

## Suppl. Fig 1.

Ptiwi16 is GSPATG00013703001

>Ptiwi16

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MSGTIVVGSNIYETEFLEDEIFIHYGNKTADTISFKDGLIISPKKLELPEYKINRSEQGEV
IMAILKEAMNLEYSKLSLDGIINMFELQYVNQLKSYP SLRFQFMDKEQPSILMDVRKSY
FTVHNCYQIIKTLNTEPSAYFKNKFVLLRYPEKKMIVQIKSVVVDQEFTYKGVSLLEDYFK
QKYQLKSKSSVYLETYNTQNIKKQFLIAEFCDLKIDYIEQDATVY CQQLEYWNKIINSS
SFIKFLNQYKLIIRNEQVEFKQQYLEPGNLV LNNQTKSIFQIVASRTSSEFEYYYTDFFL
KNASTFTTSLTINKLILVTREITELKDNFNIFLEKFDLFIKSRQYKISQPEIYDINNLL
NEILEKYVTEDSYVLFVGDQKTDFTSARNTLLSKAIPNQLINLPIQDQEINRLLAIMTAN
LGSVPWSIKEINGQINNKKSAVLGIWKSDNSFSACL SINKYLNKYISSFGDLQILTL
STYYGTHKTLANQIIIFAEEDYTDIMEQRIQGLIQLIQDQGLAESTQKPEIVMVQVKDAS
EERYFTYFERNFESY YCKNPQVGCIVPINEKQGRFSFHSQRGEQGCLQAQQLQISSNRR
EIAQLYYHLIFLNF DLSSITYLPAPLHYAKKLSKHQLQSEYKKAIEKGHMLFV
```

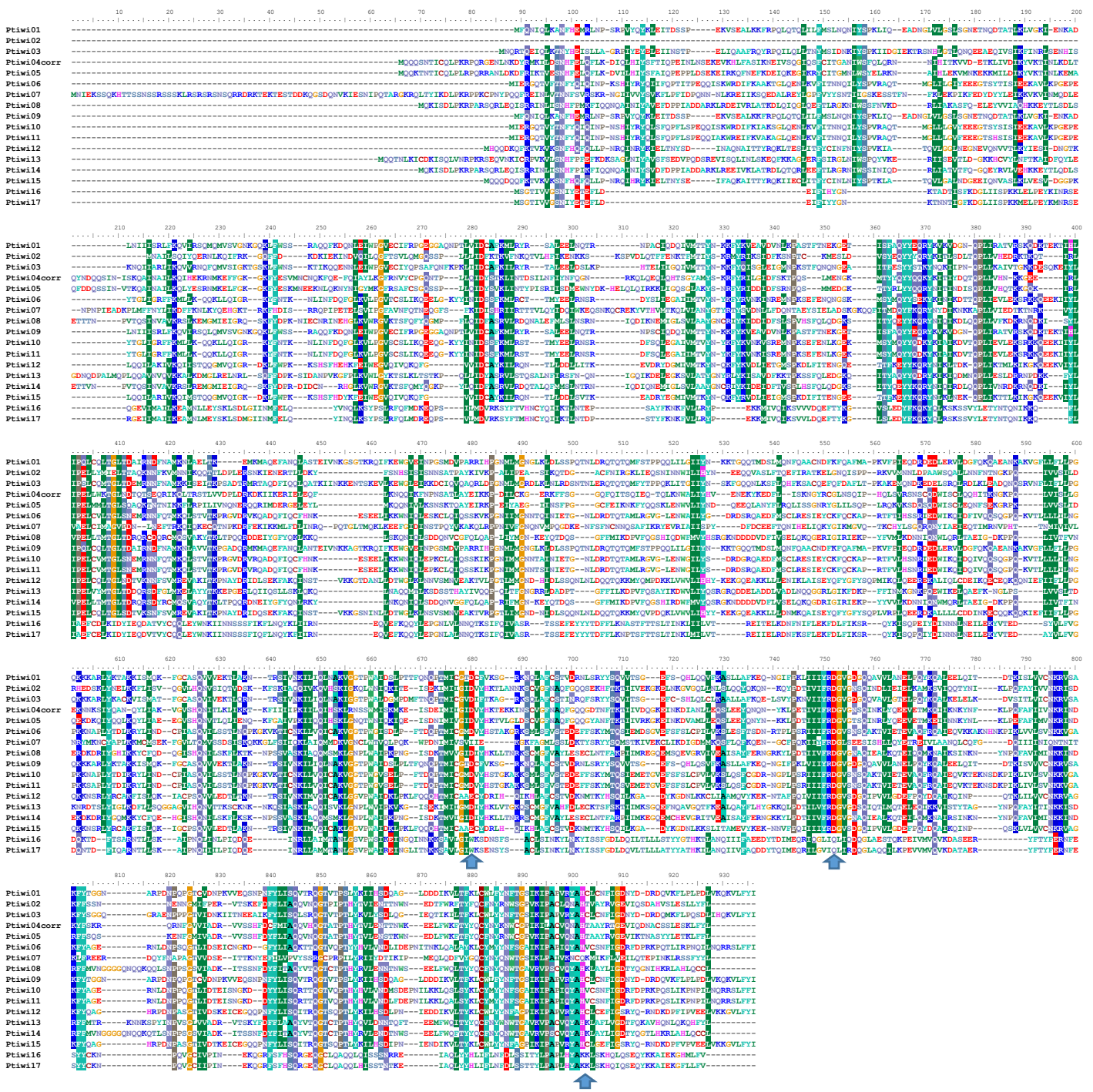
Ptiwi17 is the second part of GSPATP00016985001 as this automatically annotated genes was artificially fused with the 5'-upstream ORF

The corrected amino acid sequence is:

>Ptiwi17

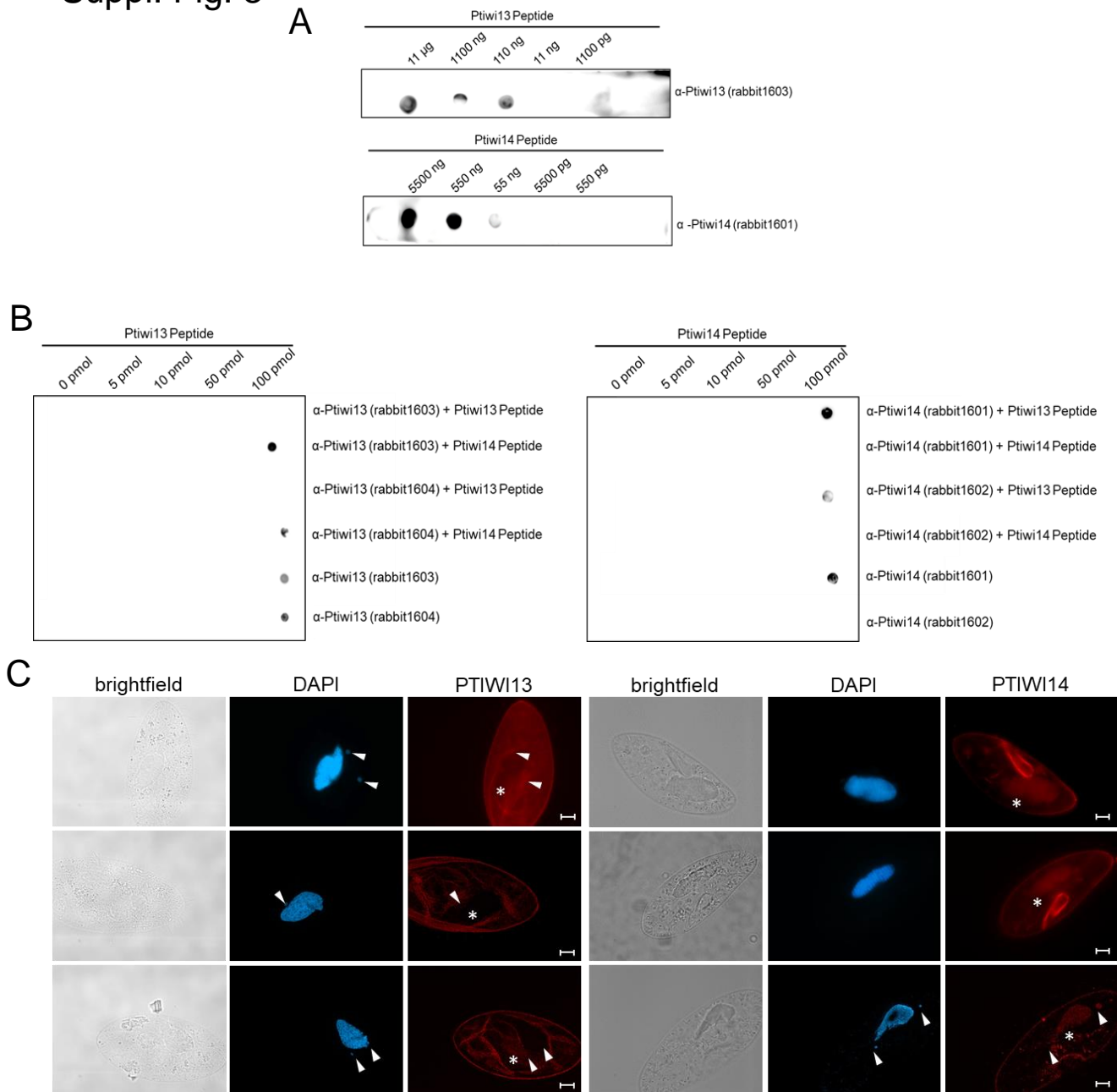
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MSGTIVVGSNIYETEFLEDEIFIYYGNKTNNTIGFKDGLIISPKKMELPEYKMNRSERGEI
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FTMHNCYQIIKTLN TDPSTYFKNKFVLLRYPEKKMIVQIKSVVLDQEFTYKGLSLEDYFK
QKYQLRSKSSVYLETYNTQNIKKQFLIAEFCELKIDYIEQDVTY CQQLEYWNKIINSS
SFIQFLNQYKFIIRNEQVEFKQQYLEPGNLALNNQTKSIFQIVASRTSSEFEYYYTDFFL
KNPTSFTTSLTINKLMILVTREIIELRDNFKSFLEKFDLFIKSRQYKISQPQIYDINNLL
NEILEKYVTE DAYVLFVGDQNTDFIQARNTLLSKAIPNQIIILPIQDQEINRLLAMMTAN
LGSVPWAIREINGLITNKSAVLGIWKSENSYSACL SINKYLNKYISSFGDLQVLTLL
ATYYATHKILANQIIVFAQDDYTDIMEQRILGVIQLIRDQGLAQQILKPEVVMVQVKDAT
AERYFTYFERNFESY YCKNPQVGCIIIPINEKQGRFSFHSQRGEQGCLQAQQLHISSTNTK
EIAQLYYHLIFLNF DLSSTTYLPAPLHYAKKLSKHQIQSEYKKAIEKGFLFV
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# Suppl. Fig 2.



**Suppl. Fig. 2** ClustalX alignment of the 17 Ptiwi Amino Acid Sequences of *P. tetraurelia*. For Ptiwi04 the gene sequence was artificially corrected to a translatable orf using the paralog Ptiwi05 as a template. Blue arrows indicate conserved catalytic residues of the DDH triad.

# Suppl. Fig. 3



**Suppl. Fig. 3** Validation of antibody specificity. A) Dot blot assay using custom made antibody directed against *P. tetraurelia* Ptiwi13 and Ptiwi14. Different amounts of each indicated peptide were spotted on the membrane. Hybridization with the respective antibodies showed the reactivity with the corresponding peptide. B) Competition assay. 0 – 100 pmol of each peptide were spotted. The antibodies were mixed with the corresponding peptide in 100x excess in advance for 1 hour, then the mix was used to decorate the membrane. Blocking  $\alpha$ -Ptiwi13 with Ptiwi13 peptide results in loss of antibody binding on the membrane, while blocking with the Ptiwi14 peptide does not have an effect on the signal (mock). Same is true for validation of Ptiwi14 antibody (bottom right). C) Localisation of Ptiwi proteins in vegetative *Paramecium* cells. Cells were analyzed by indirect immunofluorescent staining using custom antibodies directed against Ptiwi13 and Ptiwi14 labeled with secondary Alexa594 - conjugated antibody (red). Representative overlays of Z-stacks of

magnified views are presented. Other panels show DAPI (in blue) and brightfield. White arrows point at micronuclei while asterisk is indicating position of the macronucleus. Scale bar is 10  $\mu\text{m}$  and exposure is 2 s.

## Suppl. Fig. 4

ID	Prediction	Pred1	Prob1	Pred2	Pred2	Pred3	Prob3	MLCS
PTWI14	NUC	NUC	10.45	CYT	10.32	CSK	9.444	20.74
PTIWI13	CYT	CYT	13.67	NUC	11.35	CSK	9.118	24.44

NUC - Nucleus

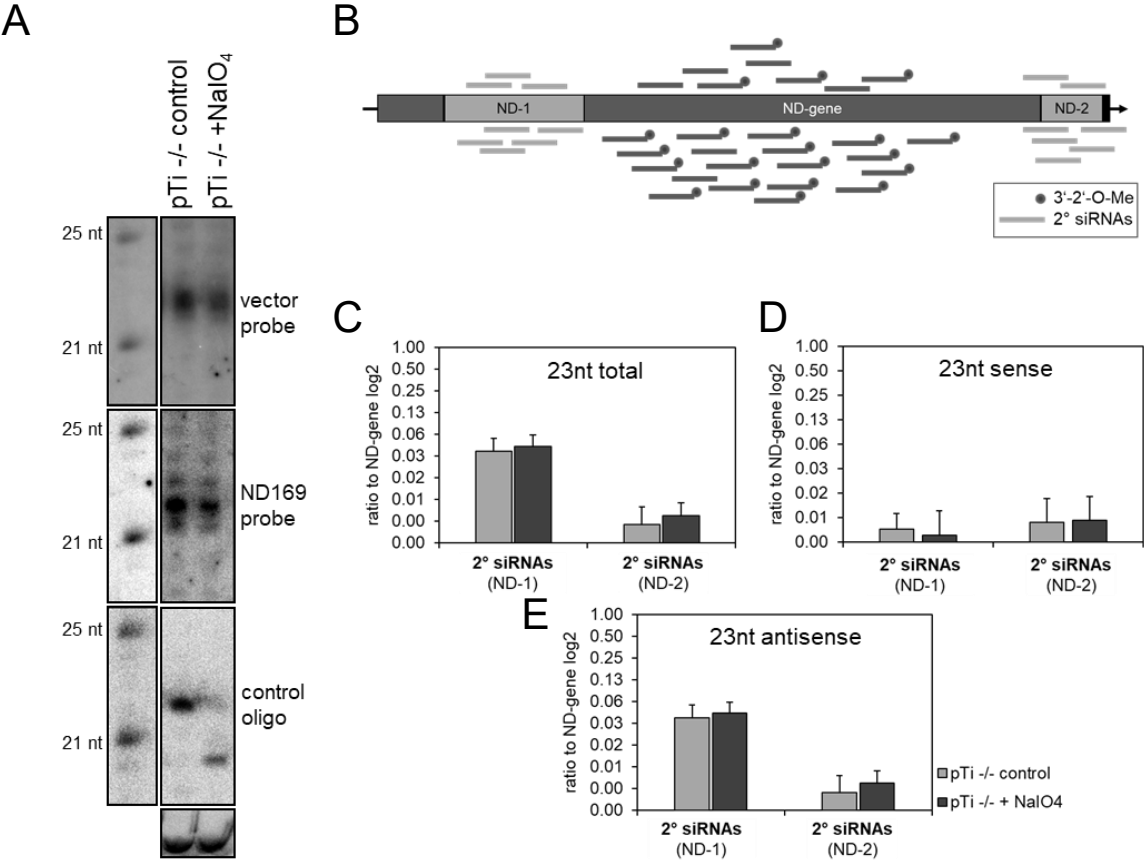
CYT - Cytoplasm

CSK - Cytoskeleton

MLCS - multi-localization confidence score

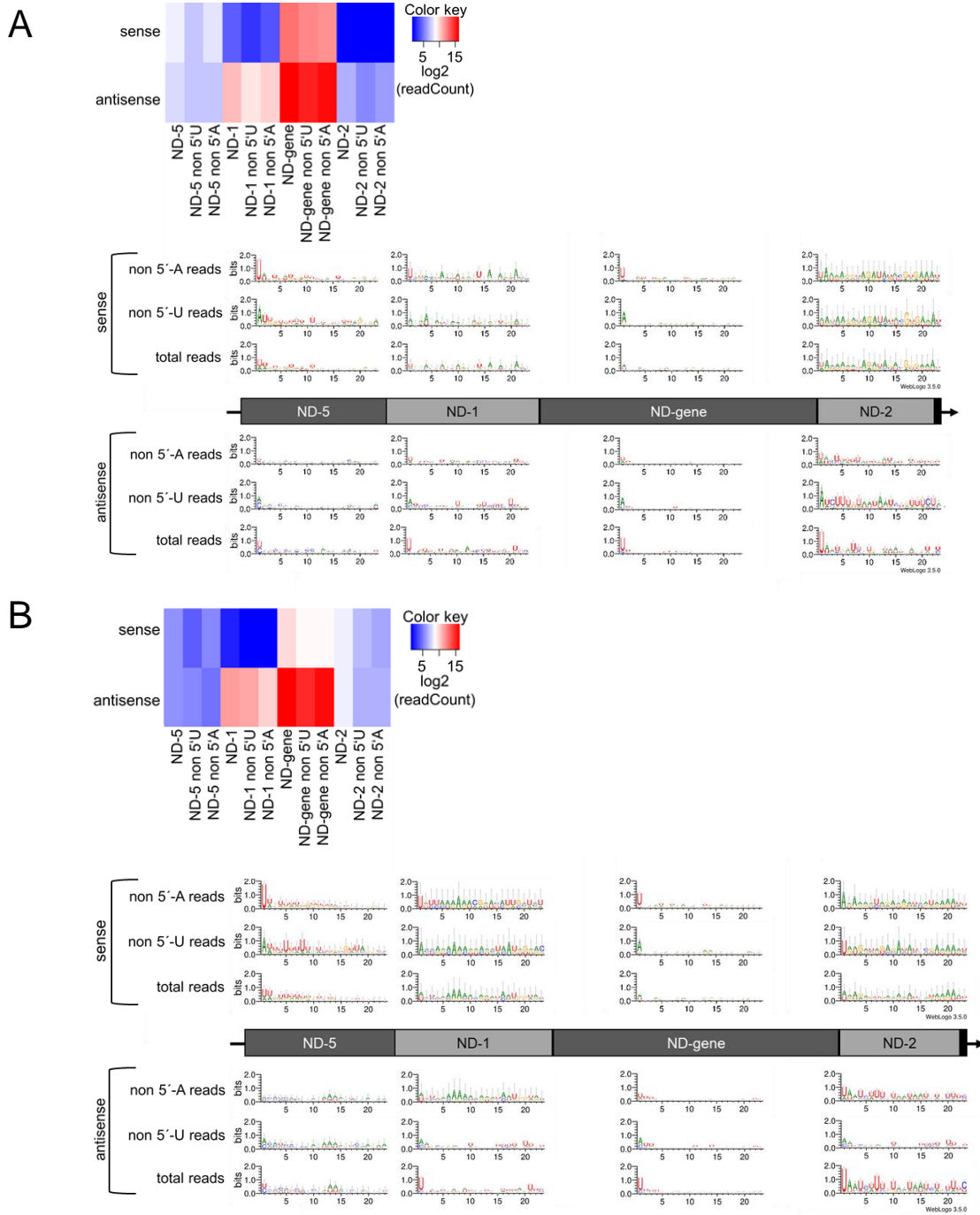
**Suppl. Fig. 4** Prediction of protein sub-cellular localisation of Ptiwi13 and Ptiwi14 using ngLoc method.

Suppl. Fig. 5



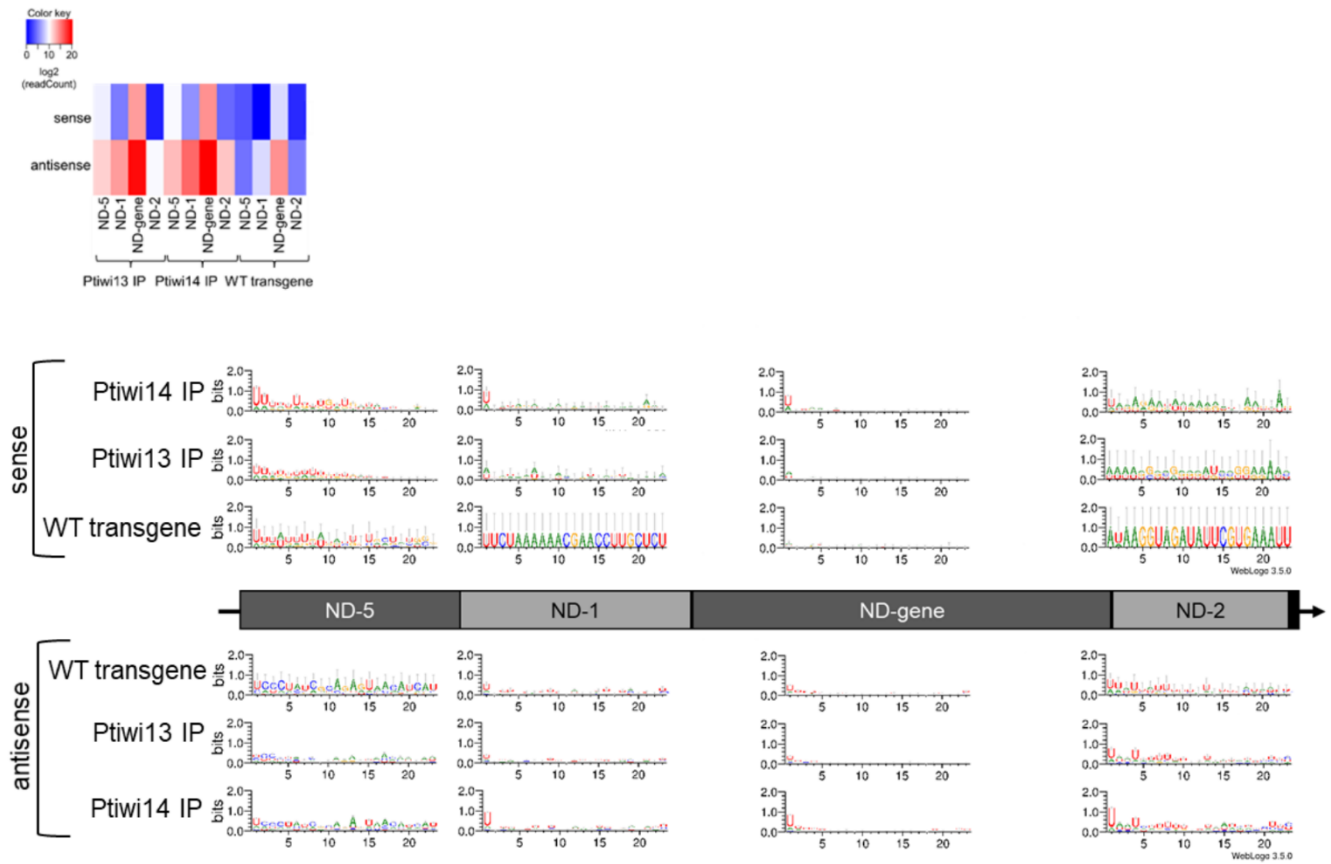
**Suppl. Fig 5** Analysis of 3' modifications of siRNAs. A) Northern blot analysis of transgene siRNAs. Untreated RNA and NaIO<sub>4</sub> treated RNA (pTi<sup>-/-</sup> + NaIO<sub>4</sub>) was blotted. Next to the marker on the left, the blot was hybridized with a transgene vector probe (outside *ND169* region, upper blot), a *ND169* probe (middle) and a as probe against the 3' unmodified spike in oligo (lower blot). B) Simplified scheme of *ND169* regions and normalization procedure visualized by arrows. Light grey regions are not part of the transgene and account for production of secondary sRNAs. C-E) Figs. show results of sRNA seq of untreated and NaIO<sub>4</sub> treated RNA normalized to the total number of 1°-*ND169* mapping siRNA. Data of two replicates were merged.

# Suppl. Fig. 6



**Suppl. Fig. 6** Sequence logos 1° and 2° siRNAs. Sequence logos of 23nt siRNAs in A) ICL KD and B) ICL KD periodate treated RNA are shown. Heat maps show log<sub>2</sub> of number of reads from each sample used for plotting the different logos.

## Suppl. Fig. 7

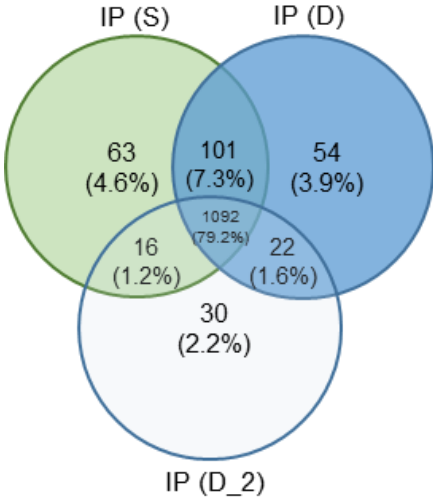


**Suppl. Fig. 7** Sequence logos of 23nt siRNAs in Ptiwi IPs. Sequences were separated by their direction (sense, top; antisense, bottom). Heat maps show log<sub>2</sub> of number of reads from each sample used for plotting the different logos.

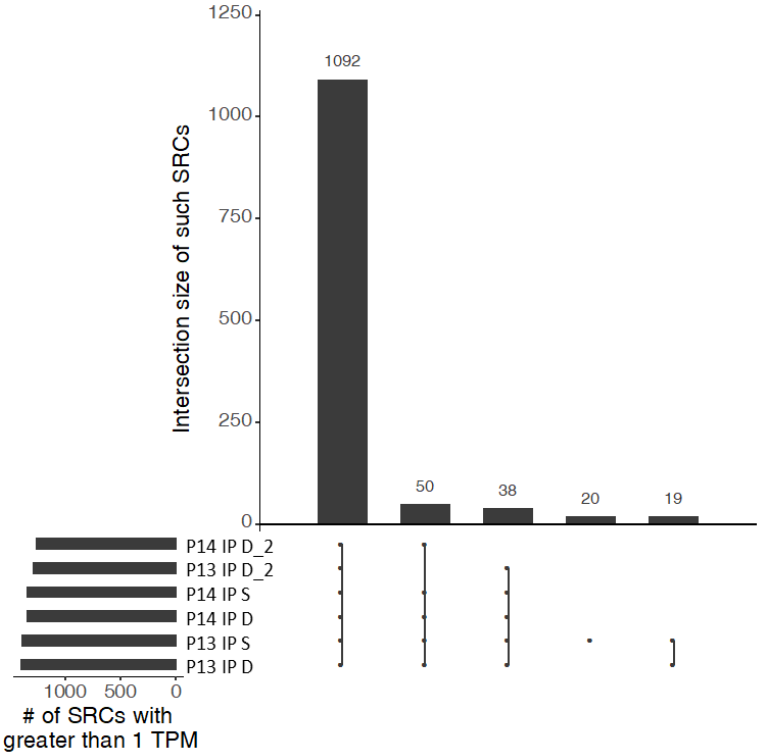


Suppl. Fig 8.

A



B



**Suppl. Fig. 8.** Analysis of Ptiwi IPs for presence of endogenous sRNAs. Samples used for this comparison are S (sonified lysate), P1 (dounced lysate with intact but permeabilized Macs) and P2 (dounced lysate with subsequent sonification for Mac destruction). A) shows a Venn diagram indicating the number of SRCs (endogenous small RNA clusters) appearing in  $\geq 1$ TPM in IP libraries. Below the percentage of covered SRCs is indicated. B) Set intersection plot of the same data.