 120×; ++ detectable at 480×; (−) not detectable at 480×.

Conclusions

We identified a novel gene, pid-1, that plays an important role during 21U-RNA biogenesis in C. elegans. PID-1 is a mostly cytoplasmic protein that likely acts during the processing of 21U-RNAs downstream from their transcription. The mature 21U-RNAs that are still made in pid-1 mutants are indistinguishable from wild-type 21U-RNAs, and, relative to these mature 21U-RNAs, 21U-RNA precursors accumulate upon loss of PID-1. Furthermore, the structure of 21U precursor transcripts is not affected by PID-1. Given these results as well as the potential nuclear import and export signals on PID-1, we suggest that PID-1 may somehow guide 21U precursors to a site where they are further processed into mature 21U-RNAs. We note that in other species, like mice or Drosophila, PID-1 orthologs cannot be identified through BLAST analysis. This may be related to the fact that, overall, the 21U-RNA pathway in C. elegans deviates significantly from piRNA pathways in other animals. However, given the still poor characterization of piRNA biogenesis in general, proteins with similar functions but with diverged sequences may operate in mammalian or fly piRNA biogenesis.

We found that pid-1 as well as prg-1 partially inhibit RNAi triggered by exogenous dsRNA. Much like what is described for other RNAi-hypersensitive mutants, we attribute this to the fact that the prg-1 pathway included proteins shared with the exo-RNAi pathway (Duchaine et al. 2006; Pavelec et al. 2009), and thus ongoing prg-1 activity in C. elegans puts a significant claim on those shared resources.

On the genetic level, we showed that the initiation of transgene silencing by 21U-RNAs depends on maternally provided 21U-RNAs. These findings are consistent with the maternal contributions of piRNA populations observed in other species, like Drosophila [Brennecke et al. 2008] and zebrafish [Houwing et al. 2007], and highlight the important role for maternally provided 21U-RNAs. These findings are consistent with the maternal contributions of piRNA populations observed in other species, like Drosophila [Brennecke et al. 2008] and zebrafish [Houwing et al. 2007], and highlight the important role for maternally provided small RNA populations in order to establish silencing in offspring. In addition, we found that the genetic background of the sperm can have an effect on the silencing of a locus. Considered together, parental influences play important roles during the establishment of the epigenetic state of an allele through the 21U pathway in C. elegans.

Small RNA library preparation

Small RNA was isolated with a Mirvana kit from Life Technologies. Following isolation, this RNA was treated with TAP and calf intestinal phosphatase followed by TAP (CIP-TAP) or NaIO4. The wild-type 21U sensor and pid-1(xf14); 21U sensor libraries (oxidized and nonoxidized)
were prepared as described before (Kamminga et al. 2012). Wild-type, pmg-1, pid-1(s14), pid-1(s35), and pid-1(s360) libraries for TAP and/or CIP-TAP were prepared as described in the Supplemental Material. Sequencing data are available at Gene Expression Omnibus, entry GSE55309.

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