

A Role for Neuronal piRNAs in the Epigenetic Control of Memory- Related Synaptic Plasticity

journal club
Tõnu Margus

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RNAi *interference*

REGULATION

- RNA interference (RNAi) is a **process** within living cells that **moderates the activity of their genes**.
- Historically, it **was known by other names**, including **co-suppression**, post transcriptional **gene silencing** (PTGS), and **quelling**.
- Two types of small ribonucleic acid (RNA) molecules – microRNA (**miRNA**) and small interfering RNA (**siRNA**) – **are central** to RNA interference.
- Small RNAs can bind to messenger RNA (mRNA) molecules and either **increase** or **decrease** their **expression**
- miRNAs were first discovered in **1993** by Victor Ambros, Rosalind Lee and Rhonda Feinbaum during a study into development in the nematode *C. elegans* regarding the gene lin-14.

Argonaute protein family

- There are **several classes of Argonaute proteins**, most famously **the Ago-class** Argonautes that bind to **miRNAs**
- **Piwi-class** Argonaute proteins are interacting with small RNAs called **piRNAs**
- There are also several classes of small noncoding RNAs that do not participate in RNAi-related pathways, for example the CRISPR (clustered regularly interspaced short palindromic repeats) RNAs (crRNAs) that are found only in prokaryotes
- CRISPR system is related to bacterial immune system defense against viruses and plasmids
- **PiRNA and CRISPR** system are very similar, however, they are **analogous** and not homologous

piRNA system

- piRNAs were definitively **discovered in 2006** by immunoprecipitating Piwi protein **from mammalian testis**
- PiRNAs are ~**26–30nt** in mammals
- The **population** of piRNAs is typically **very large** (in the **hundreds of thousands**) and complex.
- piRNAs appear to be produced by **quasi-random cleavage** of the primary piRNA transcript – therefore >
- PiRNAs are in general **difficult to predict bioinformatically** and must instead be defined biochemically
- piRNAs master loci of *D. melanogaster* were found to produce piRNAs that **repress transposable elements** in trans in **germ line** cells

A Role for Neuronal piRNAs in the Epigenetic Control of Memory-Related Synaptic Plasticity

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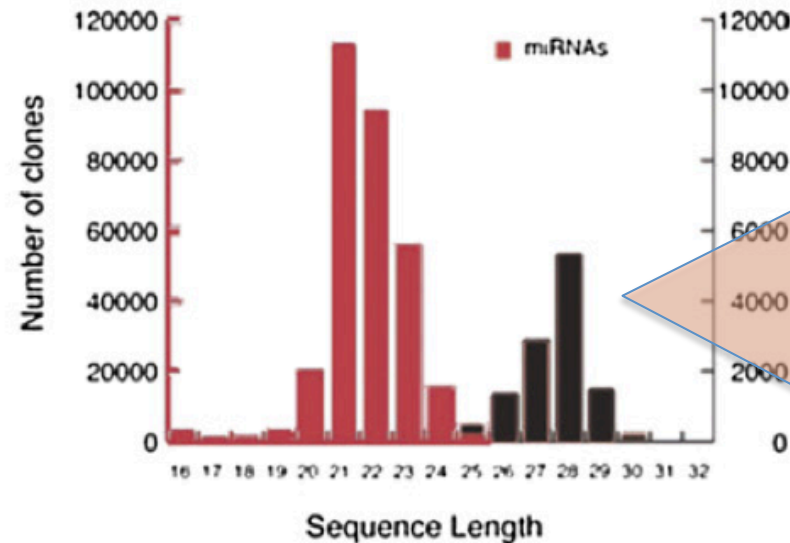
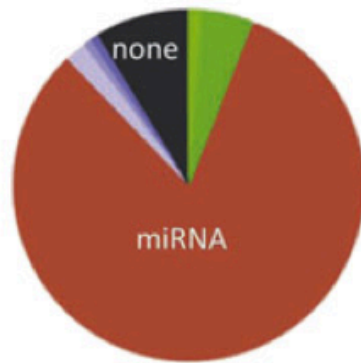
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RESULTS

pre-RESULTS



1) length of 28 nt and
2) a strong preference for a 5' U
3) mapping to the *de novo* assembled scaffold (genome) – clusters containing additional sequences with the same features, revealing a pattern that is characteristic of mammalian piRNAs

- Our previous generation of a **small RNA library** from *Aplysia* **CNS** resulted in **the majority** of sequence reads being mapped **as miRNAs**, with a minority of reads (**20%**) that mapped to the *Aplysia* genome but could not be **annotated**.

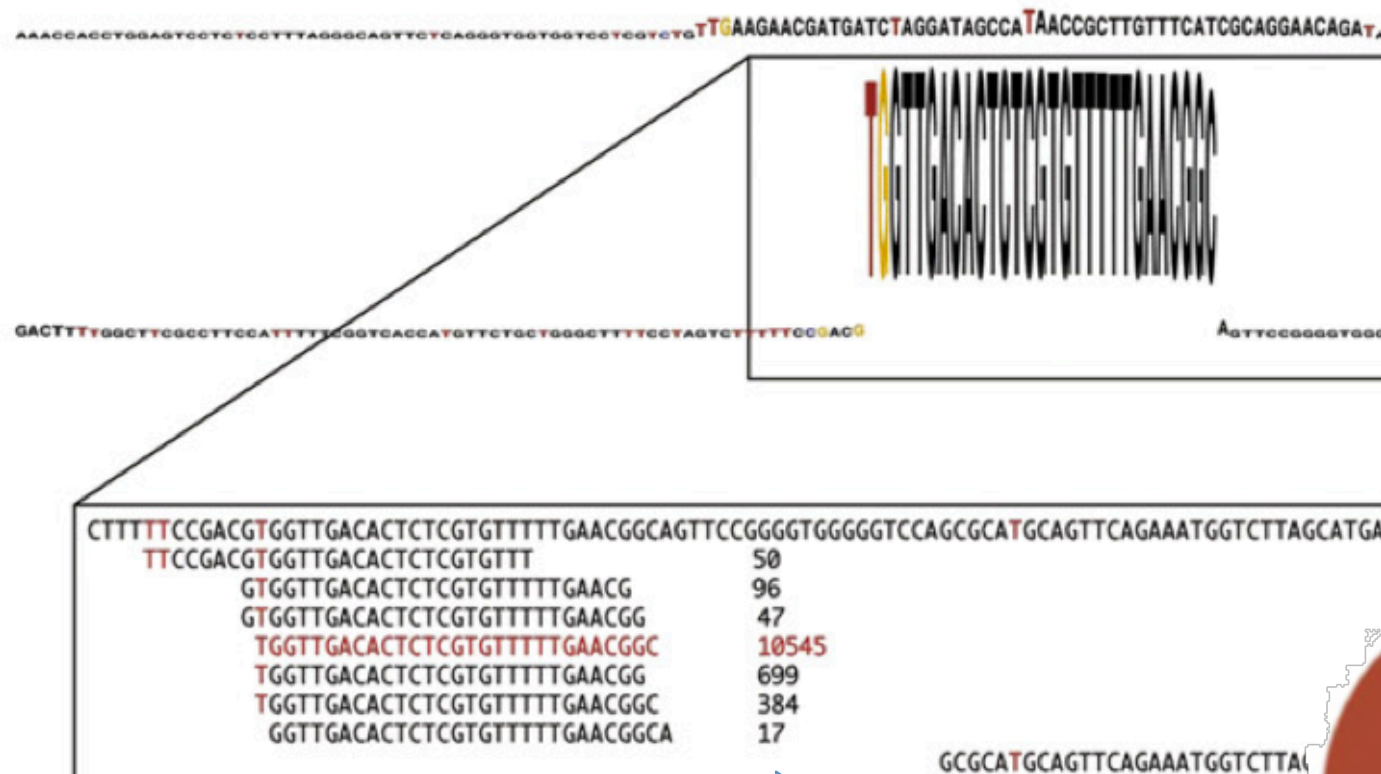
Identification of Neuronal piRNAs in *Aplysia* that Stably Associate with Piwi in Nuclear Compartments

- To more comprehensively survey piRNA expression, ten (10) different small RNA cDNA libraries using barcoded adapters were made and subjected the libraries to deep sequencing using the Illumina platform :
 - CNS adult
 - CNS juvenile
 - other tissues: ...

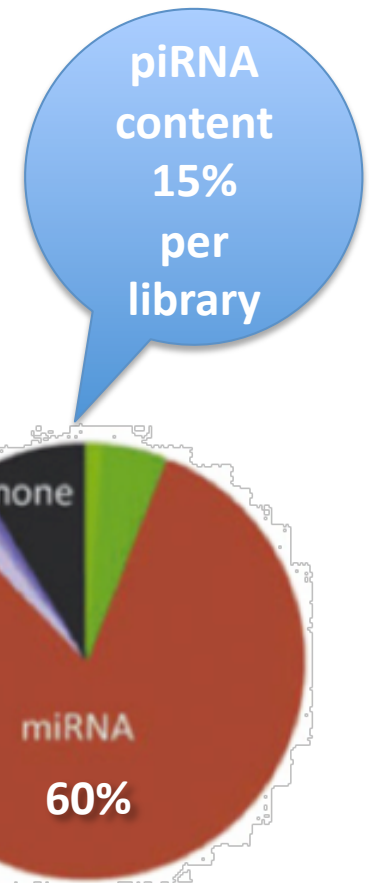
Illumina reads were mapped to *de novo* assembled contigs-scaffold(s)

RESULTS

Illumina deep sequencing reads mapped to *de novo* assembled scaffold



Region from the Figure 1B



RESULTS

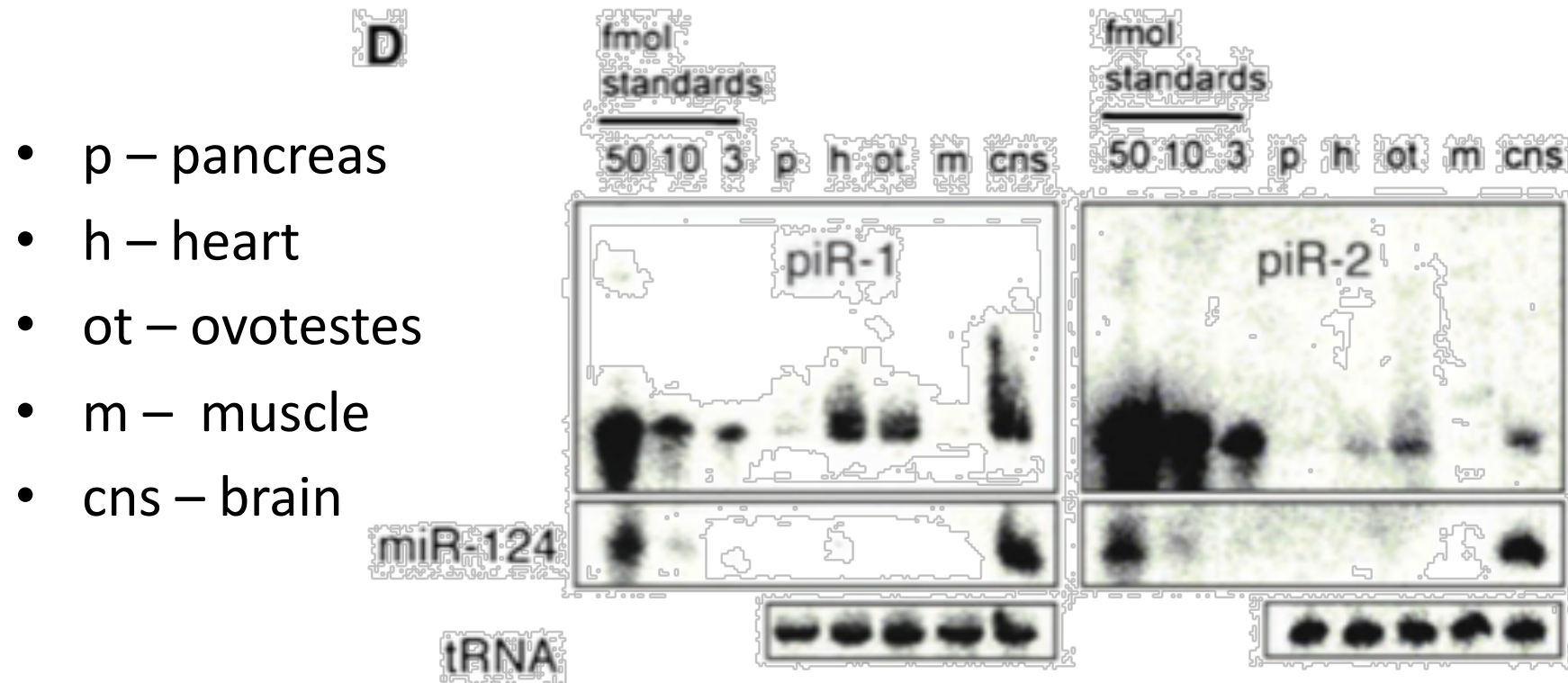
1) Prove that these piRNA's are genuine

A. Quantitative northern blots (PIR-1 & PIR-2)

B. To test, whether 3' ends are 2'-O methylated
– typical for piRNAs described in mammalian

RESULTS

To confirm the sequencing data, abundant piRNAs originating from two distinct clusters were analyzed by quantitative northern blots



>> These piRNA's are genuine

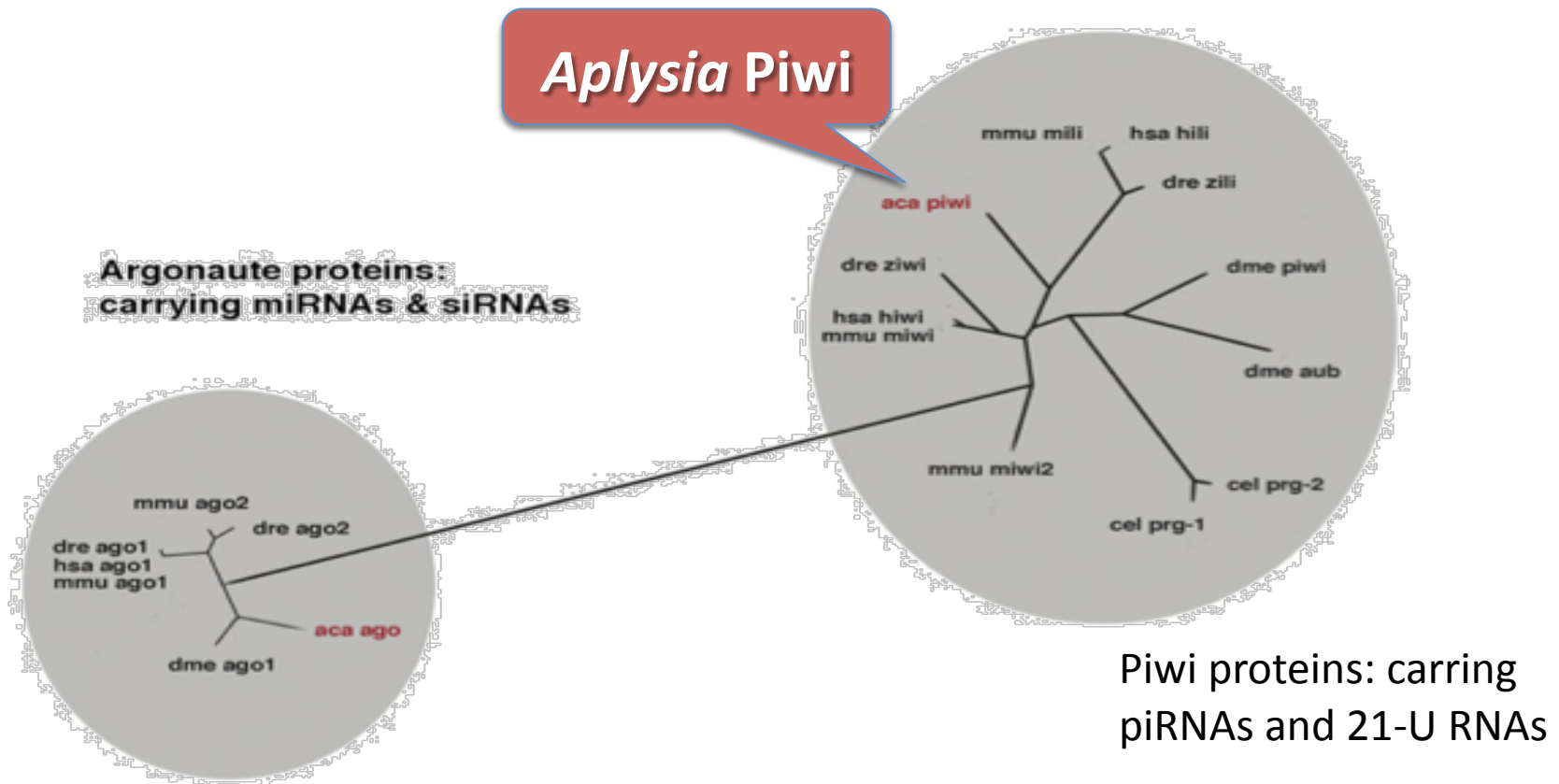
next

2) Elucidating biological role of
piRNAs in neurons

RESULTS

Aplysia Piwi Protein from CNS

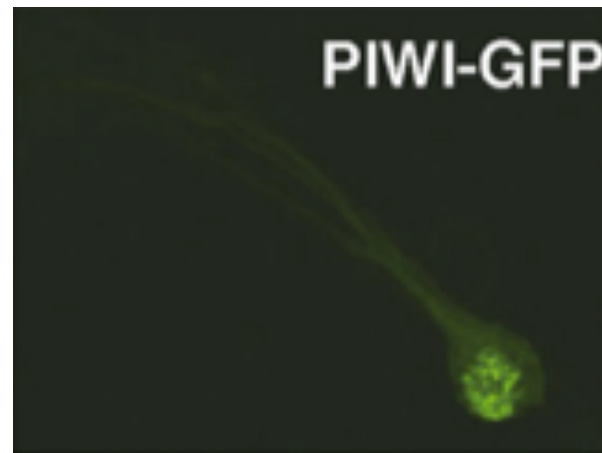
- The full-length cDNA for the **964 aa Piwi** protein was cloned
- A **polyclonal antibody** for the *Aplysia* Piwi protein was made
- The sequences of *Aplysia* Piwi protein is more closely related to vertebrate than invertebrate Piwi members



RESULTS

Piwi / piRNA interaction and cellular location

- Does Piwi protein stably interacts with piRNAs? *in vitro*
 - mix complex
 - Immuno precipitation with piwi antibodies (control miRNA + argonaut AB)
 - positive [+]
- Cellular localization *in vivo*
 - Over expression of GFP tagged Piwi protein reveals its **nuclear localization** in sensory neurons



*Pikaajaline mälu püsib aastaid –
komponendid uuenevad päevades
Kuidas on pikaajaline mälu võimalik?*

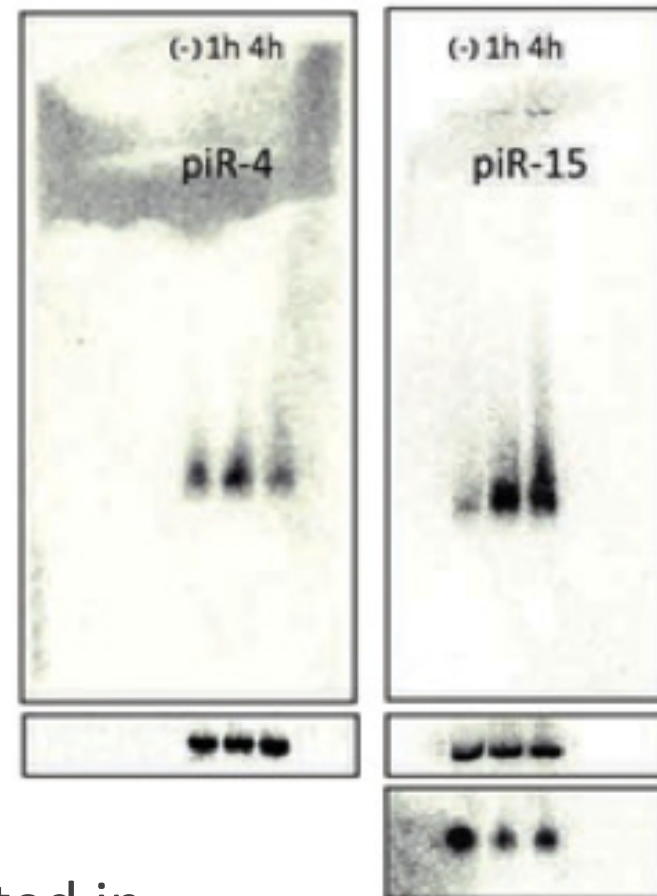
1. Prion-like proteins at synapses can adopt active, stable, and self-perpetuating conformations that preclude turnover of the protein (*Si et al., 2003, 2010; Bailey et al., 2004*)
2. Autoregulatory and positive feedback loops within protein networks can allow persistent enzymatic activity of proteins (*Lisman, 1985; Hayer and Bhalla, 2005; Song et al., 2007; Serrano et al., 2008*)
3. Epigenetic mechanisms such as DNA methylation can alter gene expression and thus the intrinsic properties of neurons in a long-term fashion, perhaps on the order of years (*Crick, 1984; Davis and Squire, 1984; Weaver et al., 2004; Miller et al., 2010; Feng et al., 2010*)

RESULTS

Serotoniin indutseerib piRNA'de eksperssiooni

vaata ka Figure 3B

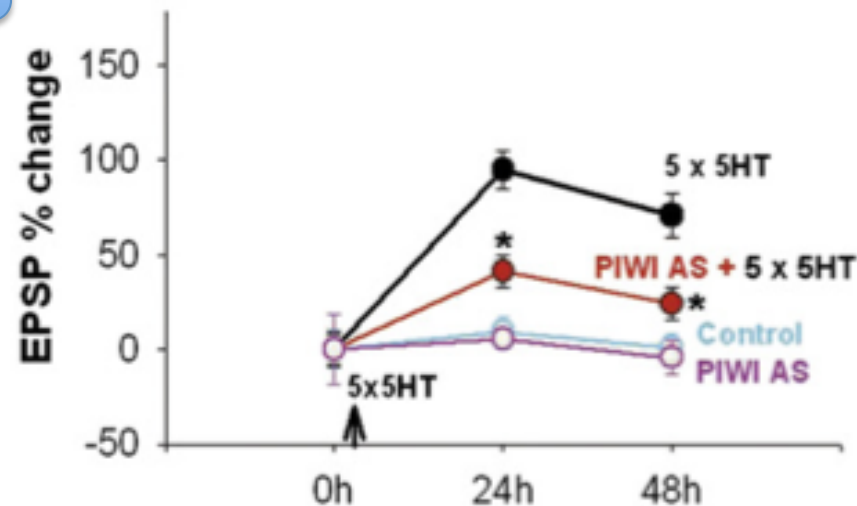
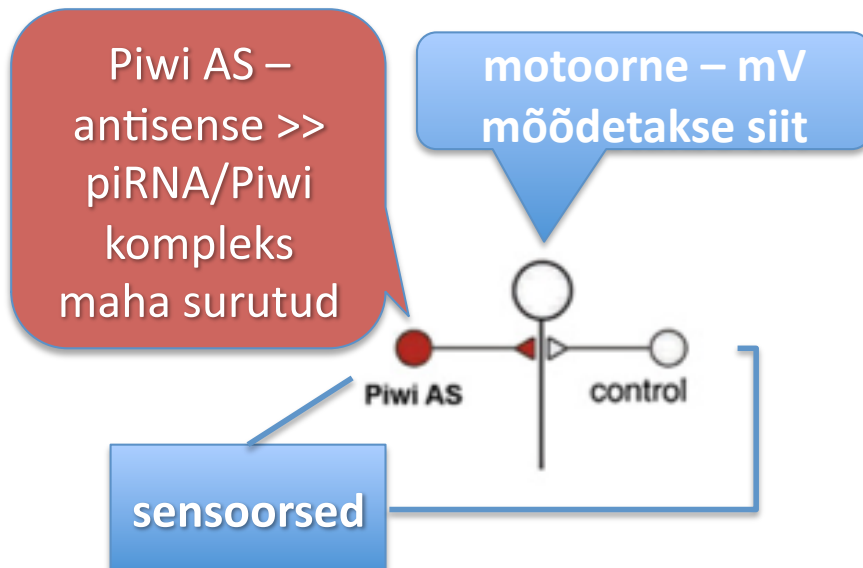
- Serotonin (5HT), a neuromodulator that is important for learning and memory
- A subset of the selected piRNAs was significantly upregulated (Figure 3A) aca-piR-4 and aca-piR-15 are examples of piRNAs that were robustly induced by 5HT



miRNAs were rapidly downregulated in neurons in response to neuromodulators and to neuronal activity (Rajasethupathy et al., 2009).

RESULTS

piRNAs role in memory-related synaptic plasticity in cultured neurons – response to 5HT

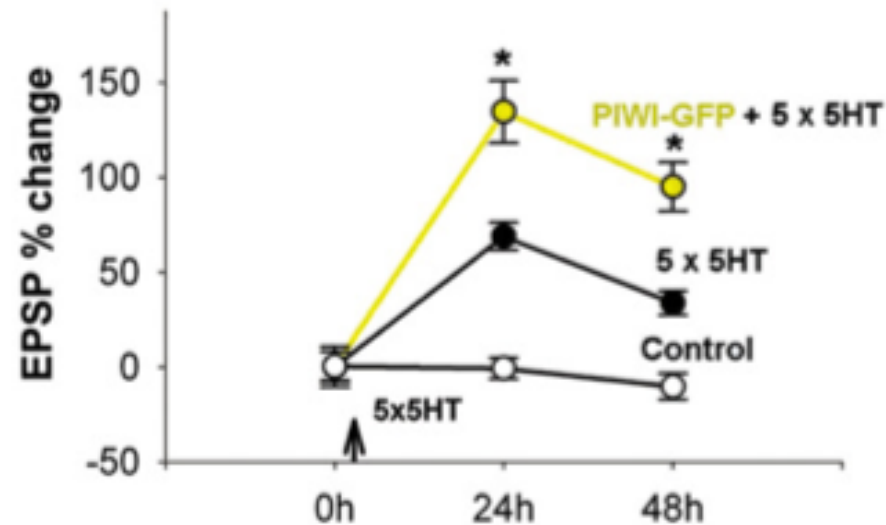
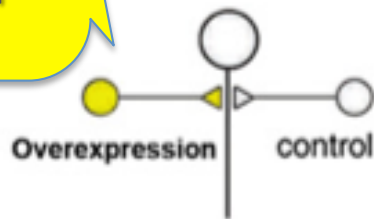


- The cocultures used in these experiments consisted of **two sensory** neurons that each synapse on a **single target motor** neuron
- *Mõõdetakse mV e. elektrilist pinget ja arvutatakse >*
- **EPSP** – excitatory postsynaptic potential
- **LTF** – memory-related long-term facilitation (LTF)

RESULTS

Overexpression of Piwi had the opposite effect

Piwi GFP
üleekspress-
seeritud
piRNA/Piwi
kompleksi
on palju



- Overexpression of Piwi-GFP (n = 22) caused a significant enhancement of 5HT-dependent long-term synaptic facilitation with respect to untreated controls (n = 40) as measured at 24 and 48 hr
- $F(2,78) = 44.04$; $p < 0.001$ repeated measures ANOVA; $p < 0.001$ Newman-Keuls post hoc test at both 24 and 48 hr

RESULTS

Overexpression of Piwi had the opposite effect

Piwi GFP

üleeksp

seerit

piRNA/

kompl

on pa

**TAKEN TOGETHER, THEY CONCLUDE
THAT SEROTONINE INDUCES THE
ACTIVITY OF PIWI-ASSOCIATED
PIRNAS, WHICH IN TURN ACT TO
ENHANCE LTF**

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- $F(2,78) = 44.04$; $p < 0.001$ repeated measures ANOVA; $p < 0.001$ Newman-Keuls post hoc test at both 24 and 48 hr

RESULTS

3) Which gene(s) is/are regulated through Piwi/piRNA?

- They screened many (i. e. antibodies were available for protein) plasticity-related genes for changes in expression levels after knockdown of Piwi
 - ✓ Assay – pleural ganglia were incubated with antisense 2' -O-methyloligoribonucleotides conjugated with penetratin to inhibit Piwi. Proteins extracted
 - ✓ Detection method – Western plot
 - ✓ Evidence – in protein level detected with antibodies

RESULT - inhibition of Piwi led to a reproducible 2-fold up-regulation of the **CREB2**
(also supported on mRNA level)

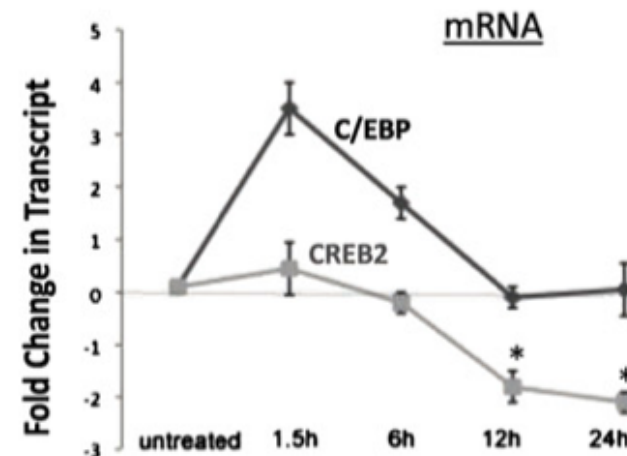
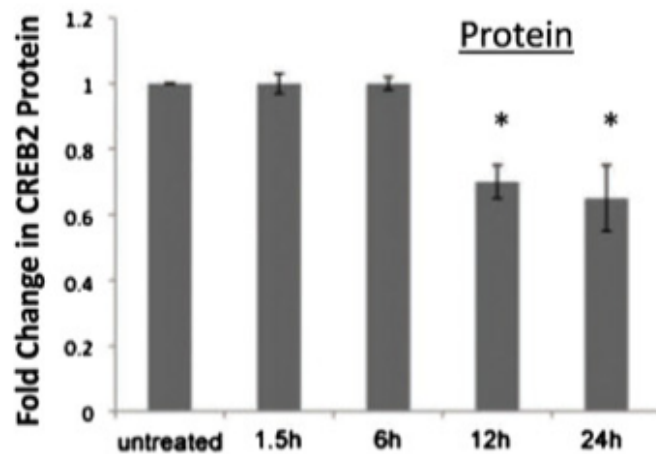
RESULTS

4) Elucidating the mechanism of CREB2 regulation by Piwi

- Piwi have nuclear localization
- Piwi have effect on both CREB2 protein and RNA levels, therefore
- Whether 5HT acts on CREB2 at the level of transcription?

RESULTS

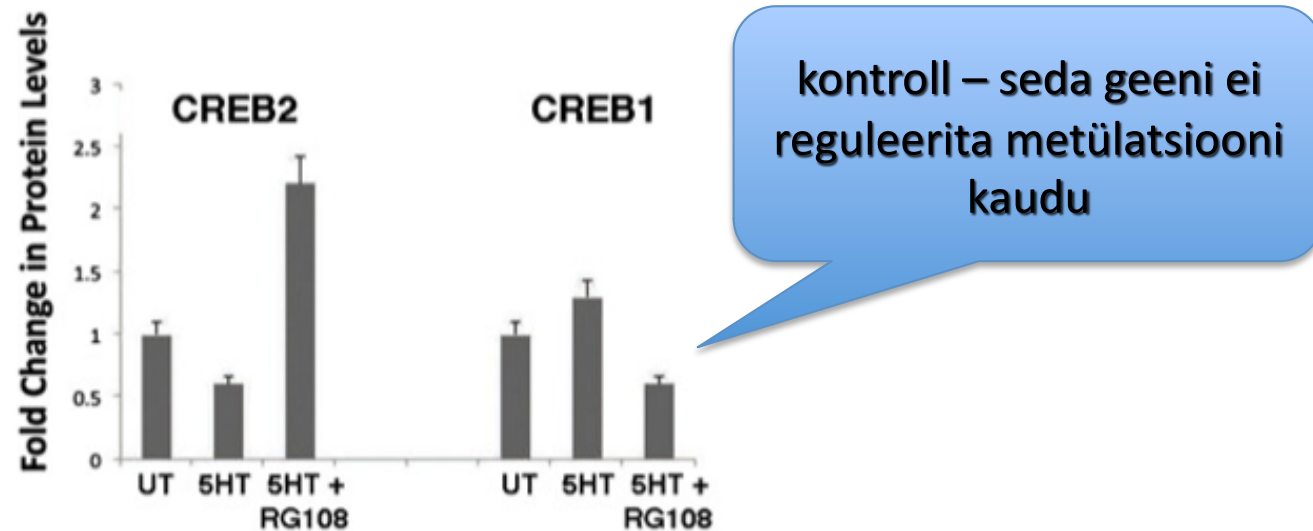
Elucidating the mechanism of CREB2 regulation by Piwi



- Protein levels begin to drop at 12 hr and continue to remain low for up to 48 hr without returning to the initial baseline level of expression - ***a stable 5HT-dependent repressive state is established***
- Piwi and piRNAs have known roles in epigenetic regulation in the germline through **DNA methylation**.

RESULTS

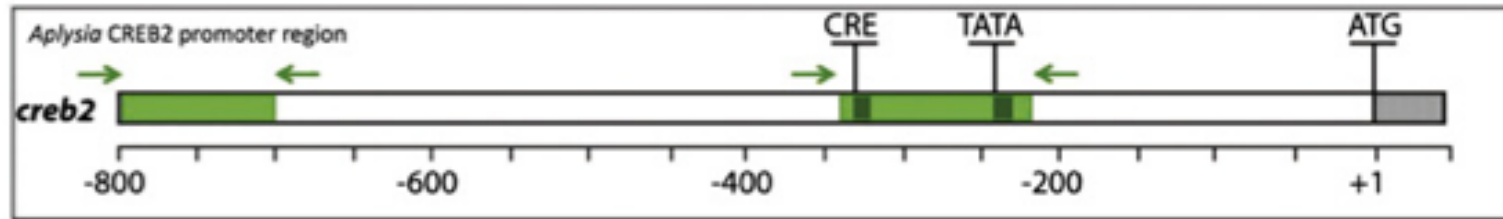
Inhibiting Aplysia DNA methyltransferase (DNMT) by inhibitor RG108



- in the presence of 5HT, 12 hr later, the 5HT-dependent long-lasting downregulation of CREB2 was abolished.

RESULTS

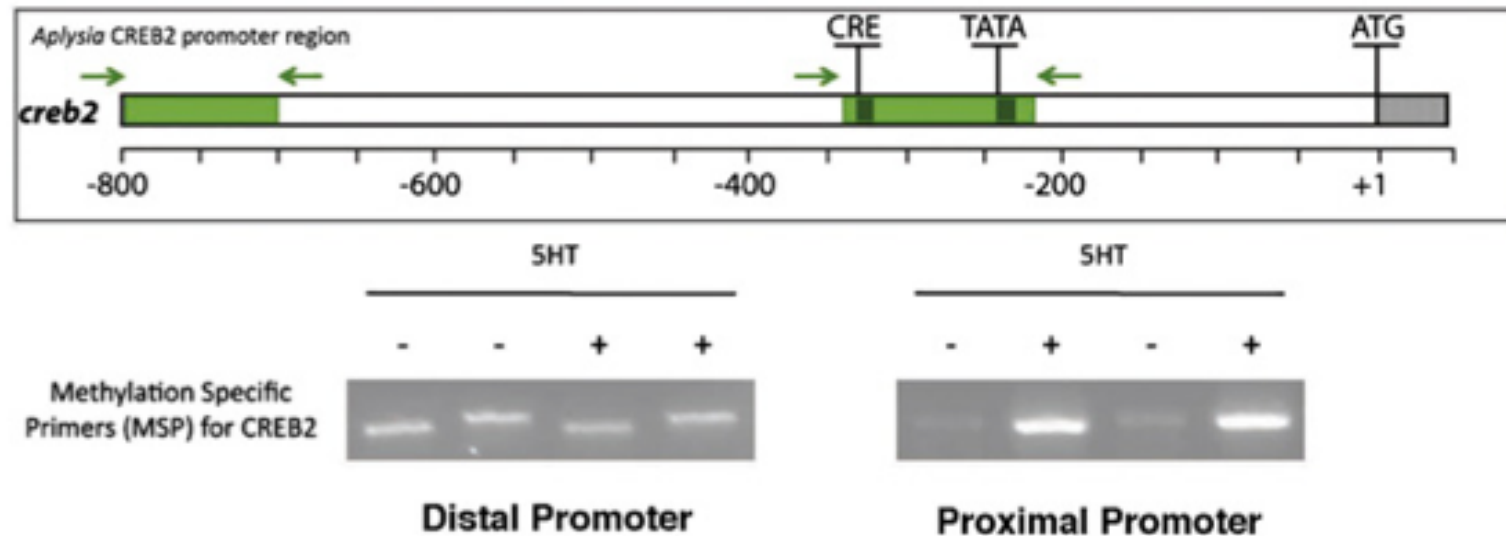
Two predicted CpG islands in CREB2 leader/promoter region



- They found two predicted CpG islands, one that is proximal to the translational start (~200 bp upstream) site and other is more distal (~700 bp upstream)

RESULTS

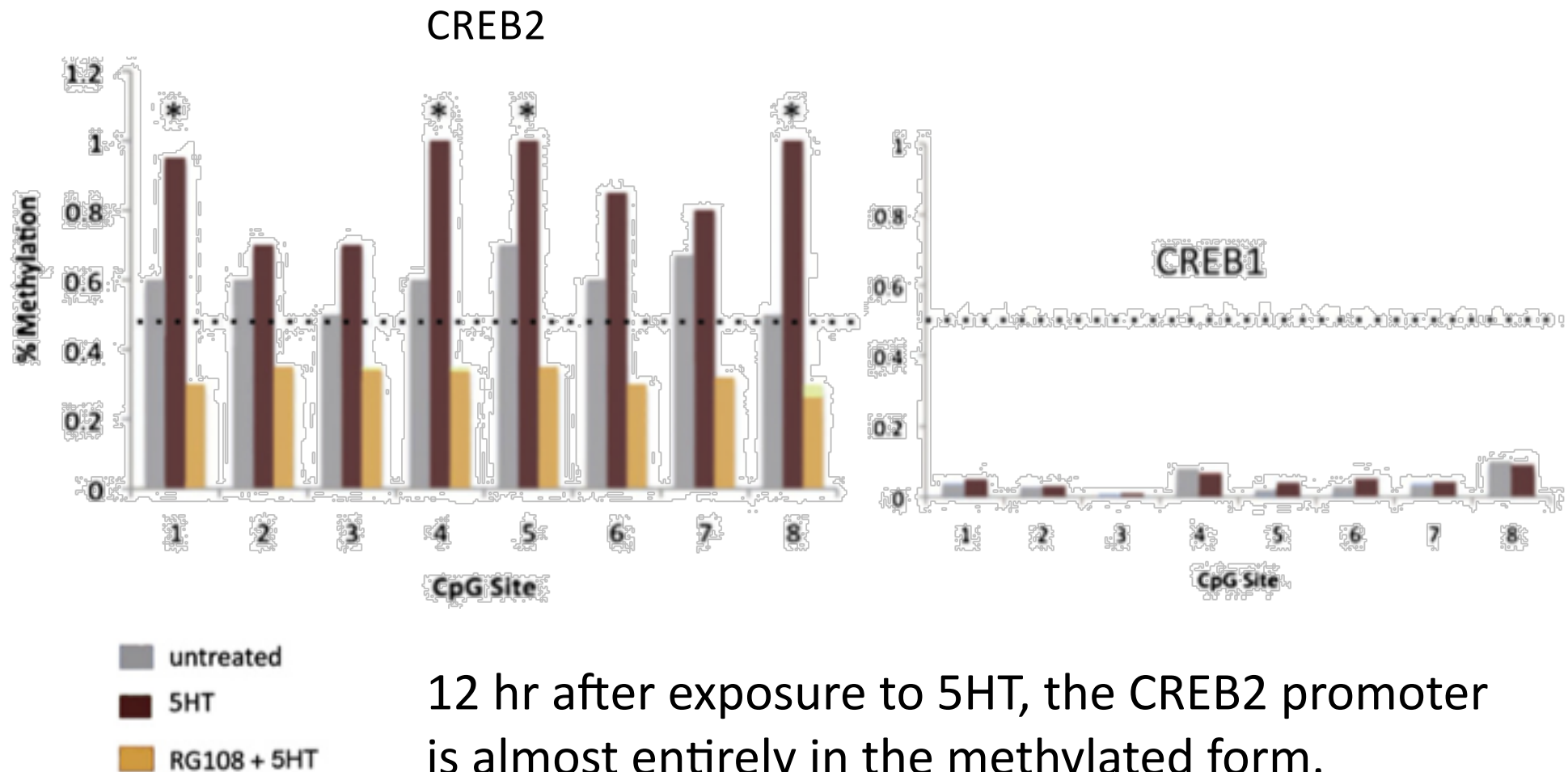
CREB2 Is Methylated at Its Promoter in Response to 5HT-Induced Synaptic Plasticity



- They used methylation-specific primers (MSP; designed to detect only the methylated copies of genomic CREB2) to estimate proportion of methylated DNA

RESULTS

CREB2 Is Methylated at Its Promoter in Response to 5HT-Induced Synaptic Plasticity



RESULTS

3) Which gene(s) is/are regulated
through Piwi/piRNA?

>>

CREB2 gene

baseline is 50% methylated

incubating with 5HT leads to 100% methylation

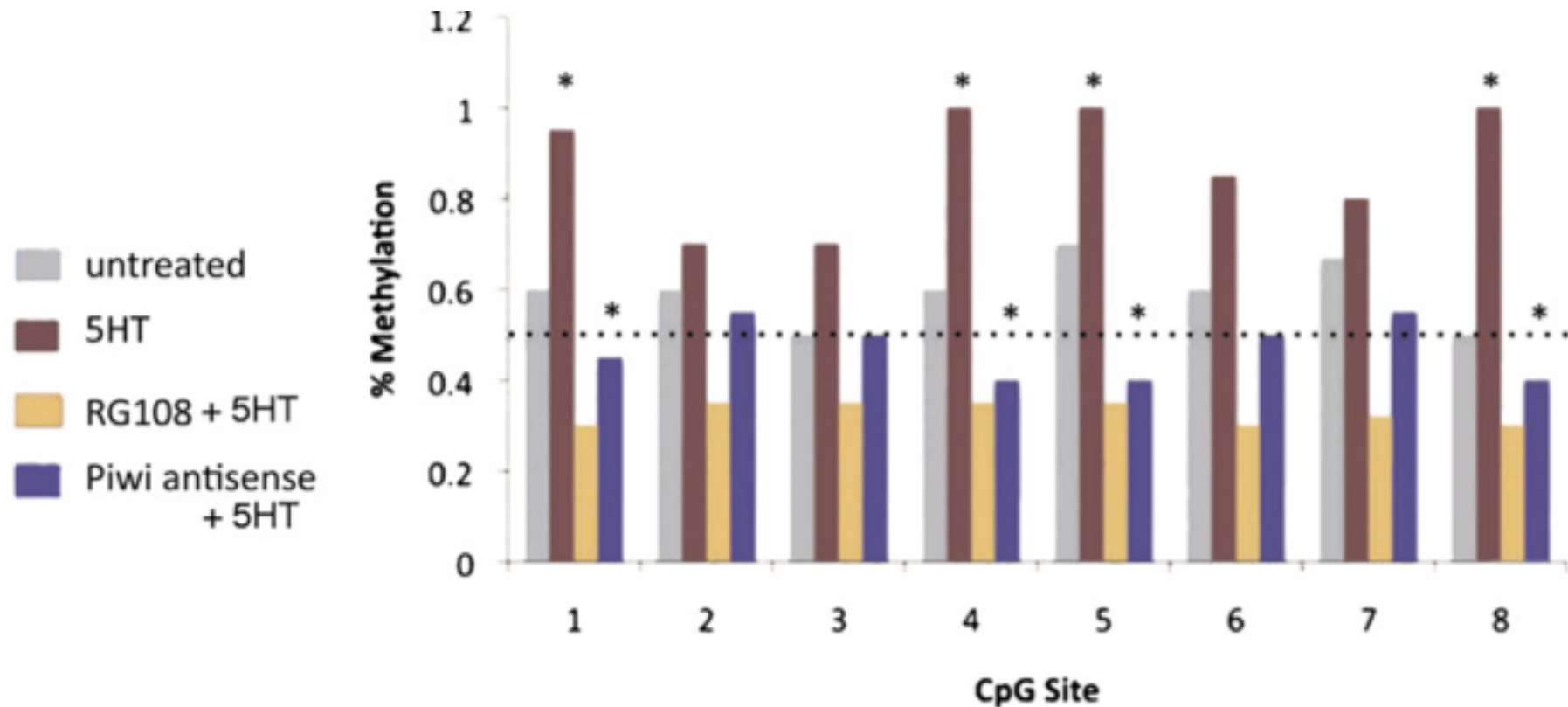
RESULTS

4) Which components exactly was required for the observed serotonin-dependent methylation of CREB2 in neurons?

- Whether Piwi was required?
- Which piRNA was required?

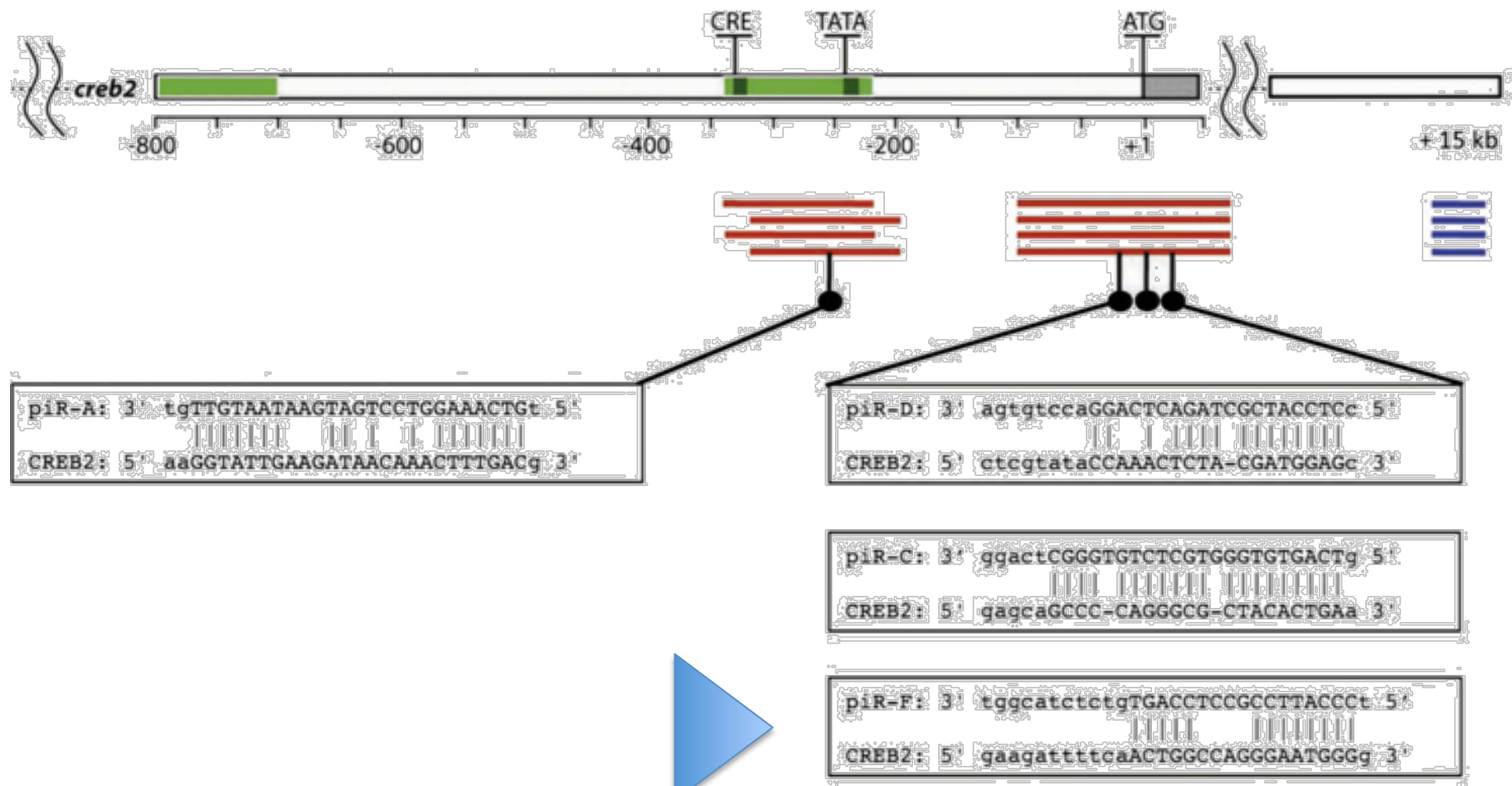
RESULTS

inhibition of Piwi completely abolished the serotonin-dependent increase in methylation at the promote



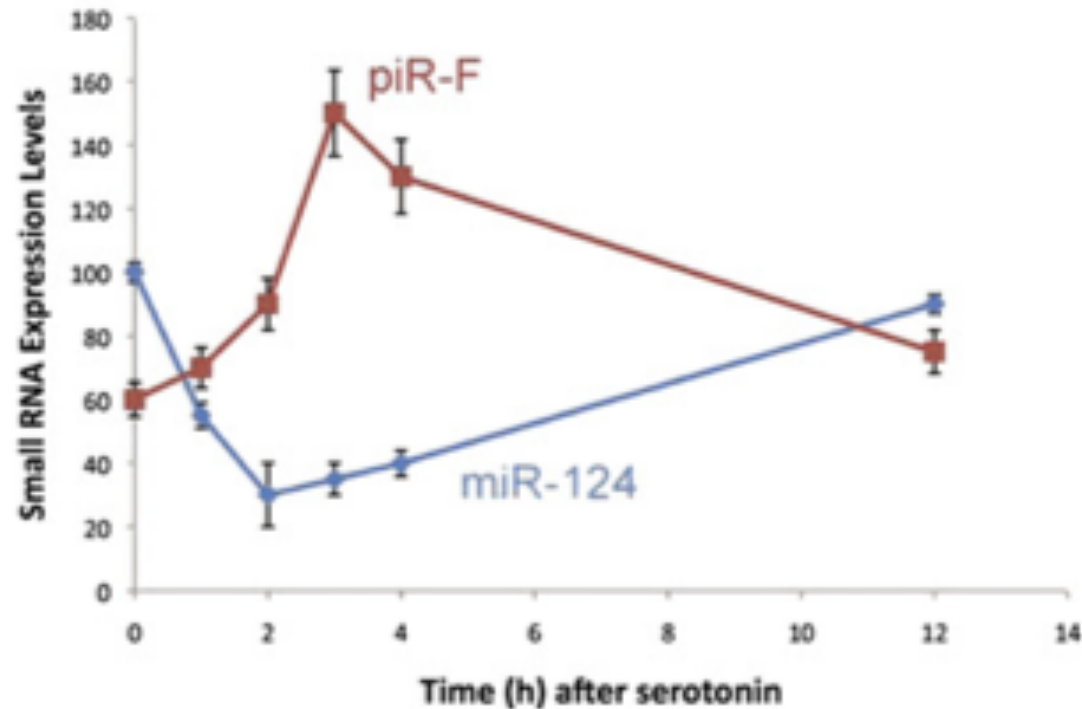
RESULTS

Through a series of knockdown experiments using 2'-O-methyl oligoribonucleotides specific to each of the four piRNAs, they observed that piRNA **aca-piR-F** had the strongest effect on CREB2 expression



RESULTS

aca-piR-F levels peaked at 3–4 hr after exposure to 5HT and dropping back to baseline at 12 hr

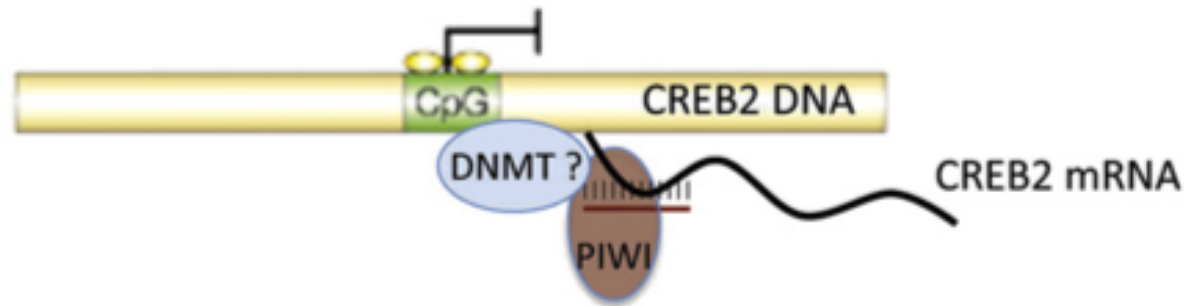


RESULTS

Piwi/piRNA Complexes Control the Methylation State of the CREB2 Promoter

proposed model

Piwi/piRNA complex binds the CREB2 nascent transcript:



The observed stable silencing of CREB2 by the Piwi/piRNA complex (Figure 6) but also noticed that when placed in the context of Figures 4A and 4B in which transient knockdown of Piwi reverses CREB2 silencing, is suggestive of active demethylation at the CREB2 promoter.

Conclusions

1. piRNA's in neurons are genuine
2. piRNA's interact with Piwi proteins and they have **nuclear localization** in sensory neurons
3. Piwi/piRNA Complexes Enhance Memory-Related Synaptic Plasticity by Regulating the Transcriptional Repressor **CREB2**
4. **CREB2** Is Methylated at Its Promoter in Response to 5HT-Induced Synaptic Plasticity
5. Piwi/piRNA Complexes Control the Methylation State of the **CREB2** Promoter

TÄNAN TÄHELEPANU EEST!