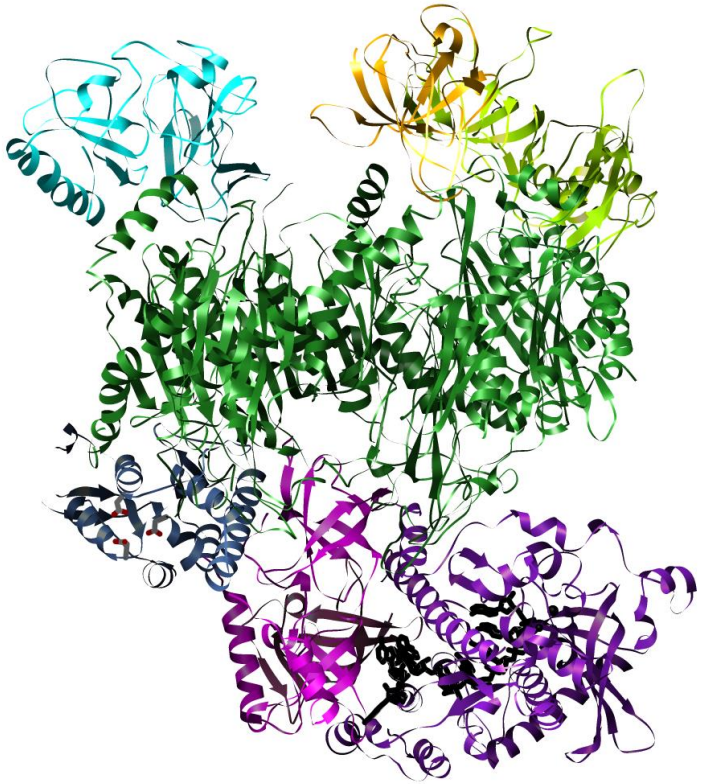
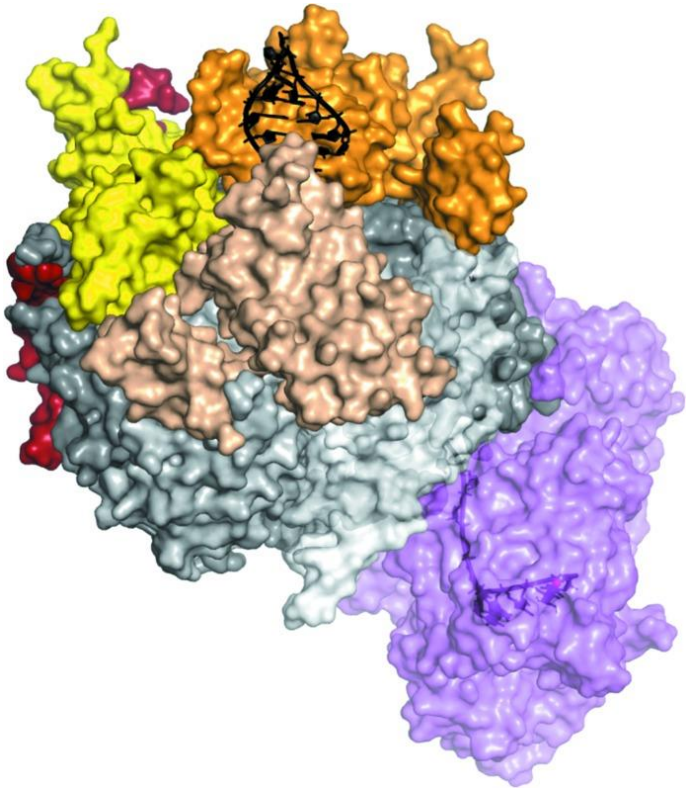
Biochemical methods for analysis of activities and functions of enzymes involved in RNA metabolism (with particular emphasis on the exosome complex)



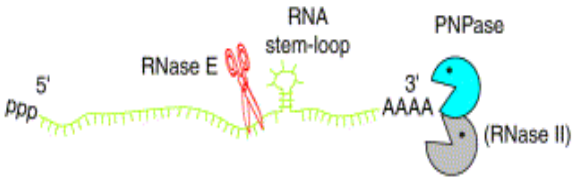


Rafał Tomecki
Laboratory of RNA Processing and Decay IBB PAS and Faculty of Biology UW

Lecture within „Molecular techniques of RNA analysis" course; 10.01.2025

RNases involved in RNA metabolism

Endoribonucleases: *e.g.* RNase E (prokaryotes)



Exoribonucleases

5′→3′: processive hydrolytic Xrn1
3′→5′: processive (exosome core with Dis3) or distributive (Rrp6)

- processive phosphorolytic: PNPase (prokaryotes; organelles of eukaryotic cells) and exosome complex in *Archaebacteria*
- processive hydrolytic: RNase R / RNase II family

Hydrolysis : RNA + H_2O → ribonucleoside monophosphates (rNMP)

Phosphorolysis : RNA + PO_4^- ↔ ribonucleoside diphosphates (rNDP)

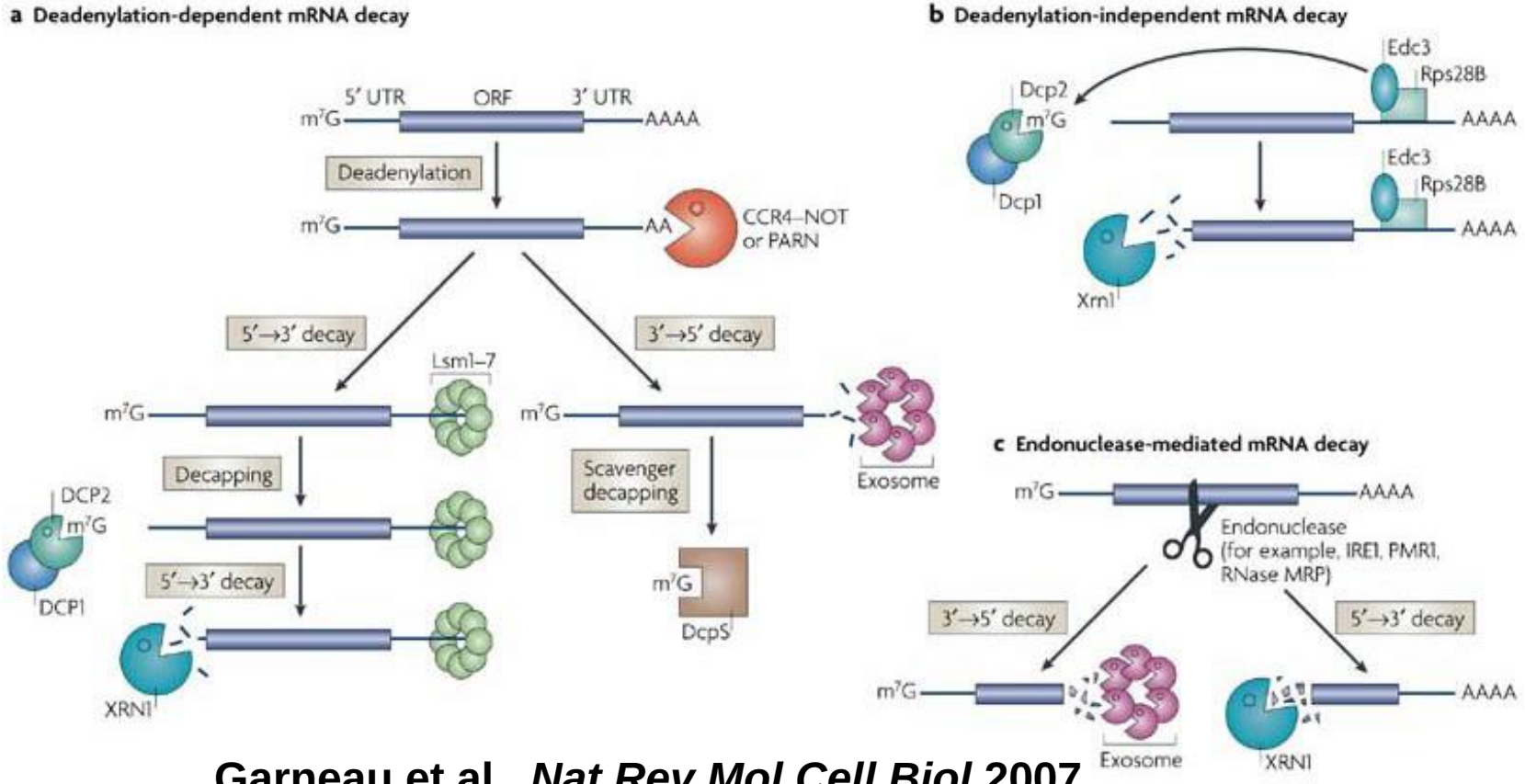
H_2O; PO_4^- - nucleophiles attacking phosphodiester bond
Catalysis occurs in the presence of divalent cation (Mg^{2+}, Mn^{2+}, Zn^{2+}) as a cofactor (activation of the nucleophilic attack)

Eukaryotic mRNA metabolic pathways

mRNA decay in the cytoplasm is usually initiated through poly(A) tail removal (deadenylation). This reaction (distributive) is controlled by a large protein complex referred to as Ccr4-Not1. After deadenylation, mRNA can be degraded via two different pathways:

– in the 3'-5' direction (exosome complex)

– in the 5'-3' direction (5' cap removal by decapping complex and Xrn1p exoribonuclease activity)



Garneau et al., *Nat Rev Mol Cell Biol* 2007

Major eukaryotic RNA-degrading enzymes

Xrn1 – 5'-3'

The enzyme

working on its own

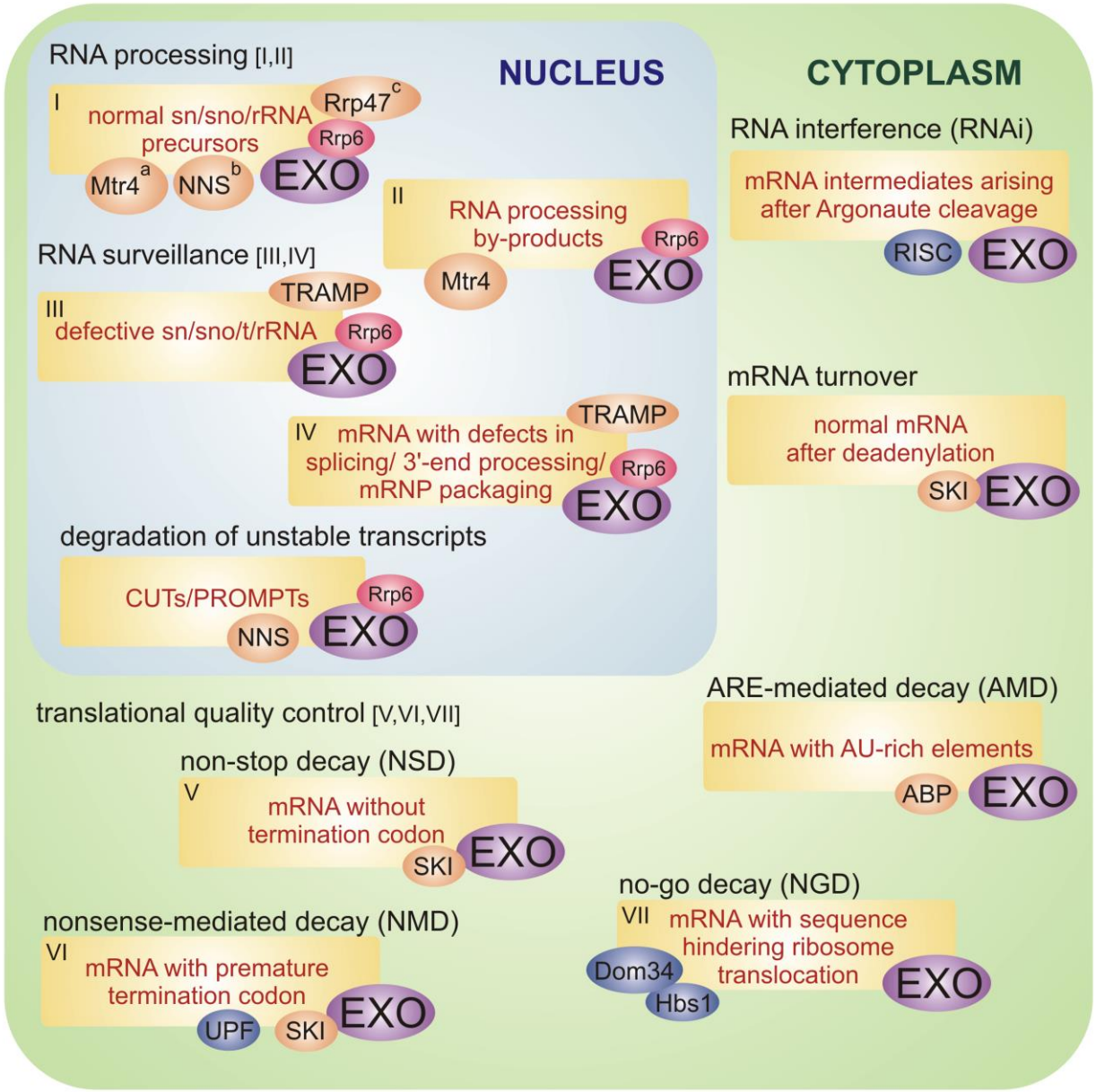
Chang et al.,
***Nat Struct Mol Biol* 2011**

Exosome – 3'-5' Large multiprotein complex

In the yeast nucleus collaborates with the TRAMP complex (poly(A) polymerase Trf4/5, RNA helicase Mtr4 and RNA-binding protein Air1/2); human counterpart – NEXT complex

In the yeast cytoplasm co-operates with putative GTPase Ski7p and with the SKI complex, composed of Ski2p RNA helicase and two additional proteins (Ski3p and Ski8p)

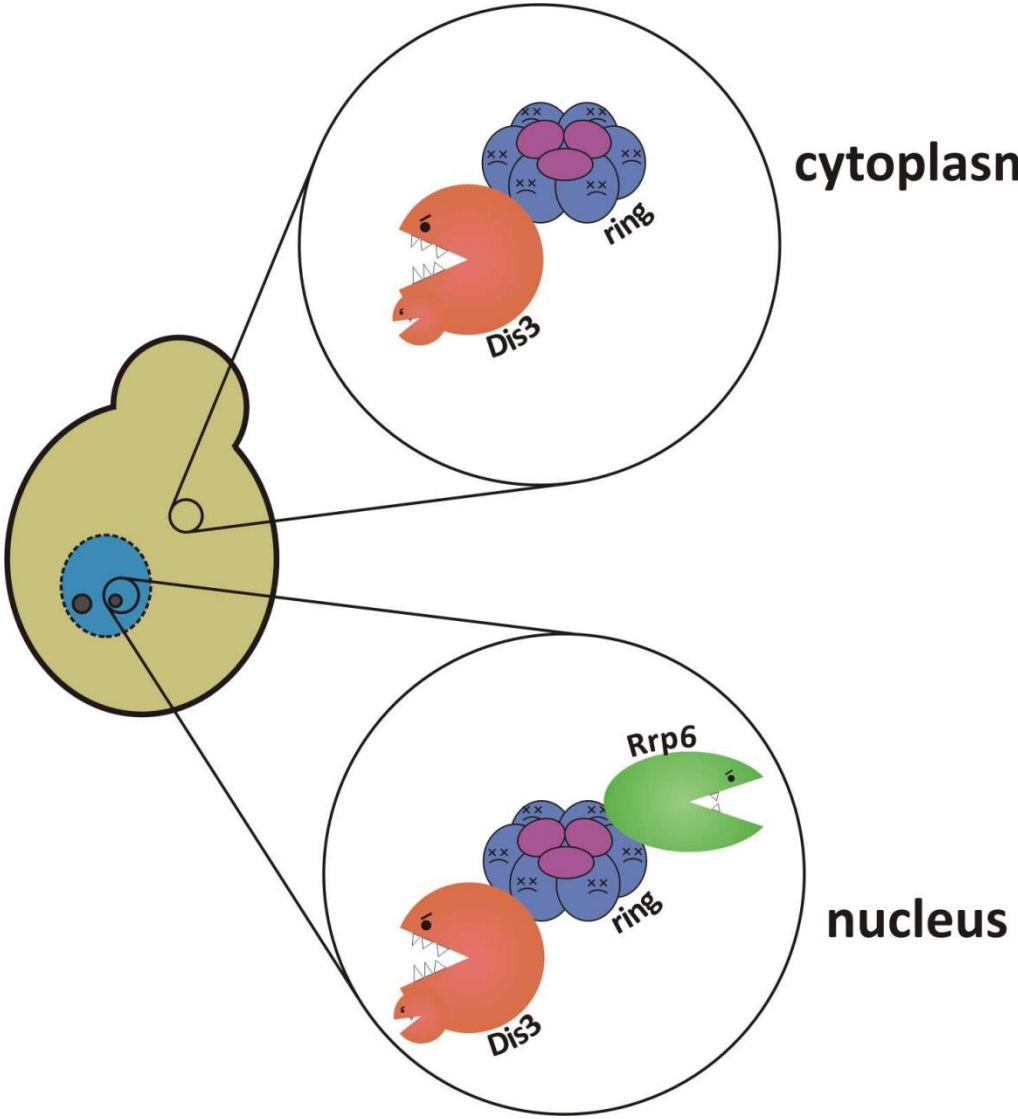
Exosome is a large 400 kDa protein complex with 3’-5’ exoribonuclease activity



The only essential 3’-5’ exoribonuclease in yeast, involved in a multitude of RNA metabolic processes, both in the nucleus and the cytoplasm

Tomecki et al., *Chembiochem* 2010

Subunit composition and intracellular localization of exosome complexes in yeast



Chlebowski et al.,
in: „RNA exosome”,
ed.: T.H. Jensen;
Landes Bioscience 2010

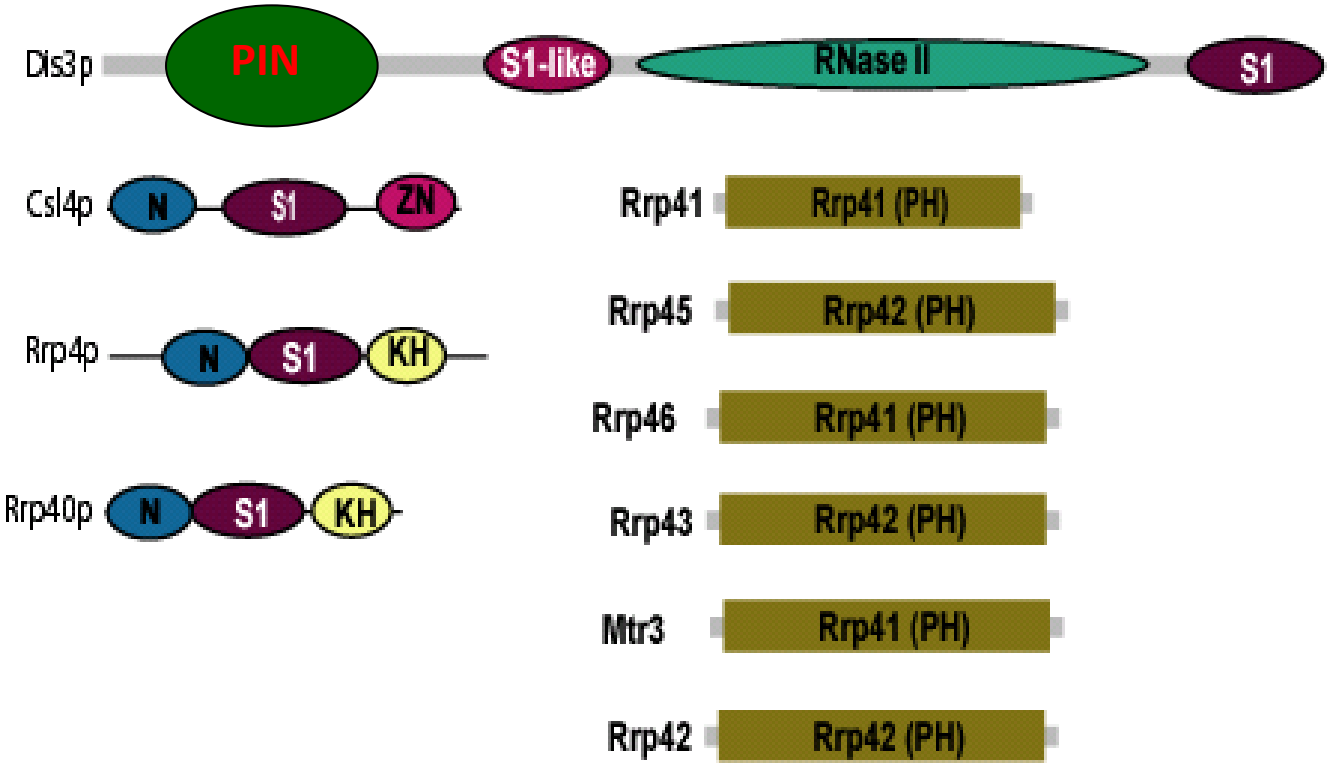
S. cerevisiae exosome core is a chimera composed of:

a) 6-subunit complex reminiscent of the ring of RNase PH/PNPase of archaebacterial origin;

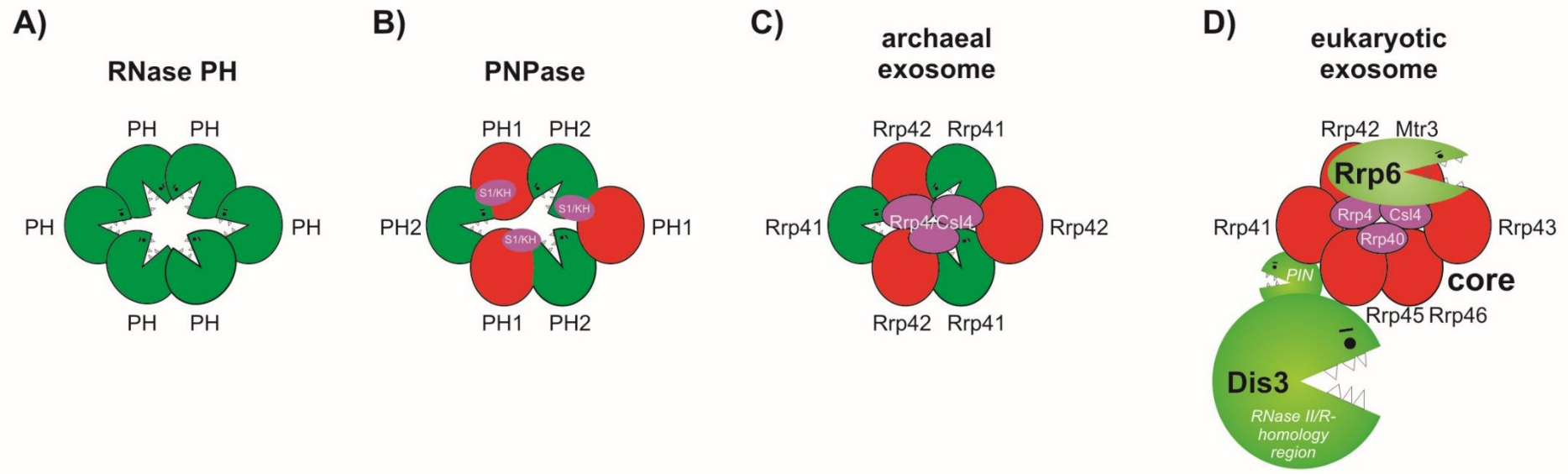
b) 3 subunits encompassing RNA-binding domains (KH and S1), which are also present in the bacterial PNPase;

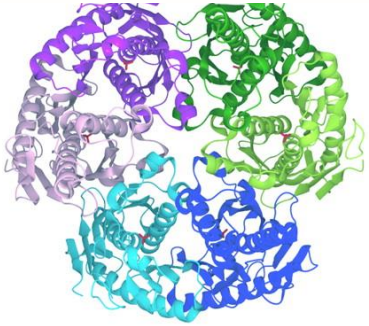
c) RNase II/R homolog, **Dis3/Rrp44** (the only catalytic subunit of the core)

All exosome core subunits are **essential** in yeast

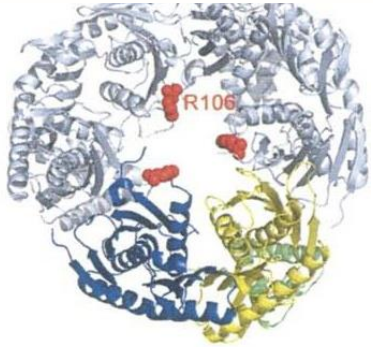


Nucleases containing RNase PH domains participate in RNA metabolism in organisms representing all kingdoms of life

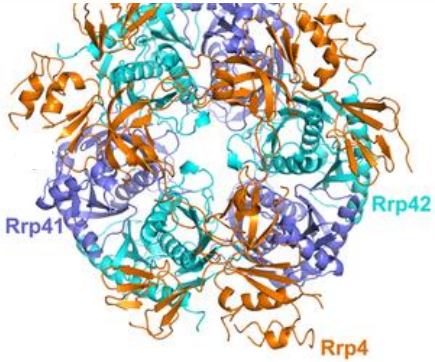




Ishii et al.,
J Biol Chem 2003



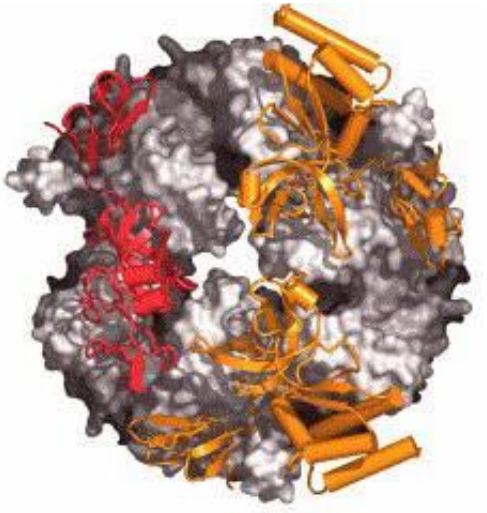
Shi et al.,
RNA 2008

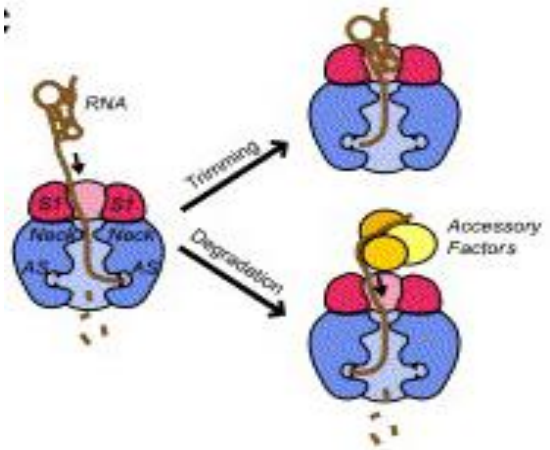


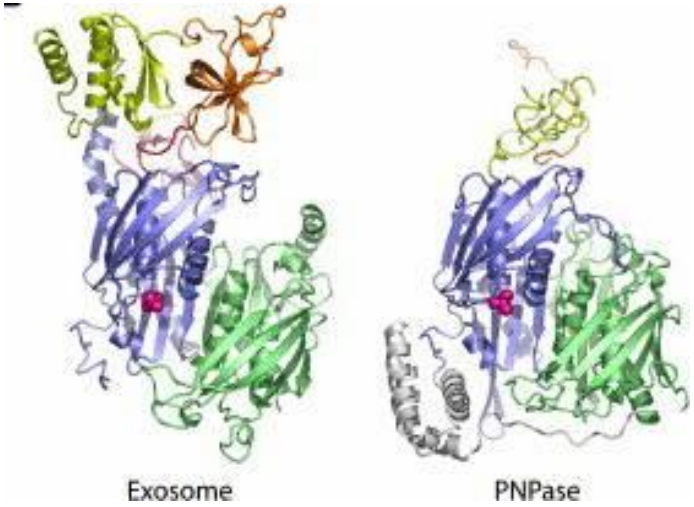
Lu et al.,
PLoS One 2008

What is the activity of the exosome complex ?

Solved crystal structures of the archaebacterial exosome complexes appeared similar to PNPase. It was suggested, that the mechanism of action of the yeast exosome may be similar to the activities of PNPase and archaebacterial exosome







Büttner et al., *Mol Cell* 2005

It was even speculated that each of the 10 subunits of the yeast exosome may display some catalytic activity

THIS IS NOT TRUE!

Biochemical ribonuclease activity assays - the exosome complex as an example [ENZYMES]

- obtaining material for research: purification of complexes or individual proteins from host cells (e.g. using TAP-tag) **OR/AND** heterologous overexpression in bacteria, and their purification in a recombinant form (optionally reconstitution of the complex from purified recombinant proteins)

- opimization of reaction conditions for particular activity: *i.a.* type and concentration of divalent cation, buffering agent, salt concentration, temperature, reaction time

- the necessity of preparing variants of the protein of interest with potential catalytic center mutations as negative controls

- possibility of narrowing down the analysis to the putative catalytic domain in case the full-length protein turns out to be insoluble

Biochemical ribonuclease activity assays - the exosome complex as an example [SUBSTRATES]

- analyzing RNA substrates labeled in various ways (at the 5’ or 3’ end; internally)

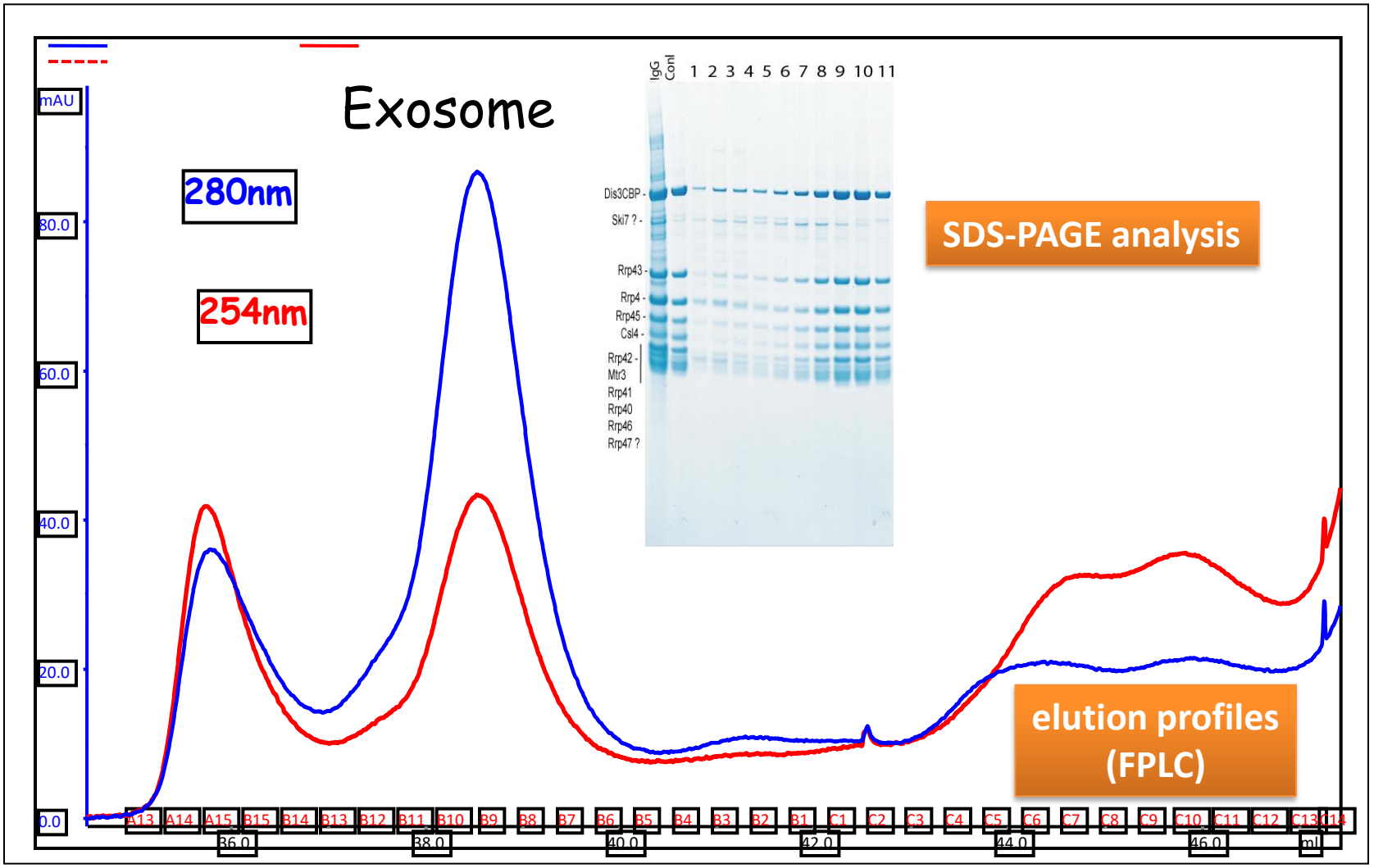
- analyzing degradation of the substrates with different structure (single-stranded: linear or circular; double-stranded)

- testing the degradation of both synthetic oligoribonucleotides and natural RNA substrates obtained in the *in vitro* transcription (IVT) reaction

- **MATCHING RESULTS OF THE *in vitro* BIOCHEMICAL ANALYSES WITH STRUCTURAL DATA AND RESULTS OF *in vivo* EXPERIMENTS**

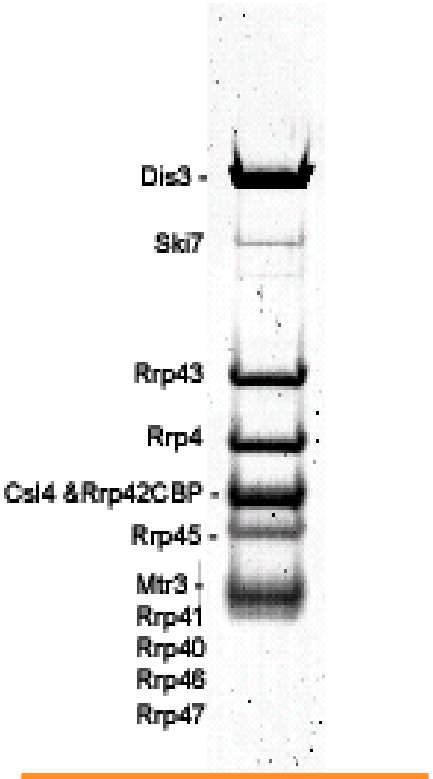
Exosome purification from yeast

Purification on IgG resin (Dis3 protein with TAP-tag as bait) + gel filtration (Superdex S-200 column). The yield was 200 µg of the purified complex per 18 litres of the yeast culture



Assaying the exosome biochemical activity

In order to assay the activity of the exosome core (9-subunit ring + Dis3p protein), the complex was purified using Rrp41-TAP fusion from the *S. cerevisiae* strain lacking *RRP6*.



SDS-PAGE analysis

In the preliminary experiments, no phosphorolytic activity was detected, but a very low hydrolytic activity was noticed. This forced the need for optimization of the parameters for assaying the biochemical activity of the complex.

Dziembowski et al., *Nat Struct Mol Biol* 2007

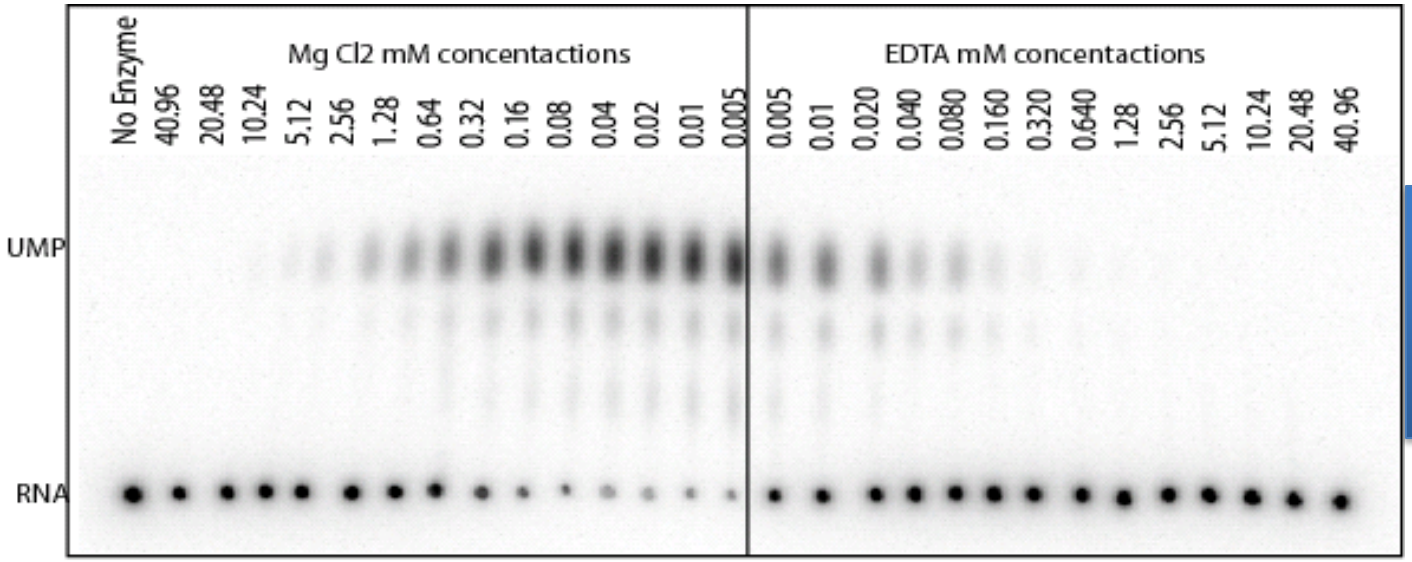
Exosome is a hydrolase

Exosome activity is dependent on the Mg^{2+} ions, but strongly inhibited at magnesium concentrations exceeding 1 mM.

An example of optimization of the divalent cation concentration in the reaction mixture

All previous *in vitro* experiments were carried out in conditions, in which the actual activity of the complex is *ca.* 100-fold lower than that in optimal conditions

No phosphorolysis (no evidence of UDP formation) was detected



TLC analysis

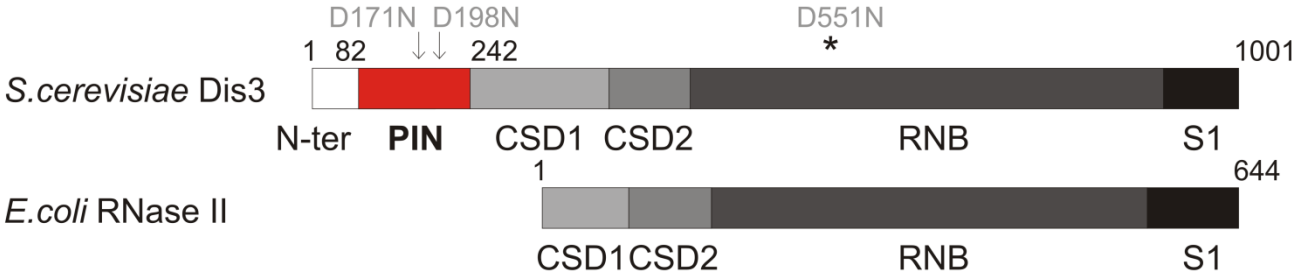
substrate synthesized by *in vitro* transcription using [^{32}P]-UTP

Analysis of the exosome activity using TLC (PEI-cellulose) at variable Mg^{2+} and EDTA concentrations (buffer: 10 mM Tris pH=8; 75 mM NaCl; 1 mM β-mercaptoethanol)

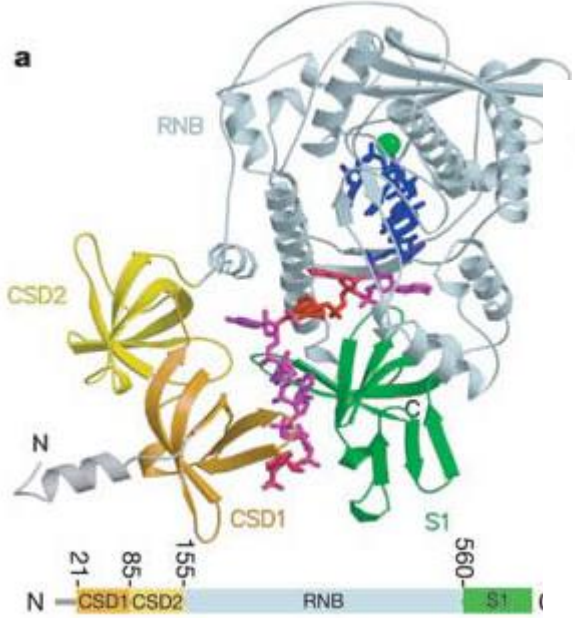
Dziembowski et al., *Nat Struct Mol Biol* 2007

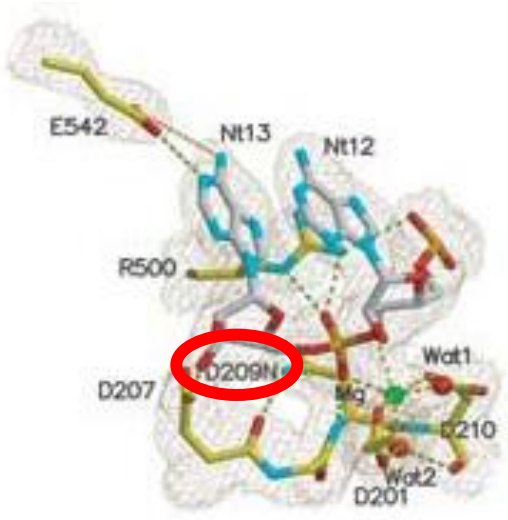
Dis3 – potential catalytic subunit

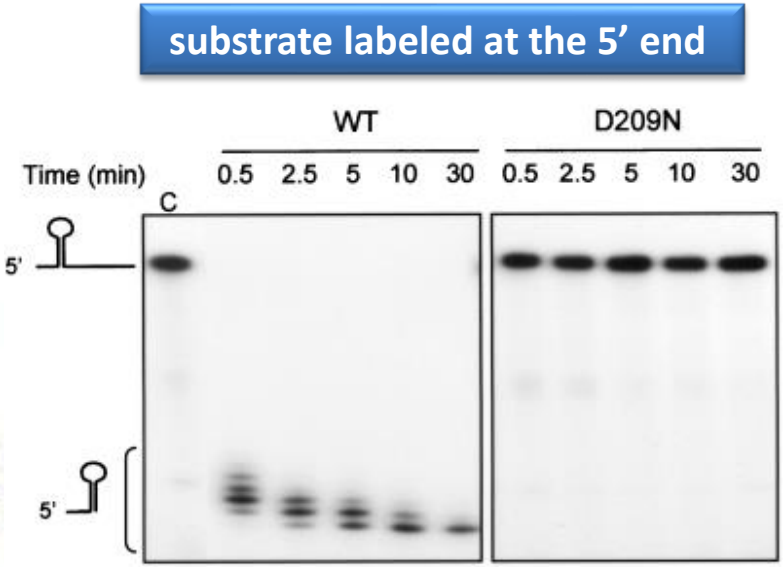
- **Organization of Dis3p functional domains in comparison to RNase II**



- meanwhile, a mutation abolishing *E. coli* RNase II (D209N) was identified [D209 residue is involved in the coordination of Mg^{2+} ion]





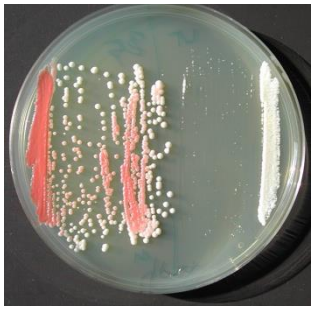


Frãzao et al., *Nature* 2006

PAGE analysis

Does an analogous mutation of the Dis3 protein affect the survival of yeast?

- homologous **aspartate (D551) in *DIS3*** was converted to asparagine by *in vivo* recombination in two yeast strains: wild-type (for phenotype analysis) and in strain with *RRP6* deletion (Δ*rrp6*) (for purification of the complex to be used in activity assays)

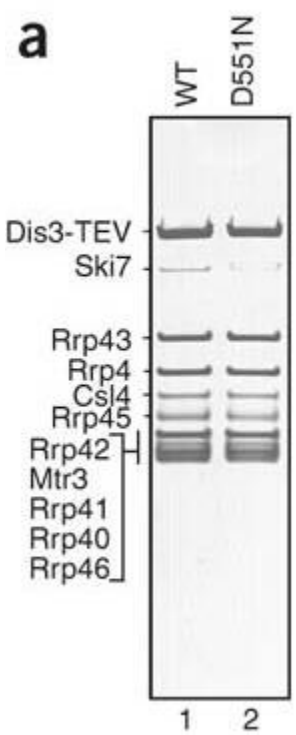
WT  **D551N**

DIS3 D551N substitution exerts a very strong growth phenotype

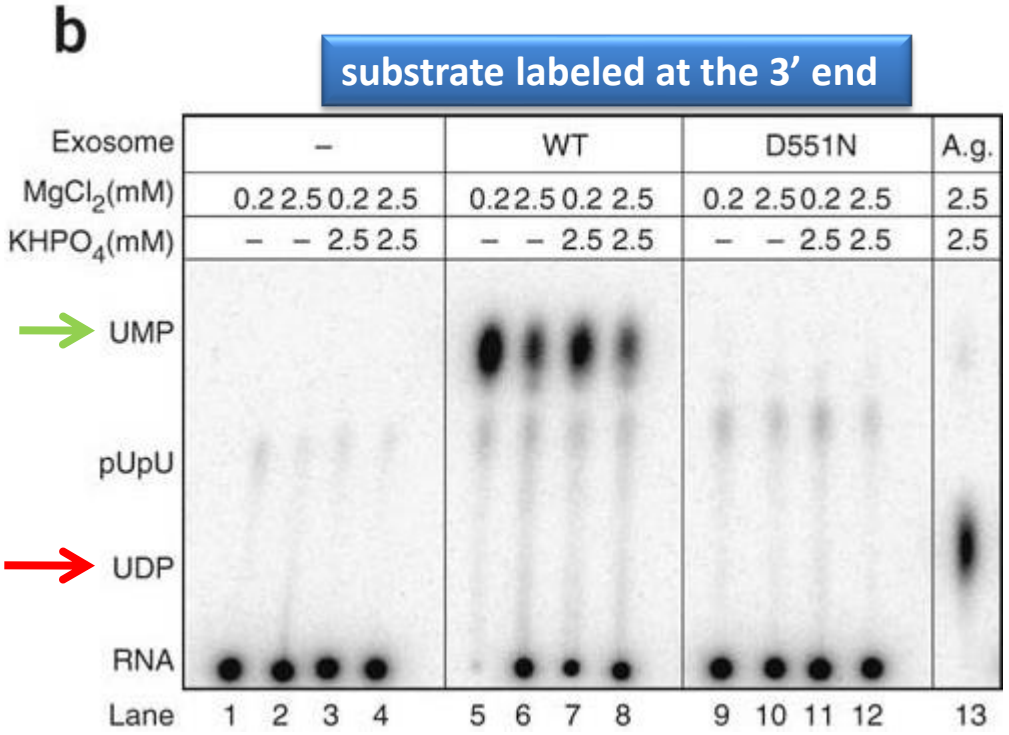
CONCLUSION: an intact D551 amino acid is indispensable for proper functioning of the cells

HYPOTHESIS: Dis3 D551N mutation abolishes catalytic activity of the exosome

Does D551N mutation abolish hydrolytic activity of the exosome?



SDS-PAGE analysis



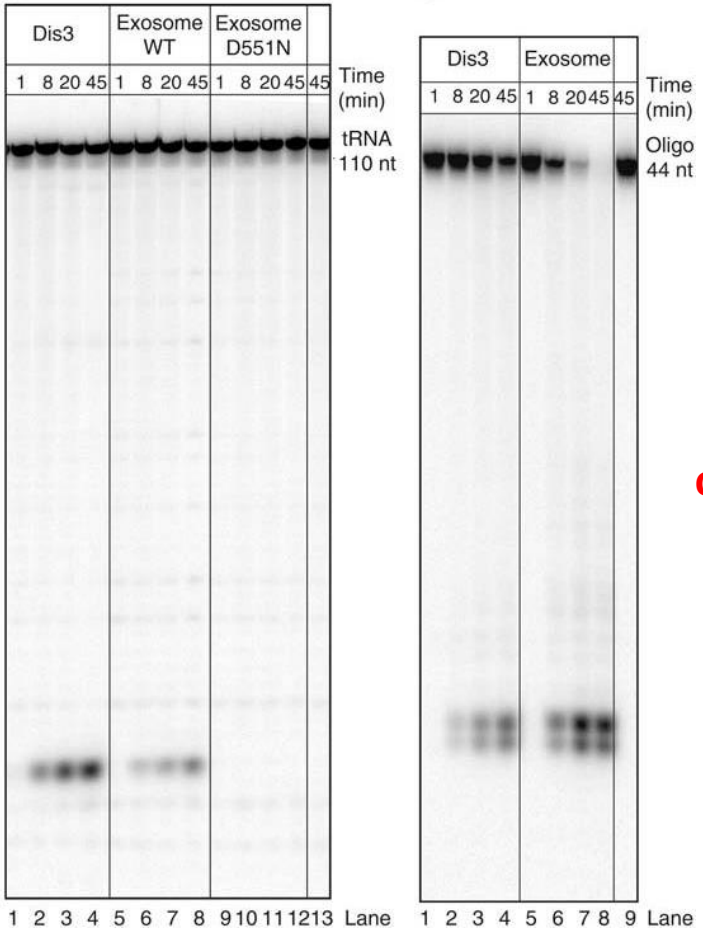
TLC analysis

Analysis of exoribonucleolytic activity of exosomes containing Dis3 WT or Dis3 D551N towards RNA substrate labeled with [^{32}P]-pUpU at its 3'-end

CONCLUSION: The presence of an intact D551 amino acid is a pre-requisite of the proper nucleolytic activity of the exosome complex

Dziembowski et al., *Nat Struct Mol Biol* 2007

The exosome complex and Dis3 alone have comparable activity towards different RNA substrates (single-stranded)



PAGE analysis

„natural" substrate (pre-tRNA)

synthetic oligoribonucleotide

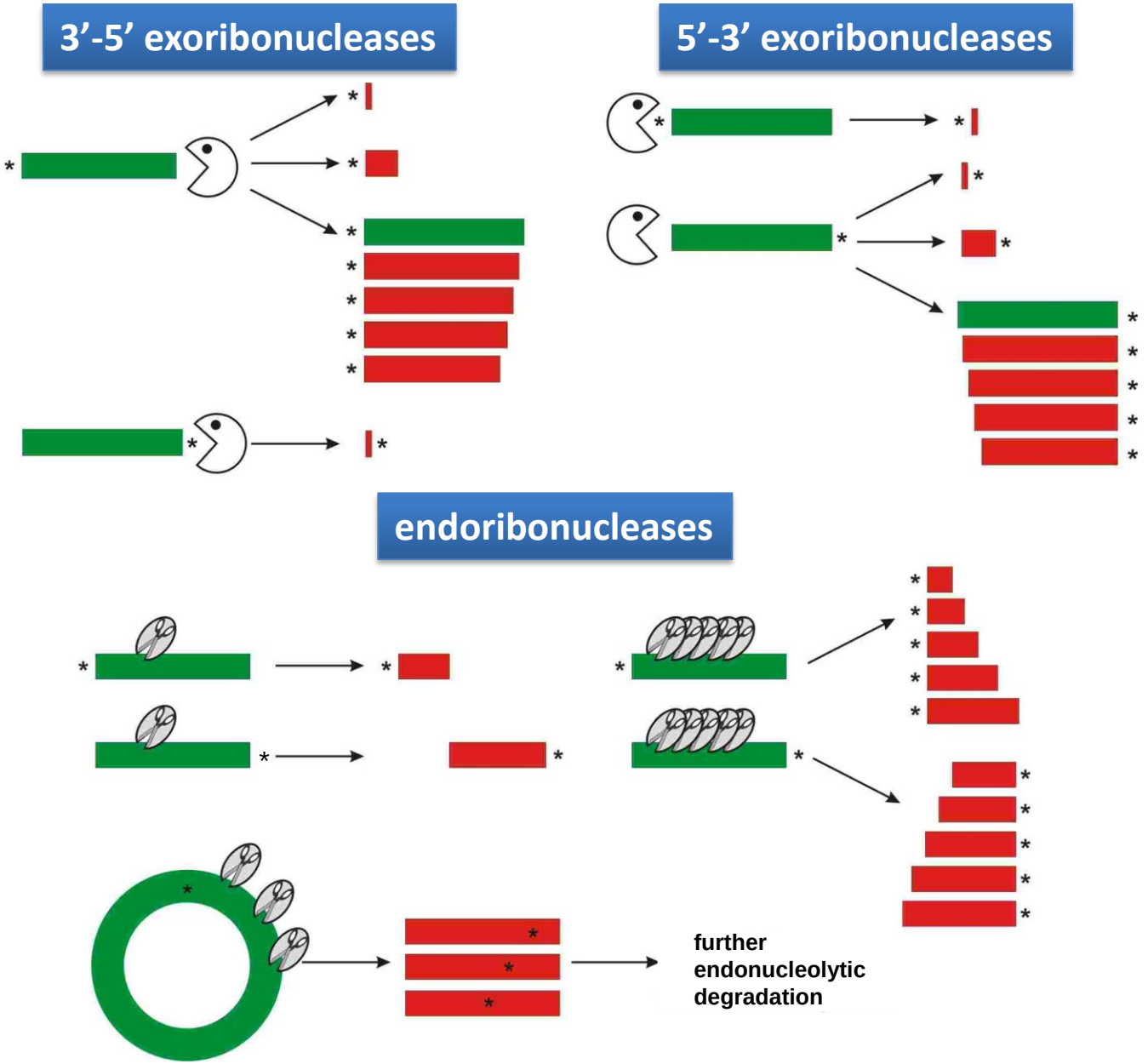
short degradation products are evidence of 3'-5' exoribonucleolytic activity

substrates labeled at the 5'-end

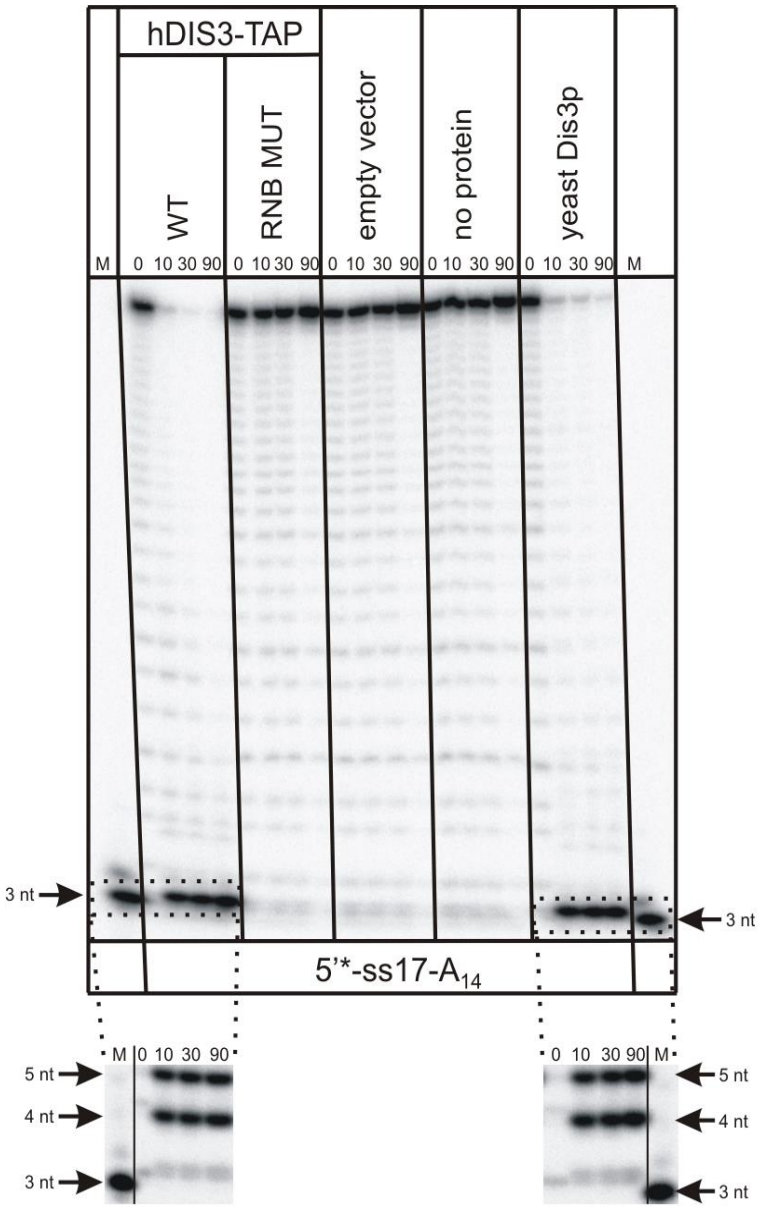
CONCLUSION: Results of *in vitro* experiments indicate, that Dis3 is a major catalytic subunit of the exosome complex

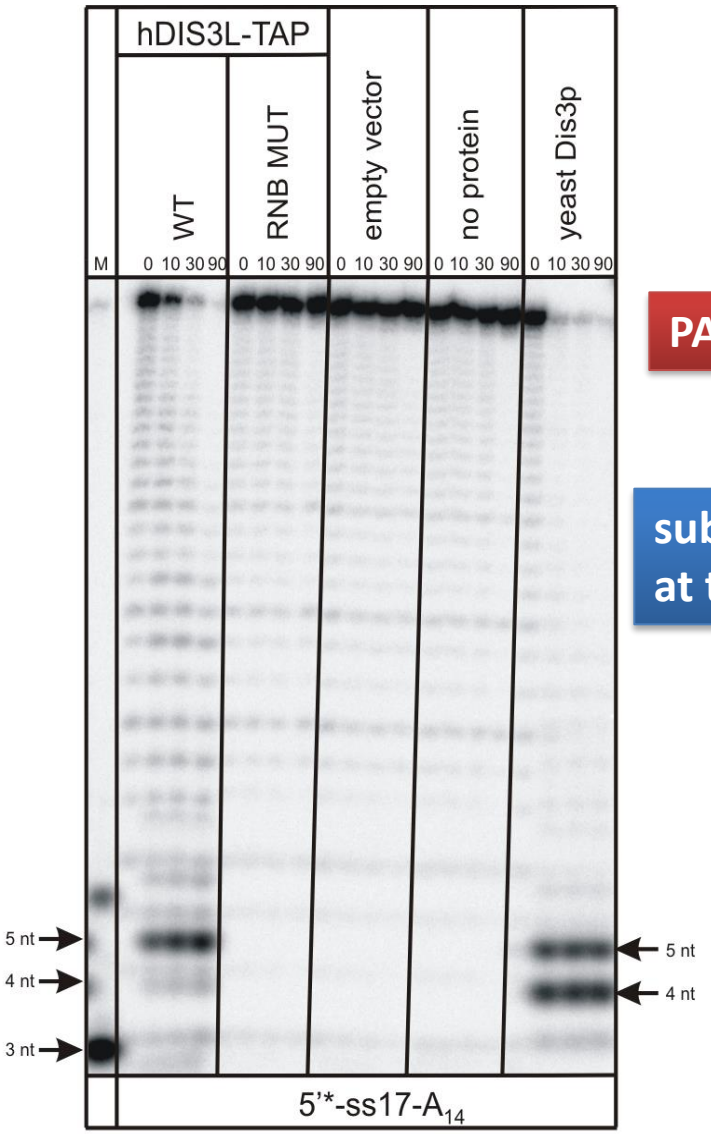
Dziembowski et al., *Nat Struct Mol Biol* 2007

How to determine what nuclease are we dealing with based on the results of biochemical experiments ?



Are human homologs of Dis3 – hDIS3 i hDIS3L – also 3’-5’ exonucleases?



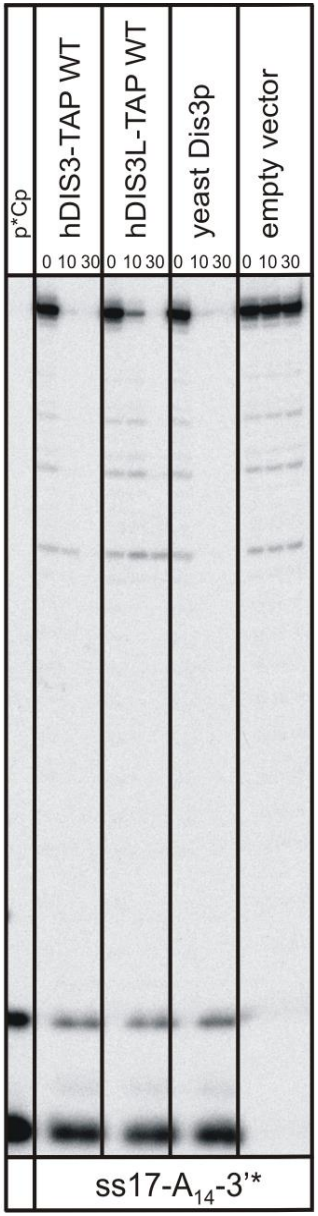


PAGE analysis

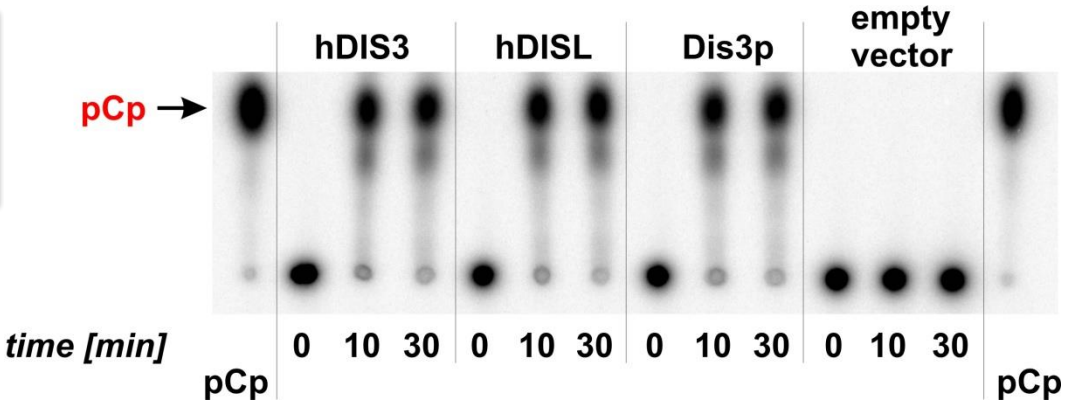
substrate labeled at the 5’ end

Tomecki et al., *EMBO J* 2010

To be absolutely certain, substrate labeled at the opposite terminus also has to be examined



substrate labeled at the 3' end

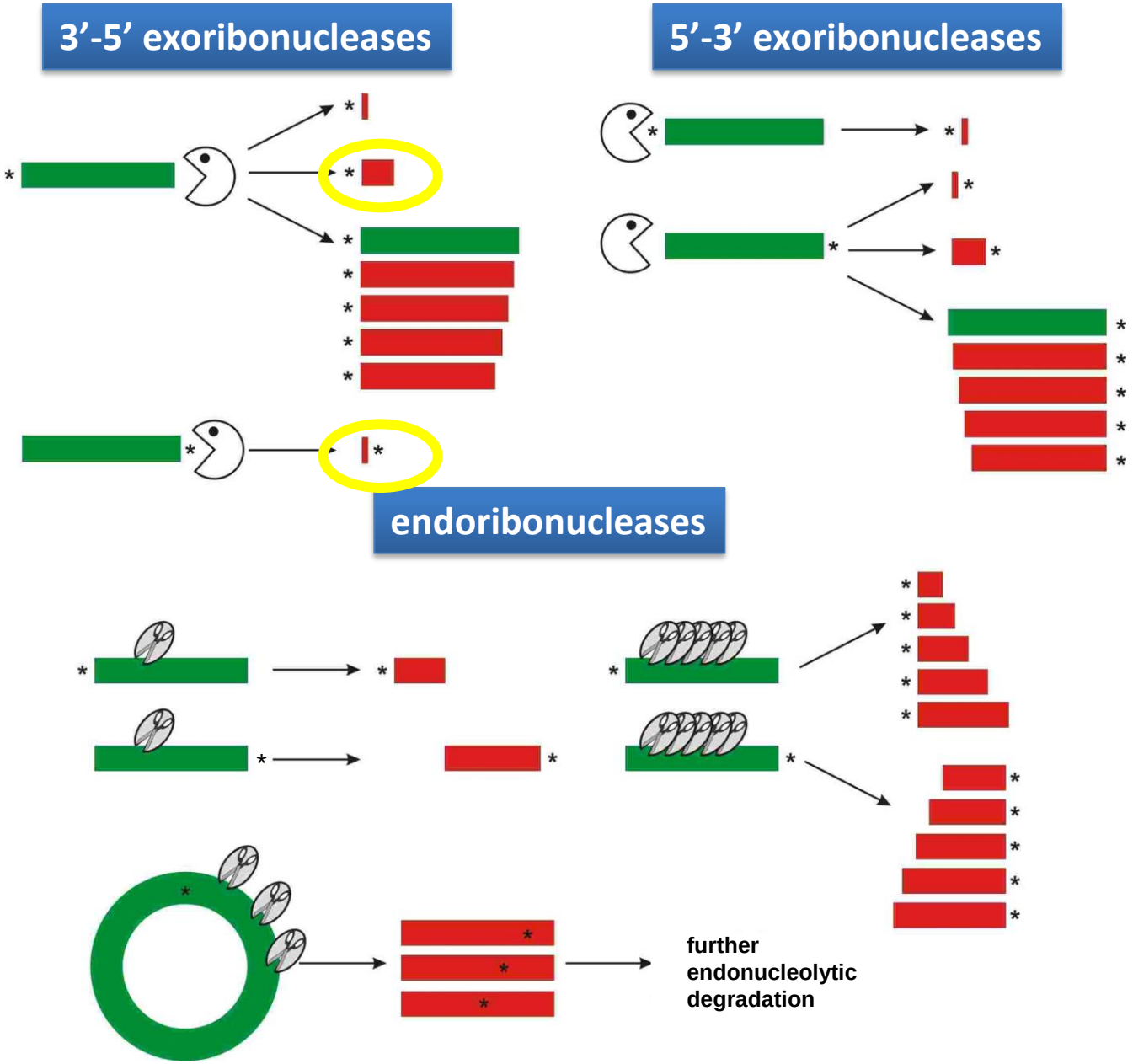


PAGE analysis

TLC analysis

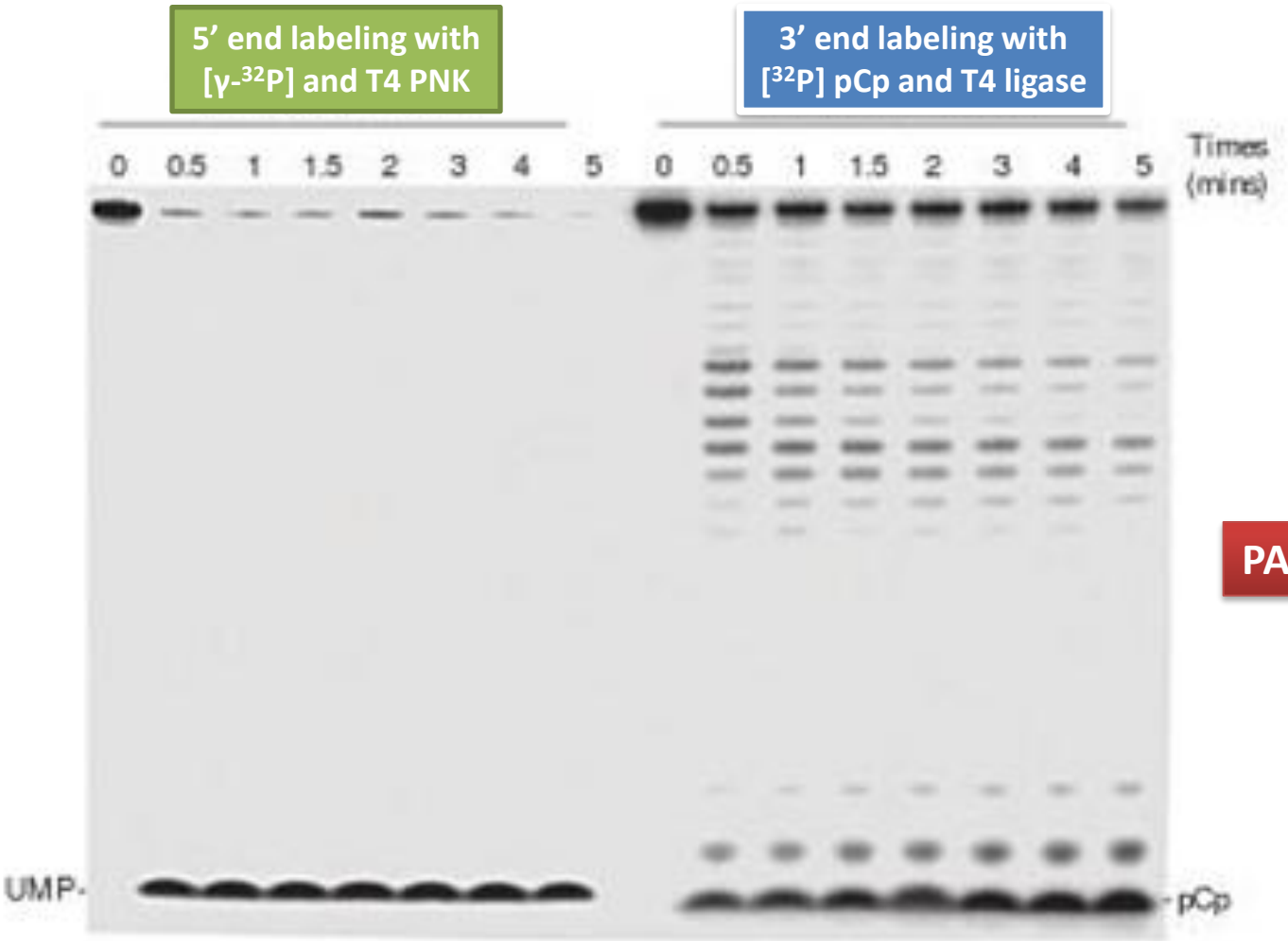
Tomecki et al., *EMBO J* 2010

Human Dis3 orthologs are 3’-5’ exoribonucleases



How about 5’-3’ exonucleases?

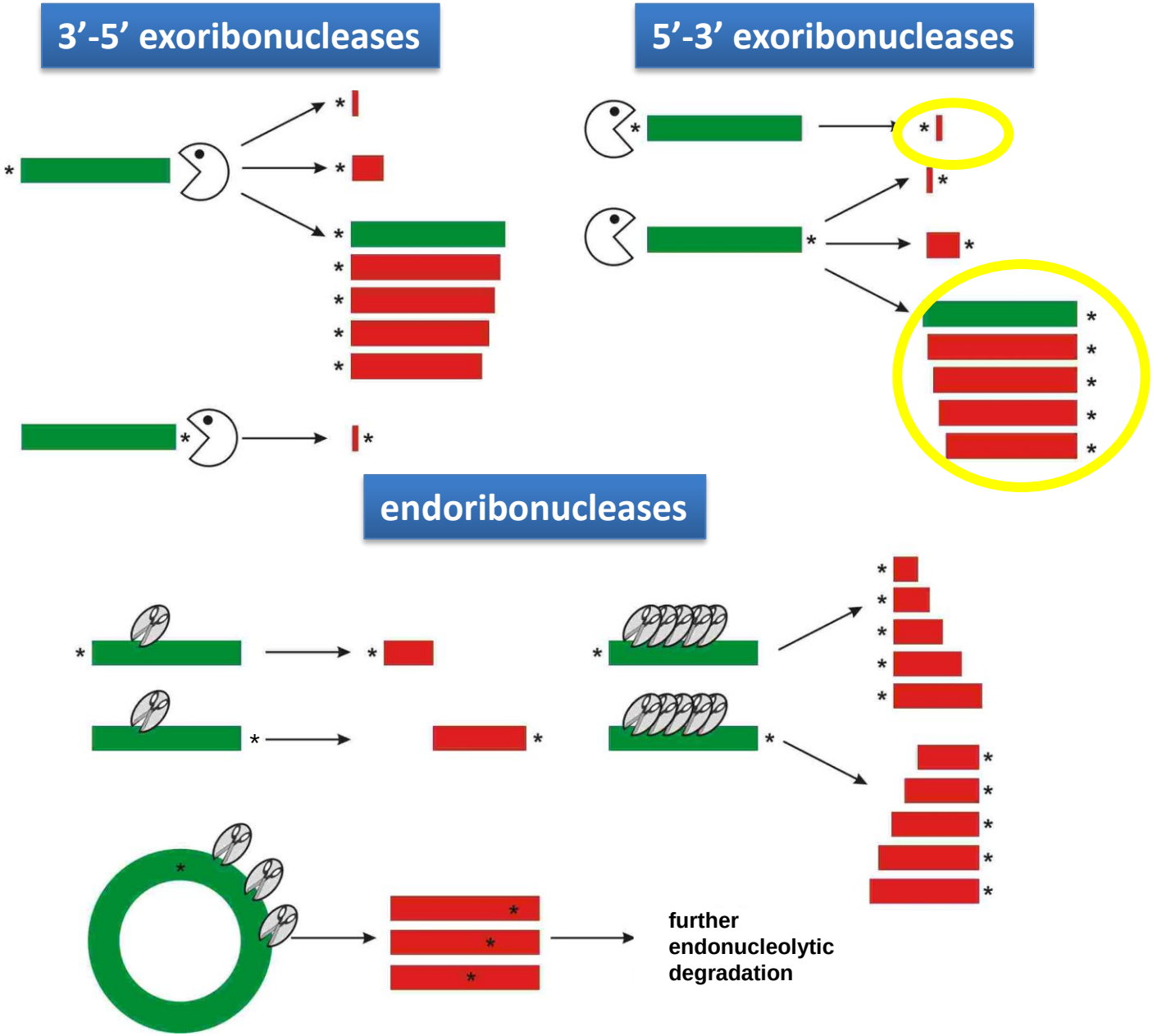
Example 1: Xrn1 from *S. cerevisiae*



PAGE analysis

Pellegrini et al., *Methods enzymol* 2008

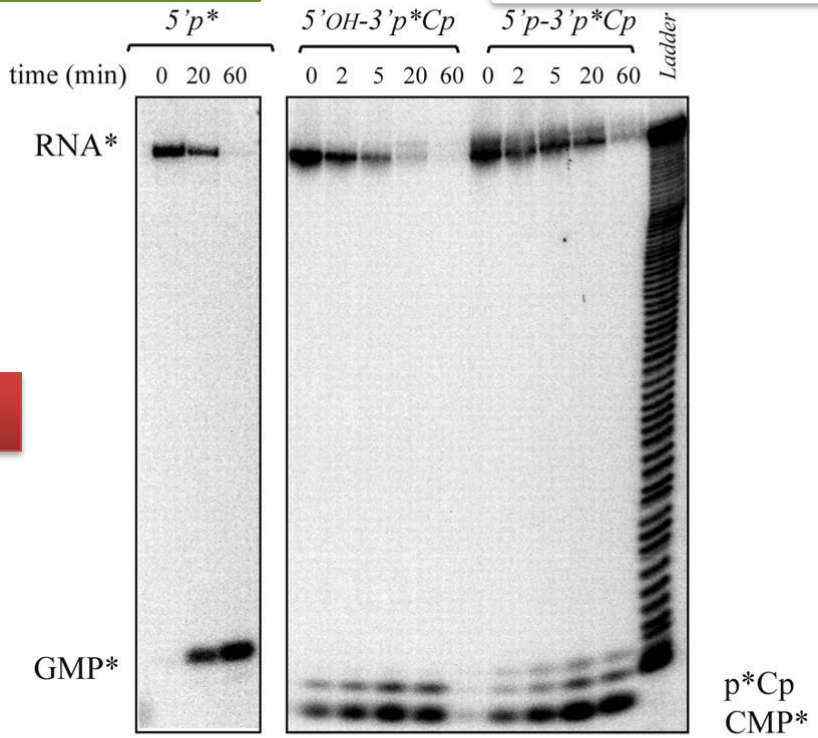
Xrn1 is a 5’-3’ exoribonuclease



Does RNase J1 work in the 3'-5' or 5'-3' direction?

5' end labeling with [γ-^{32}P] and T4 PNK

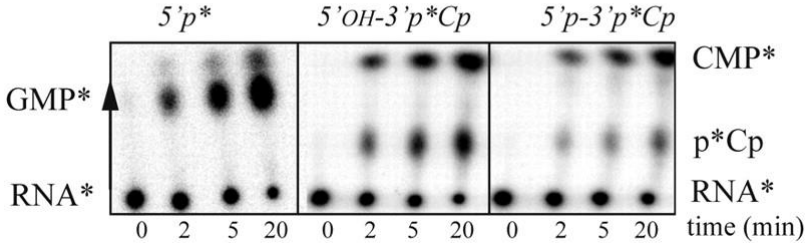
3' end labeling with [^{32}P] pCp and T4 ligase



Clouet-d'Orval et al., *J Biol Chem* 2010

PAGE analysis

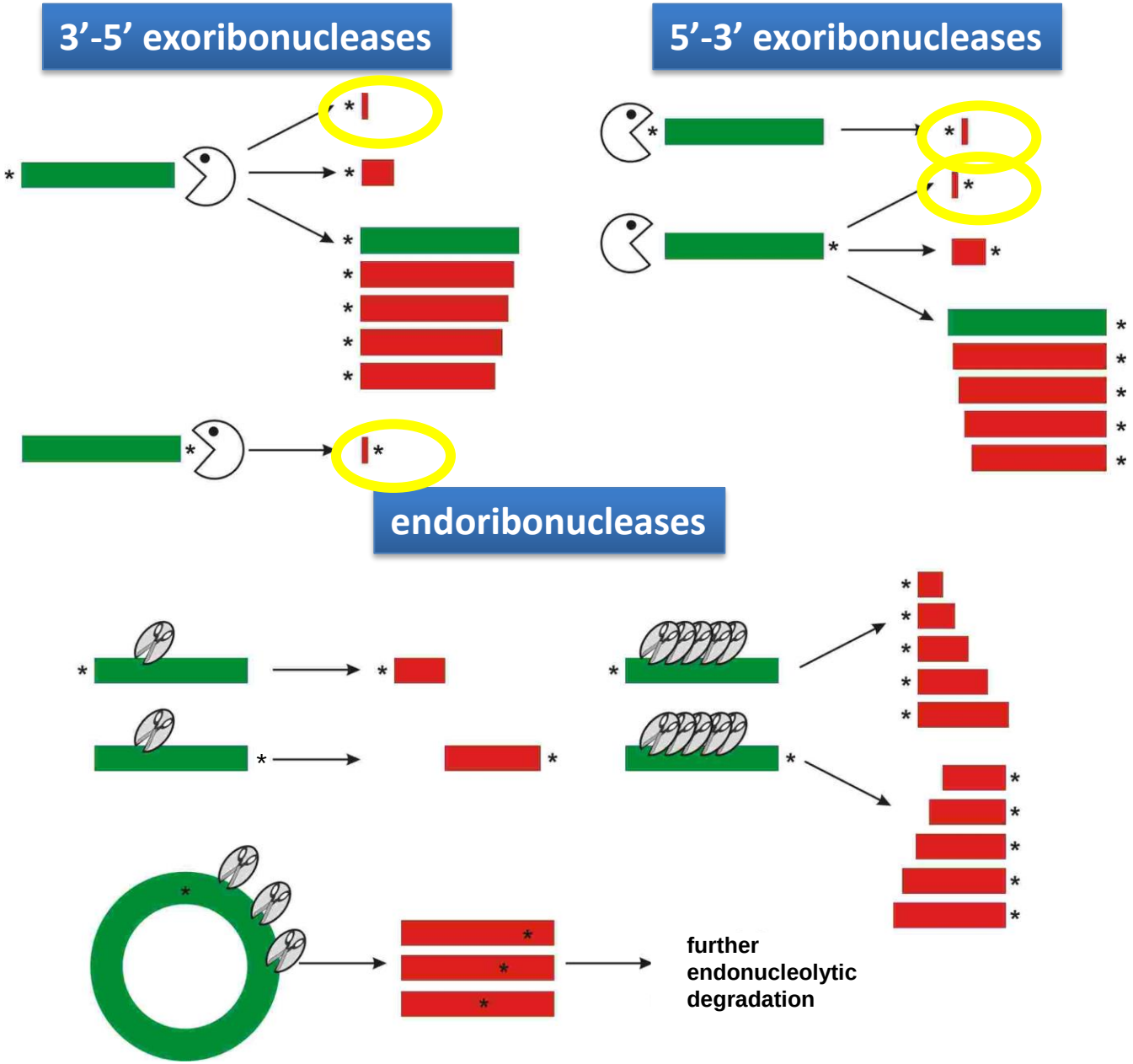
G – first nucleotide of the sR47 substrate



TLC analysis

It is impossible to say based on above results !

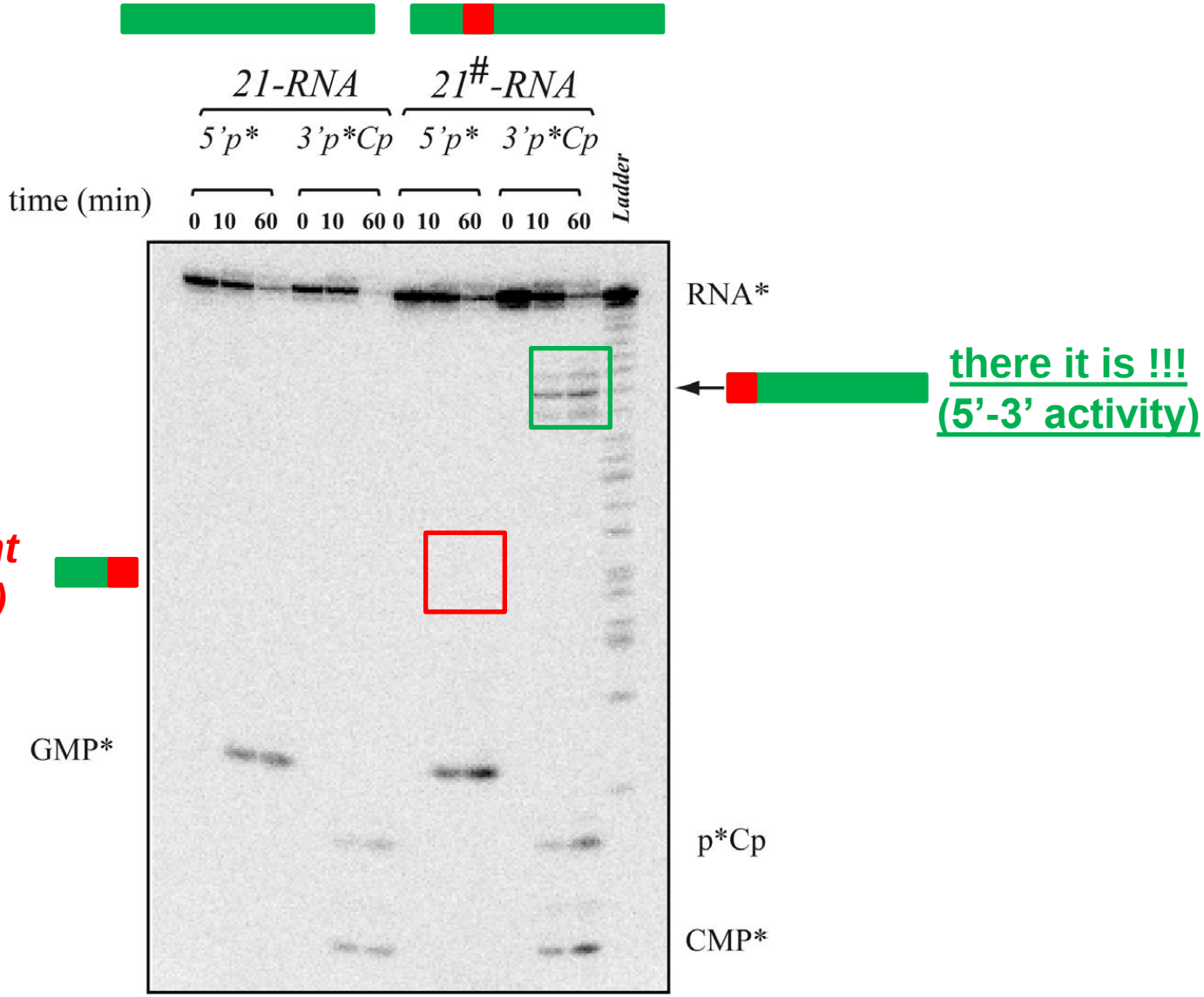
It is not always that straightforward…



How to determine this definitively ?

Asymmetric introduction of an element slowing down nucleolytic activity into the substrate

PAGE analysis



there it is !!!
(5'-3' activity)

no such fragment
(no 3'-5' activity)

Clouet-d'Orval et al.,
***J Biol Chem* 2010**

Activity of multiple ribonucleases depends on the 5’ end phosphorylation status – how to study this phenomenon?

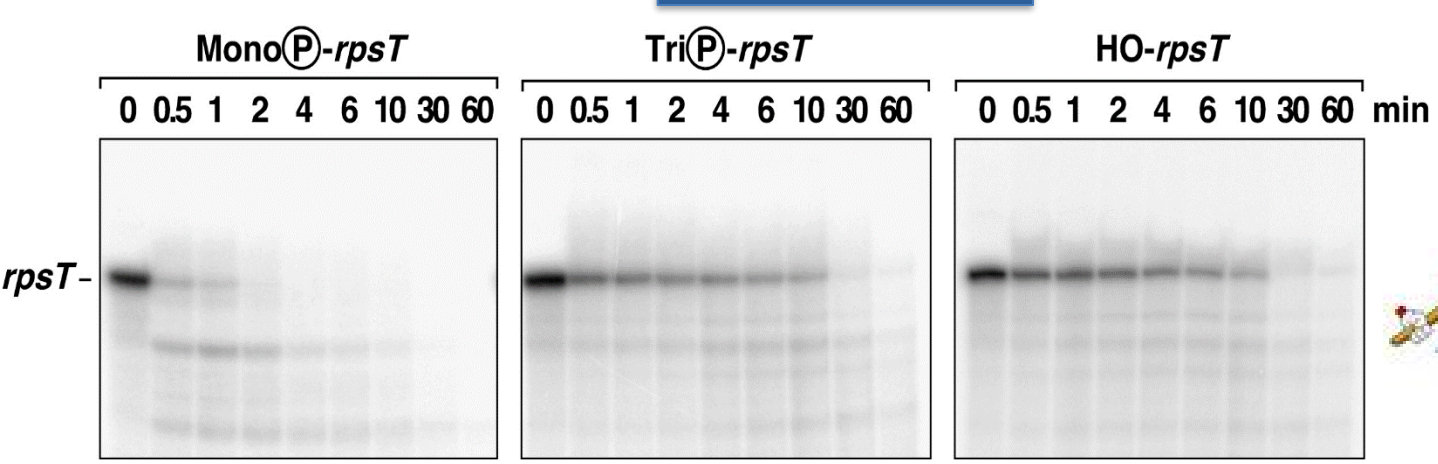
Preparation of substrates with different 5’ ends:

***In vitro* transcription in the presence of [α-^{32}P]UTP:**

1) **equal NTP concentrations – triphosphate**
2) **excess of NMP corresponding to the 1st nucleotide of the substrate – monophosphate**

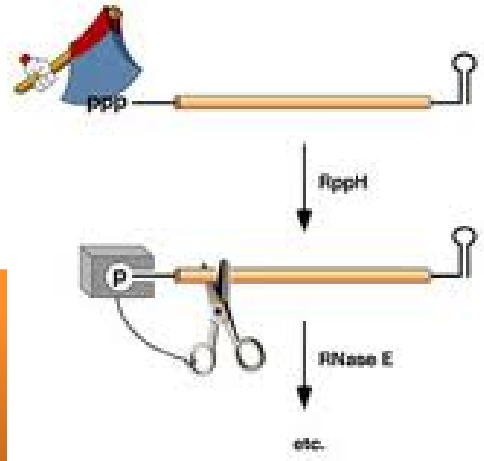
1) **substrate treatment with alkaline phosphatase – hydroxyl group**
2) **excess of cap analog (eukaryotic transcript) – guanosine cap**

B **RNase E cleavage in vitro:** *Escherichia coli*



PAGE analysis

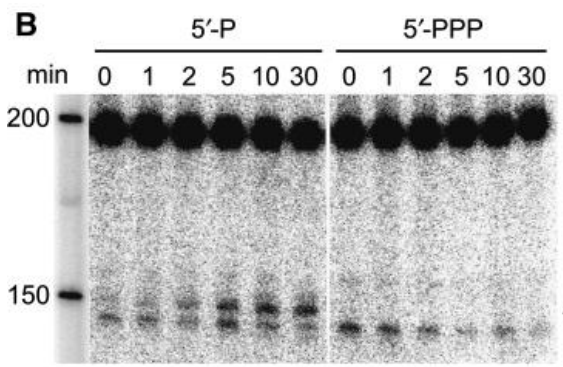
Celesnik et al., *Mol Cell* 2007



Pyrophosphate removal in *E. coli* is catalyzed by RppH pyrophosphohydrolase. This is a step initiating degradation, which precedes RNA cleavage by RNase E !

Other examples of nucleases dependent on the 5’ end sensor

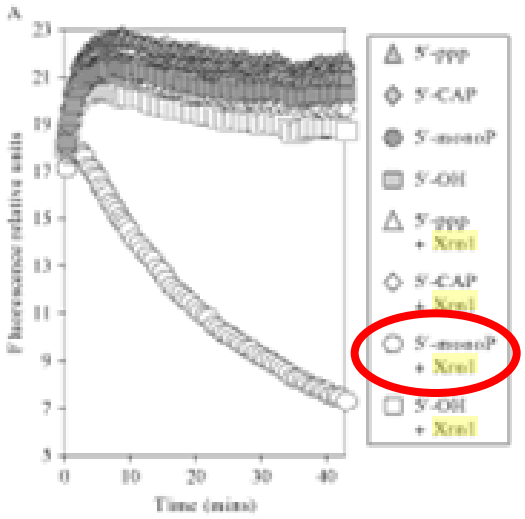
- RNase J1 *Bacillus subtilis and Archaea* (see above)
- RNase Y *Bacillus subtilis*



analiza PAGE

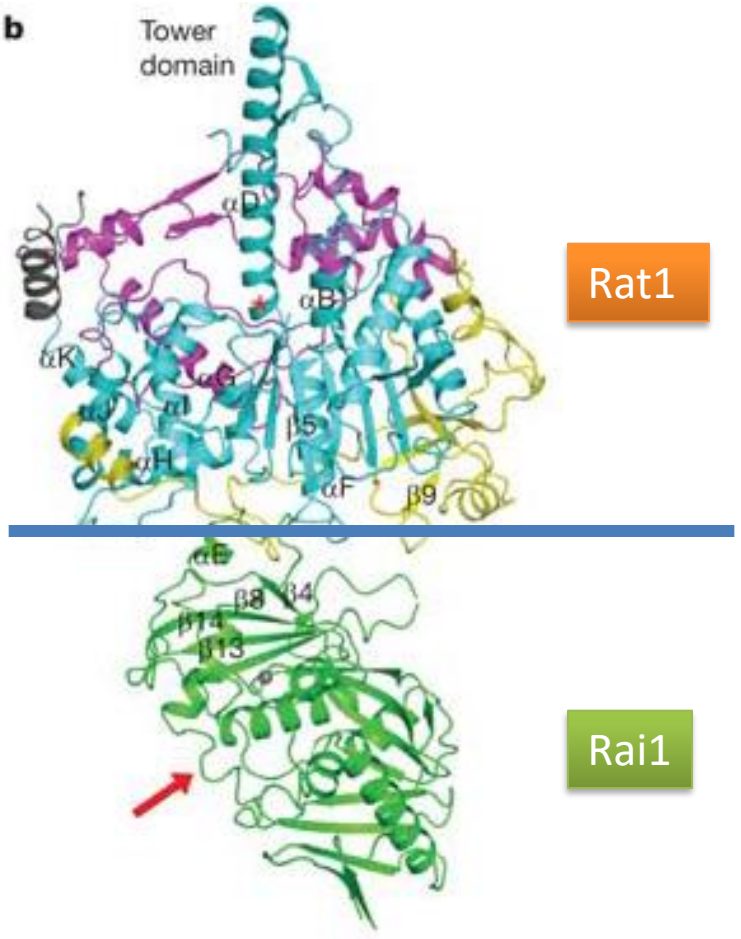
Shahbabian et al., *EMBO J* 2009

- Xrn1 (thus cap removal necessary for decay)



Pellegrini et al., *Methods enzymol* 2008

- Rat1 (collaborates with Rai1 pyrophosphohydrolase)

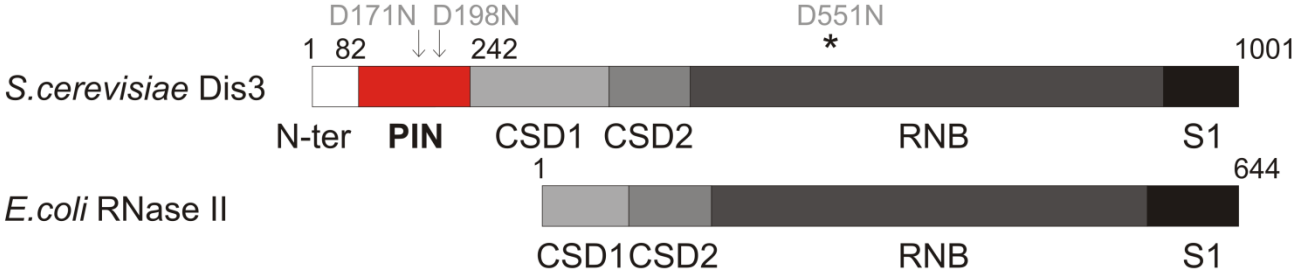


Xiang et al., *Nature* 2009

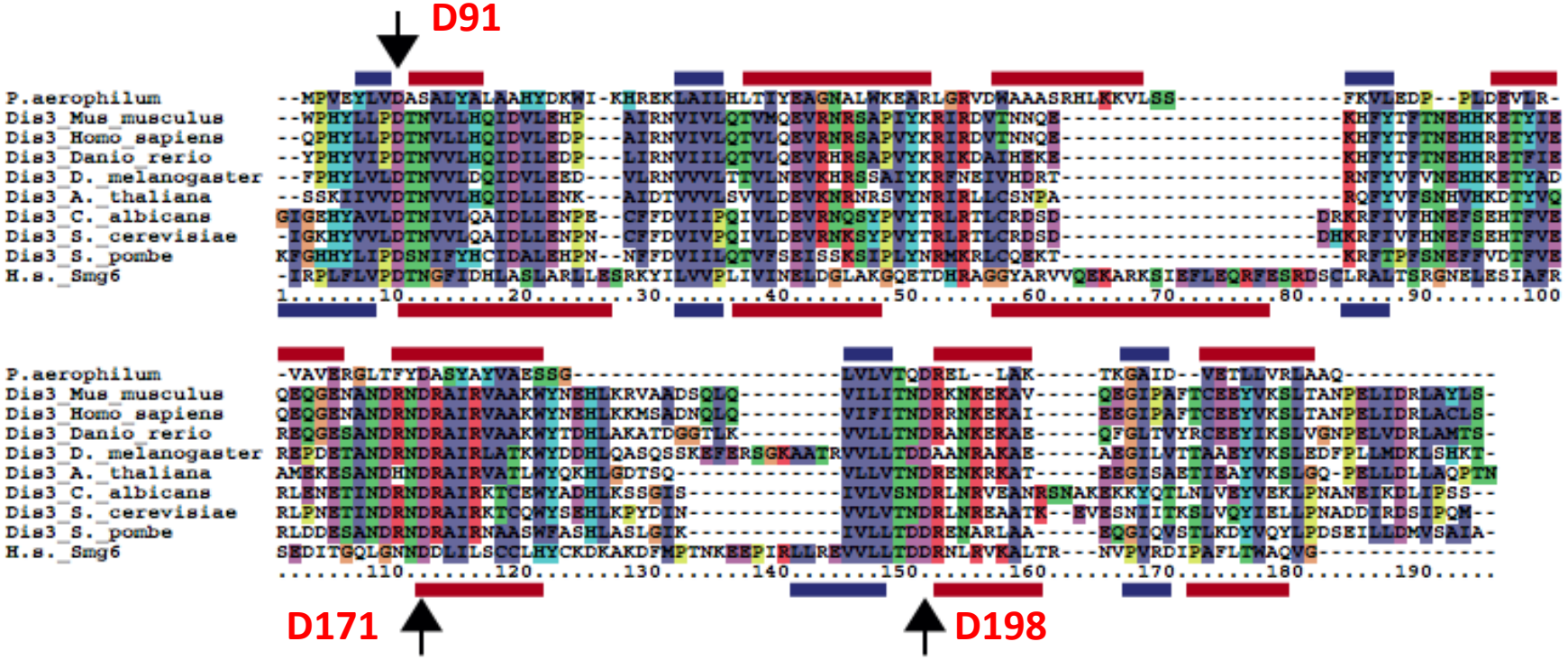
Is Dis3 exclusively a 3'-5' exoribonuclease ?

… on that it is worth dedicating some time to a thorough analysis of the amino acid sequence of the protein of interest

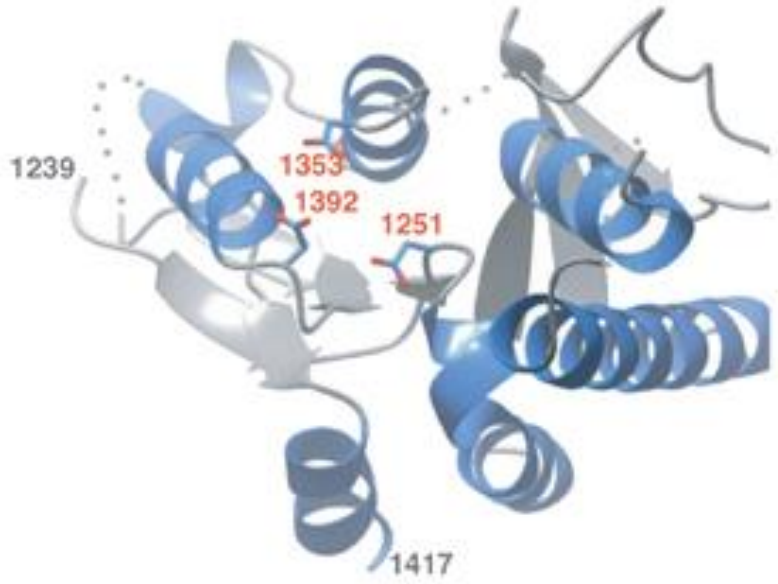
- **Organization of Dis3 functional domains compared to RNase II**



- **N-terminal PIN domain is strongly evolutionary conserved**



PIN domain-containing proteins are nucleases

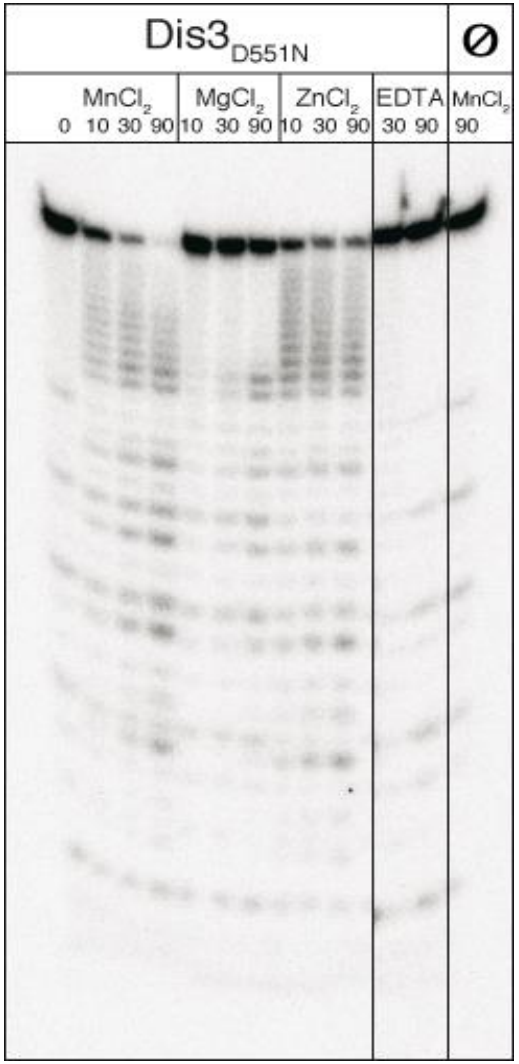


SMG6 protein structure (Glavan *et al*., EMBO J, 2006)

- T4 bacteriophage RNase H
- FEN1 endonuclease
- Nob1 protein participating in the endonucleolytic 20S pre-rRNA processing
- SMG6 protein – a component of NMD machinery, displays endonucleolytic activity

Dis3 D551N (exo-) mutant devoid of exoribonucleolytic activity is still able to degrade RNA *in vitro*

An example of optimization of the type of divalent cation used in the reaction



Preferences towards cofactors:

$[Mn^{2+}] > [Zn^{2+}] > [Mg^{2+}]$

substrate labeled at the 5' end

PAGE analysis

Lebreton, Tomecki et al., *Nature* 2008

Nucleolytic activity of Dis3 D551N (exo-) mutant requires high Mn^{2+} concentration

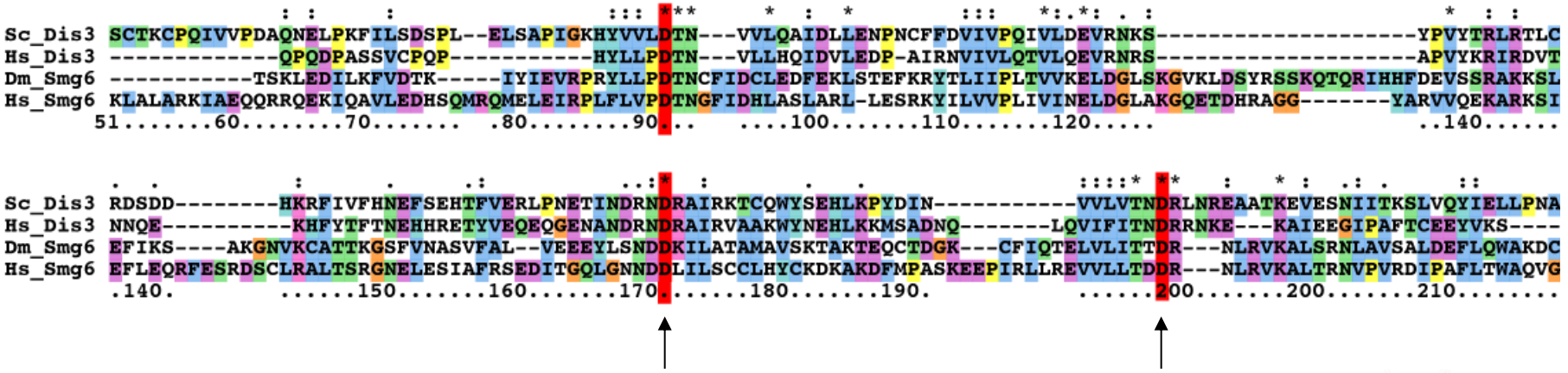
An example of optimization of the concentration of a divalent cation used in the reaction

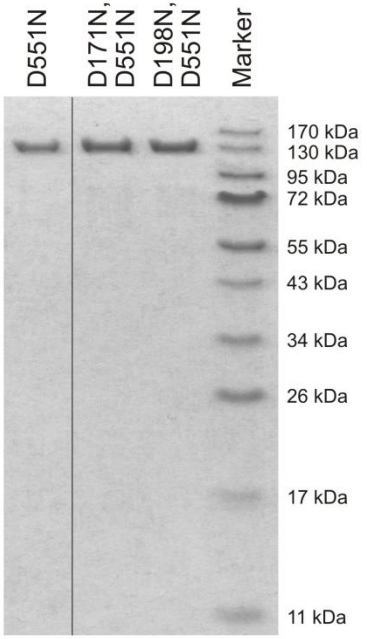
substrate labeled at the 5' end

PAGE analysis

Is the observed additional nucleolytic activity associated with the PIN domain?

Additional mutations of conserved aspartate residues within the PIN domain catalytic triad





SDS-PAGE analysis

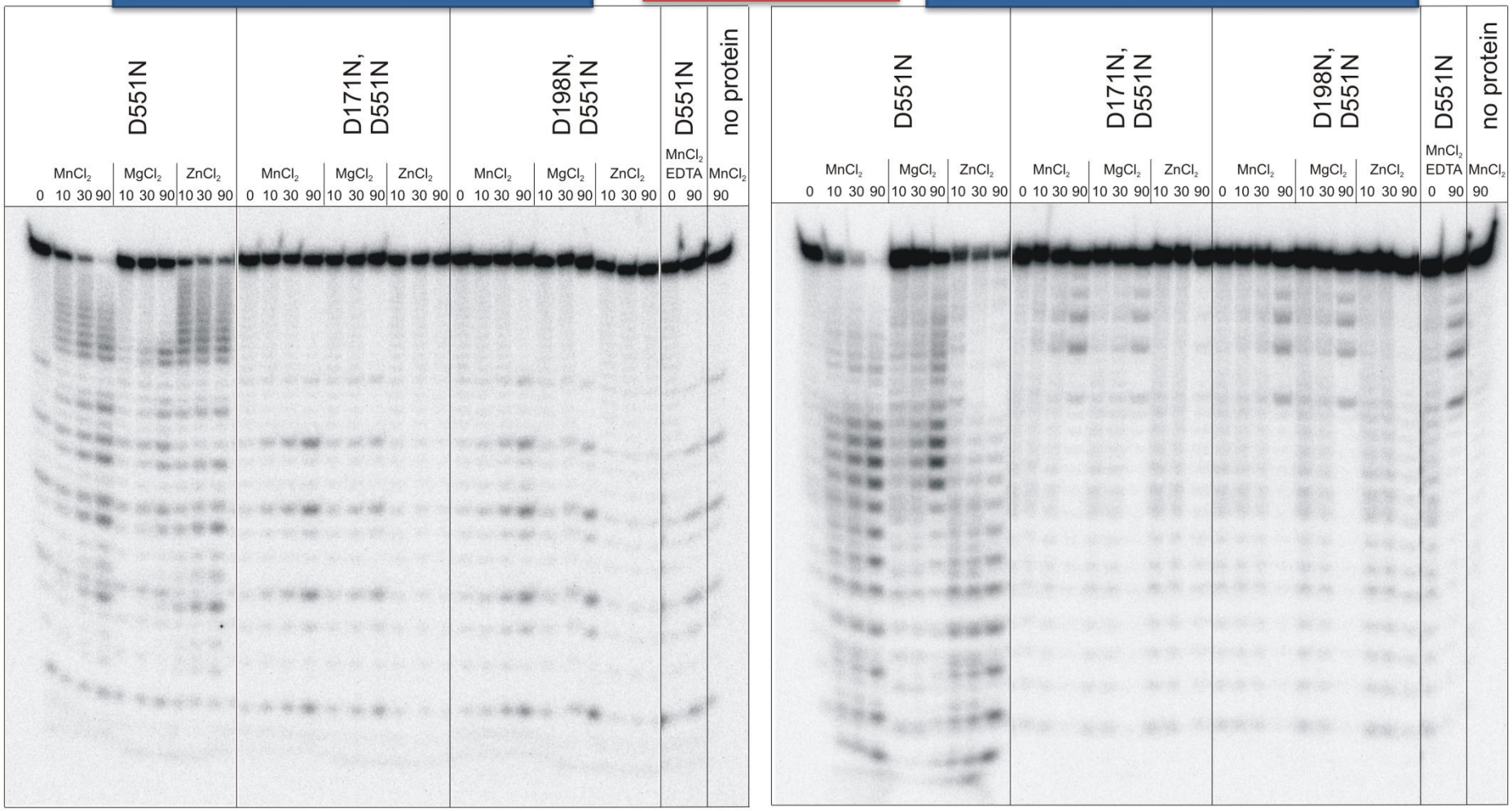
Lebreton, Tomecki et al., *Nature* 2008

The presence of catalytic aspartate residues in $Dis3_{PIN}$ is necessary for RNA degradation *in vitro*

substrate labeled at the 5' end

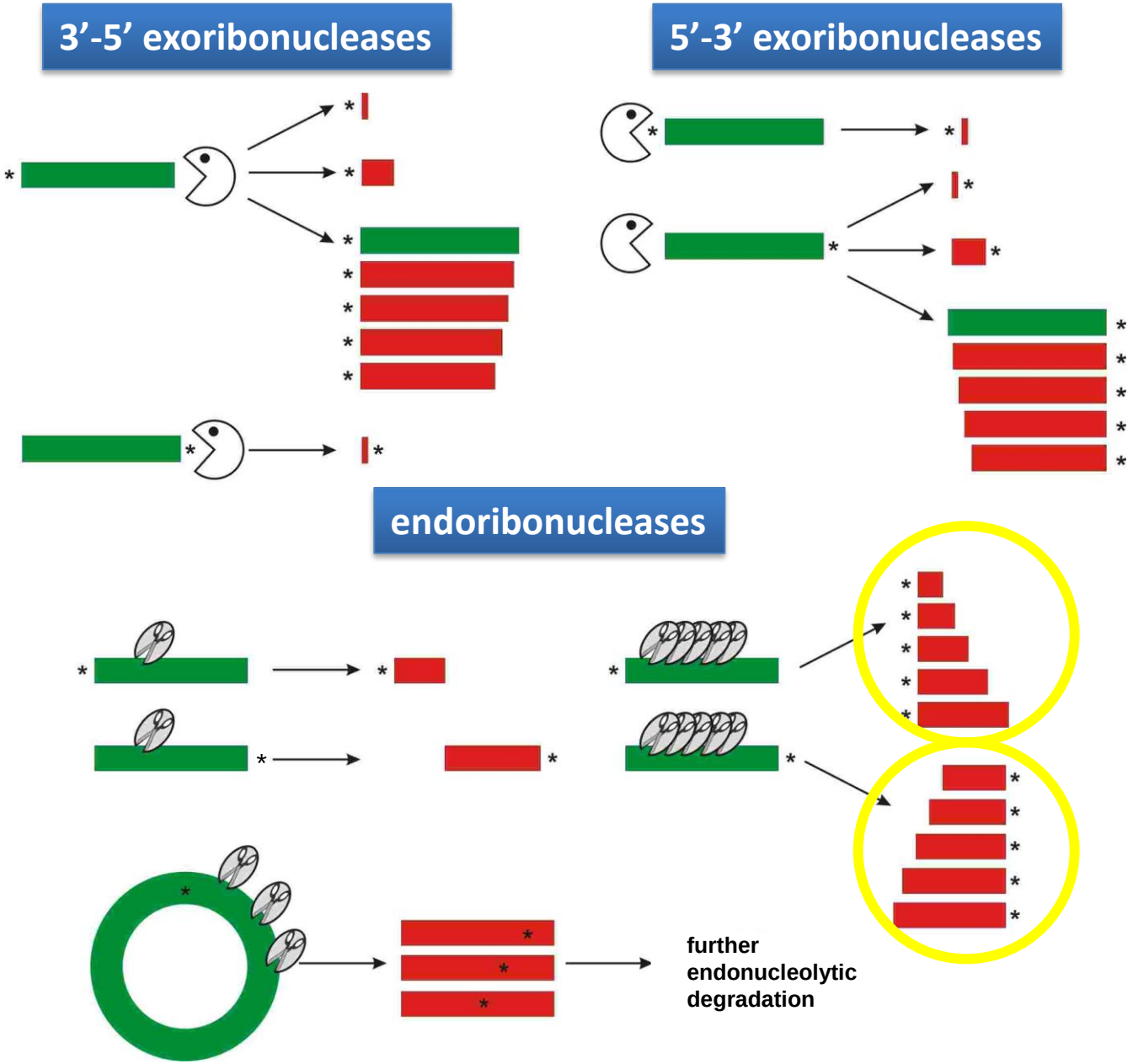
PAGE analysis

substrate labeled at the 3' end



"ladder" of degradation products observed for substrates labeled at opposite ends suggests that we observe endoribonucleolytic activity

Example of an endoribonucleolytic degradation pattern



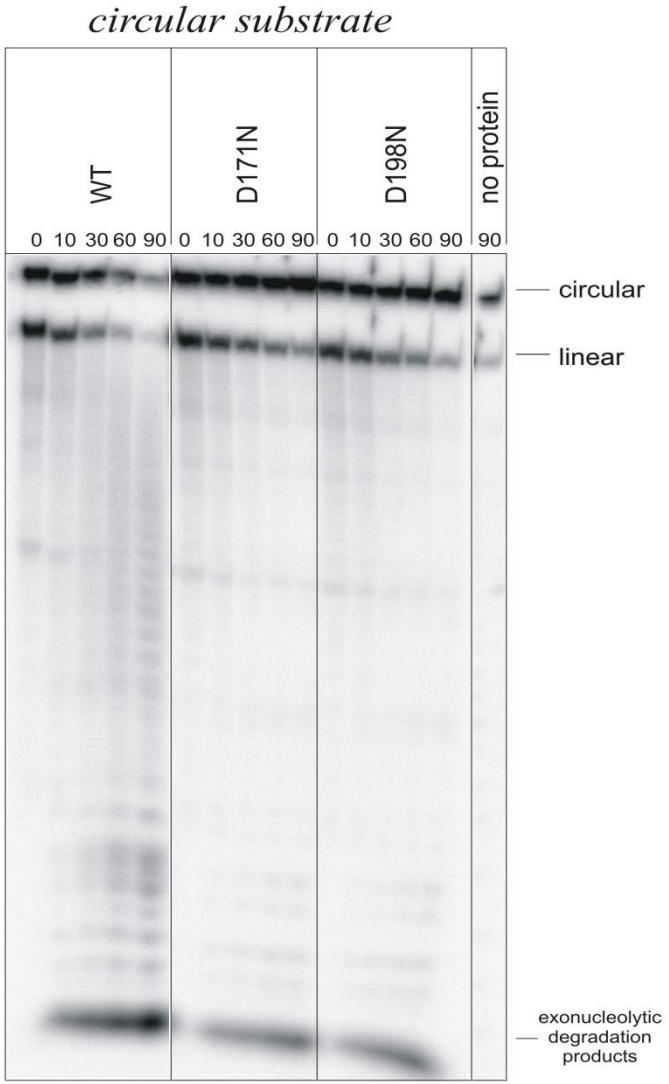
How to unequivocally confirm that the PIN domain is associated with endoribonuclease activity ?

Testing the enzyme activity towards RNA substrate without free ends (that is … CIRCULAR)

PIN domain is associated with the novel nucleolytic activity of Dis3 protein, able to degrade a broad repertoire of substrates:

- 5’ end-labeled
- 3’ end-labeled
- circular

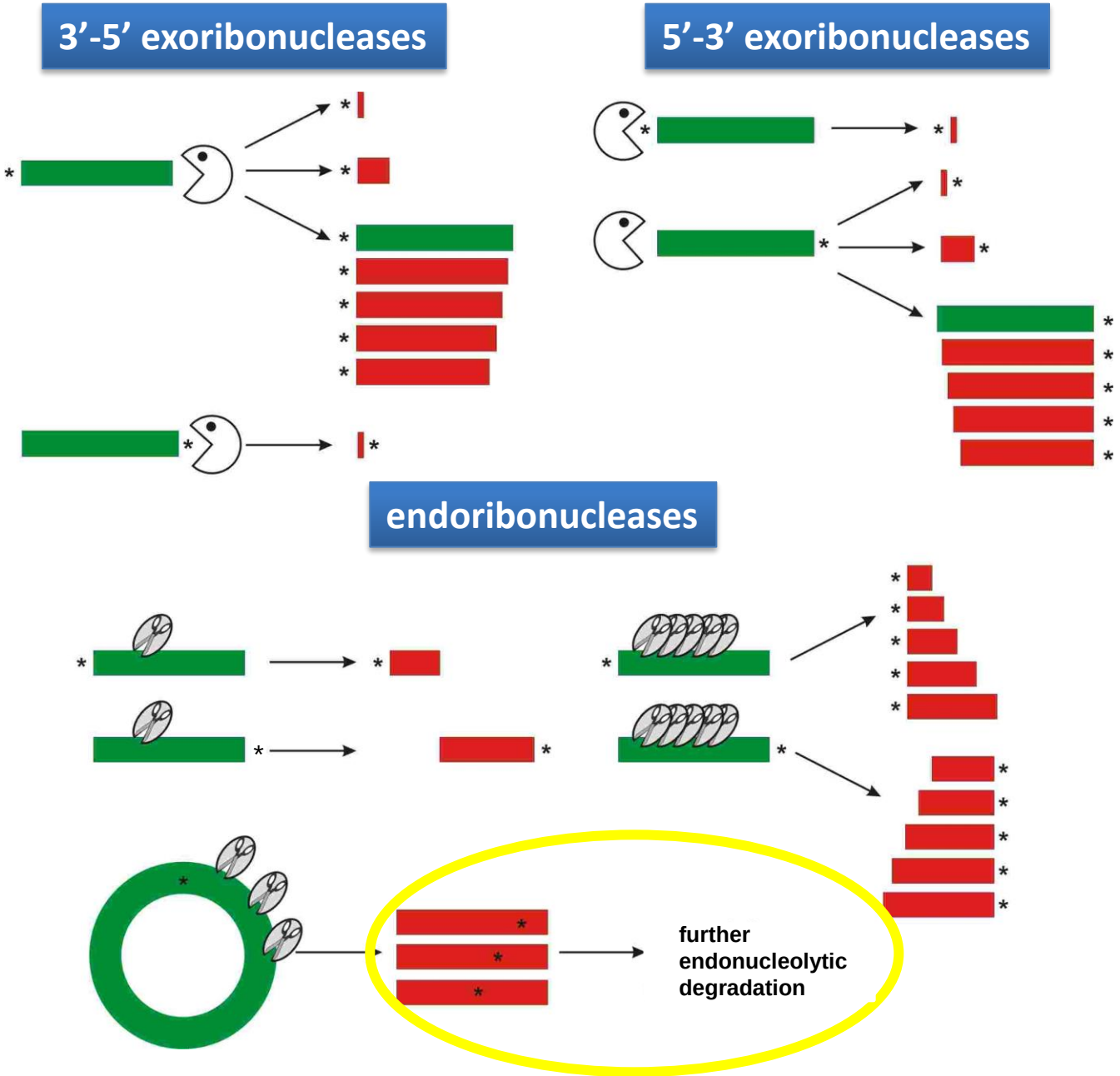
PAGE analysis



… which proves that it is an endonuclease !

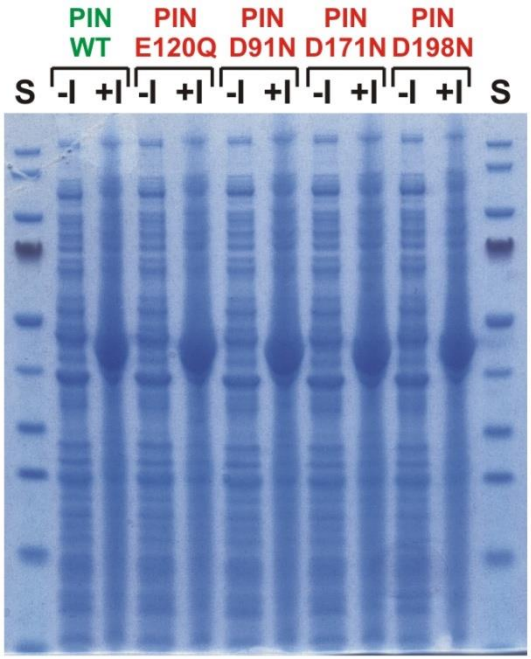
Lebreton, Tomecki et al., *Nature* 2008

Degradation of circular substrates – only endonucleases !

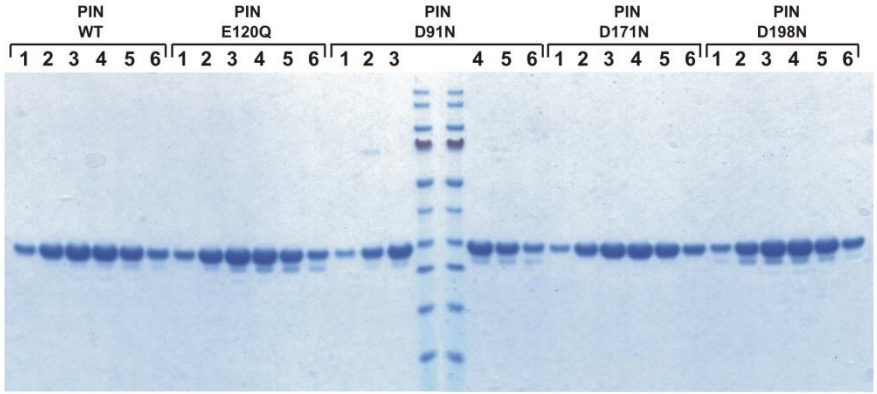


Is endoribonucleolytic activity of the PIN domain dependent on the rest of the Dis3 protein?

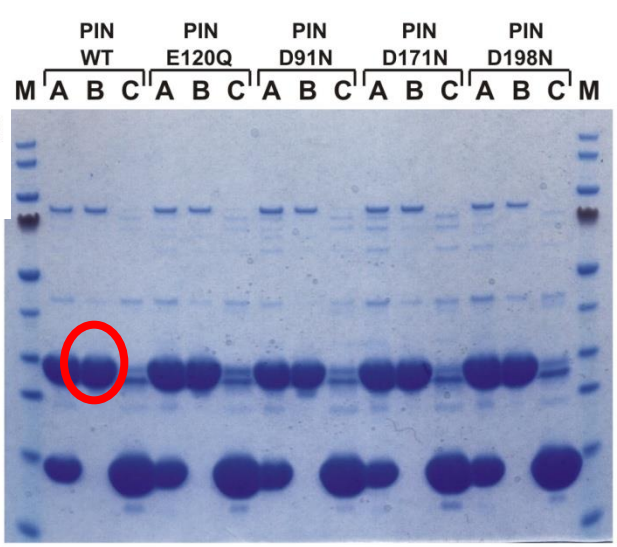
The necessity to purify a single protein domain and to examine its activity



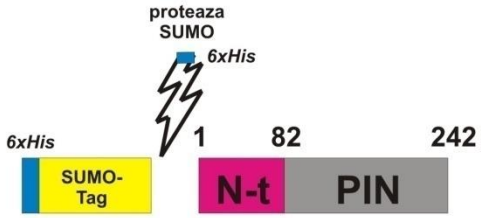
SDS-PAGE analyses



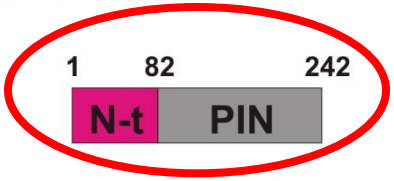
gel filtration



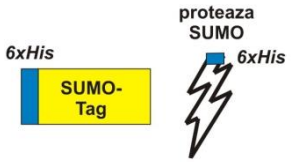
(A) eluate after 1st purification on the nickel resin and SUMO protease digestion



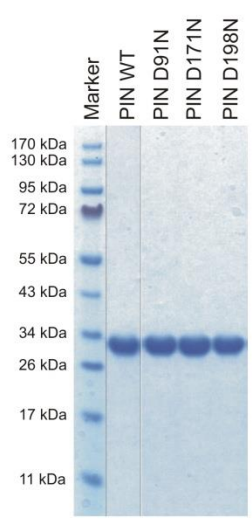
(B) flow-through: proteins that do not bind to the resin during 2nd purification (HERE: our protein)

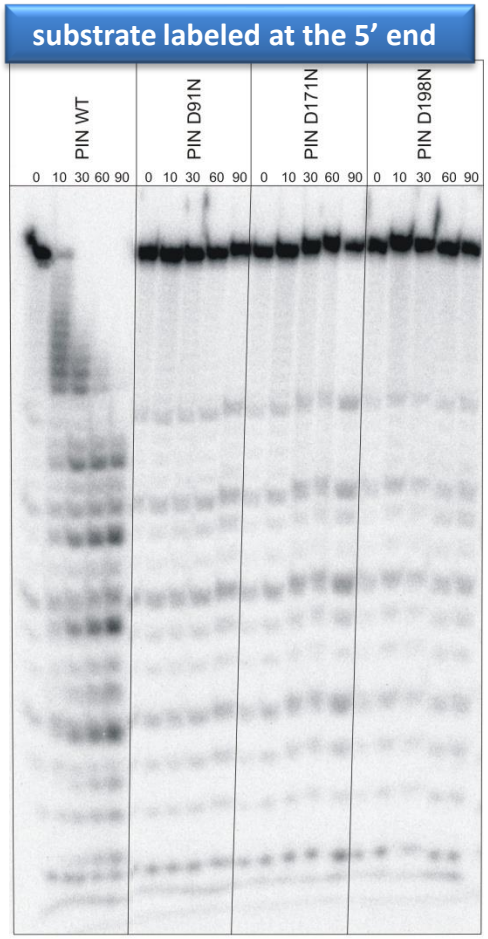


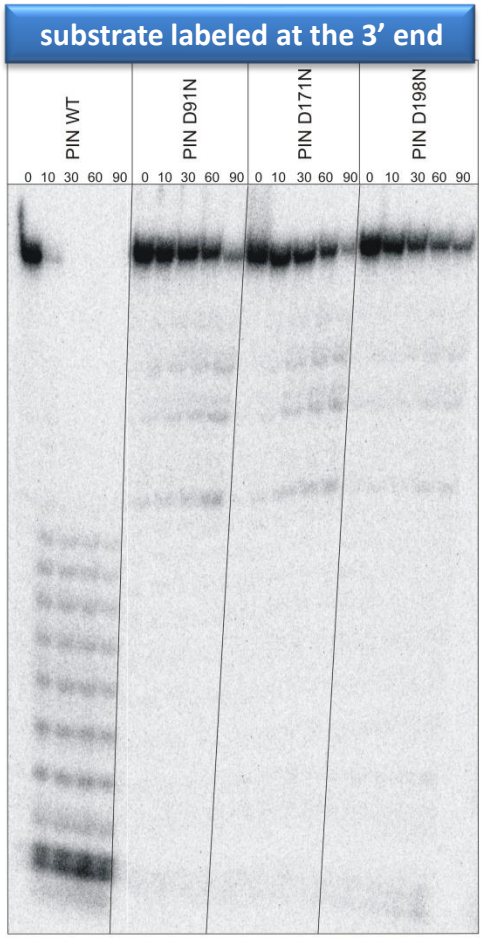
(C) eluate after 2nd purification on the nickel resin (remainders)

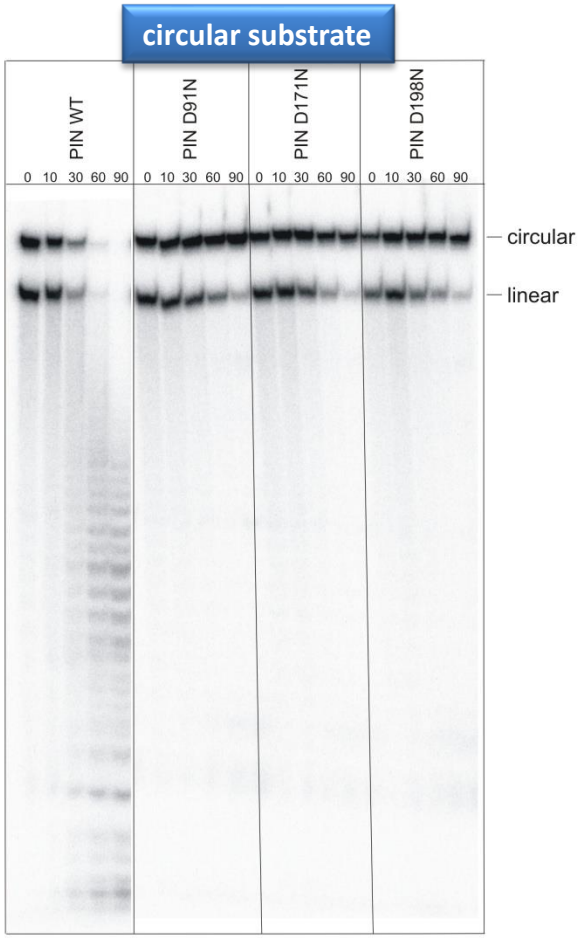


PIN domain alone displays endonucleolytic activity, which is abolished by mutations of conserved aspartate residues in the active site





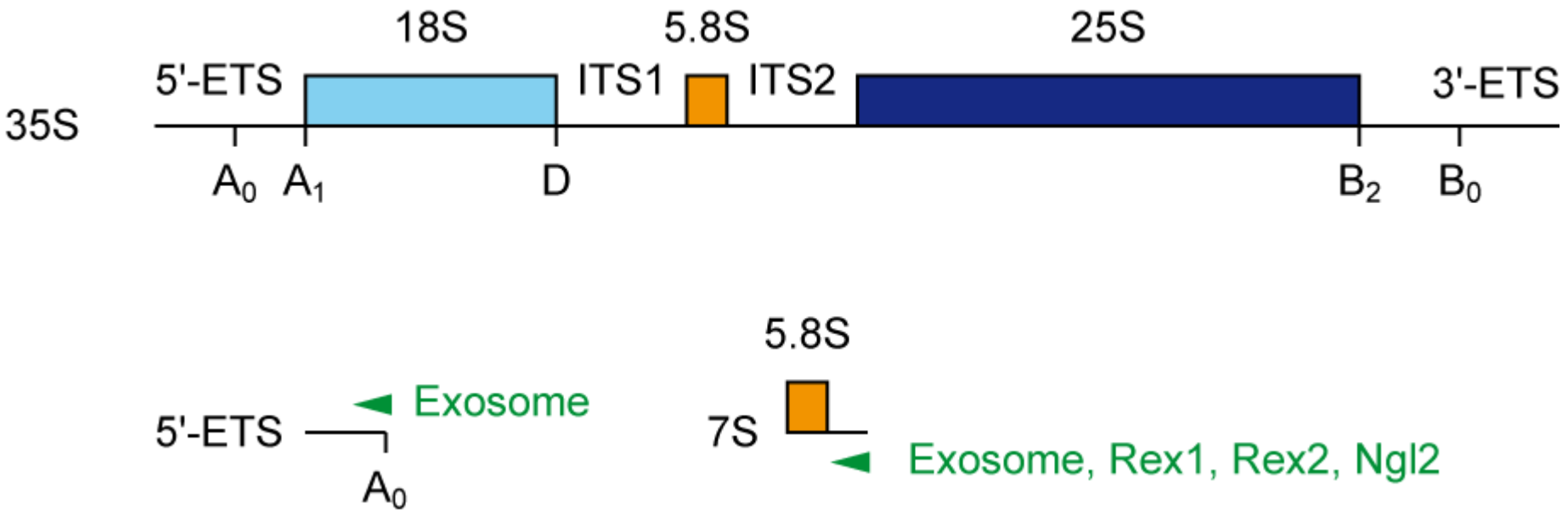




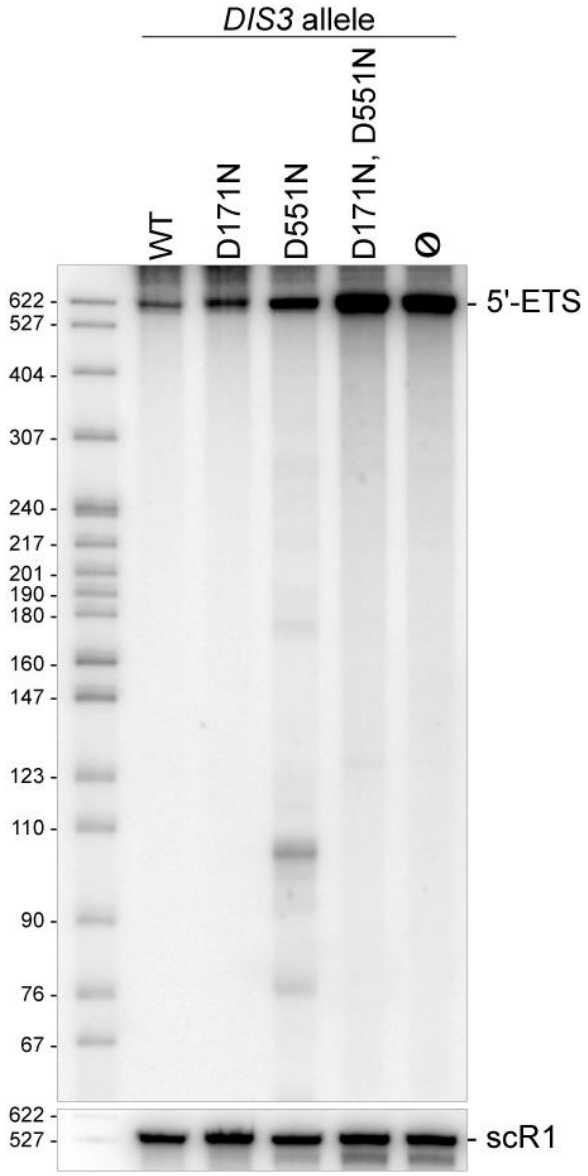
PAGE analysis

Lebreton, Tomecki et al., *Nature* 2008

Exosome substrates in pre-rRNA processing pathways



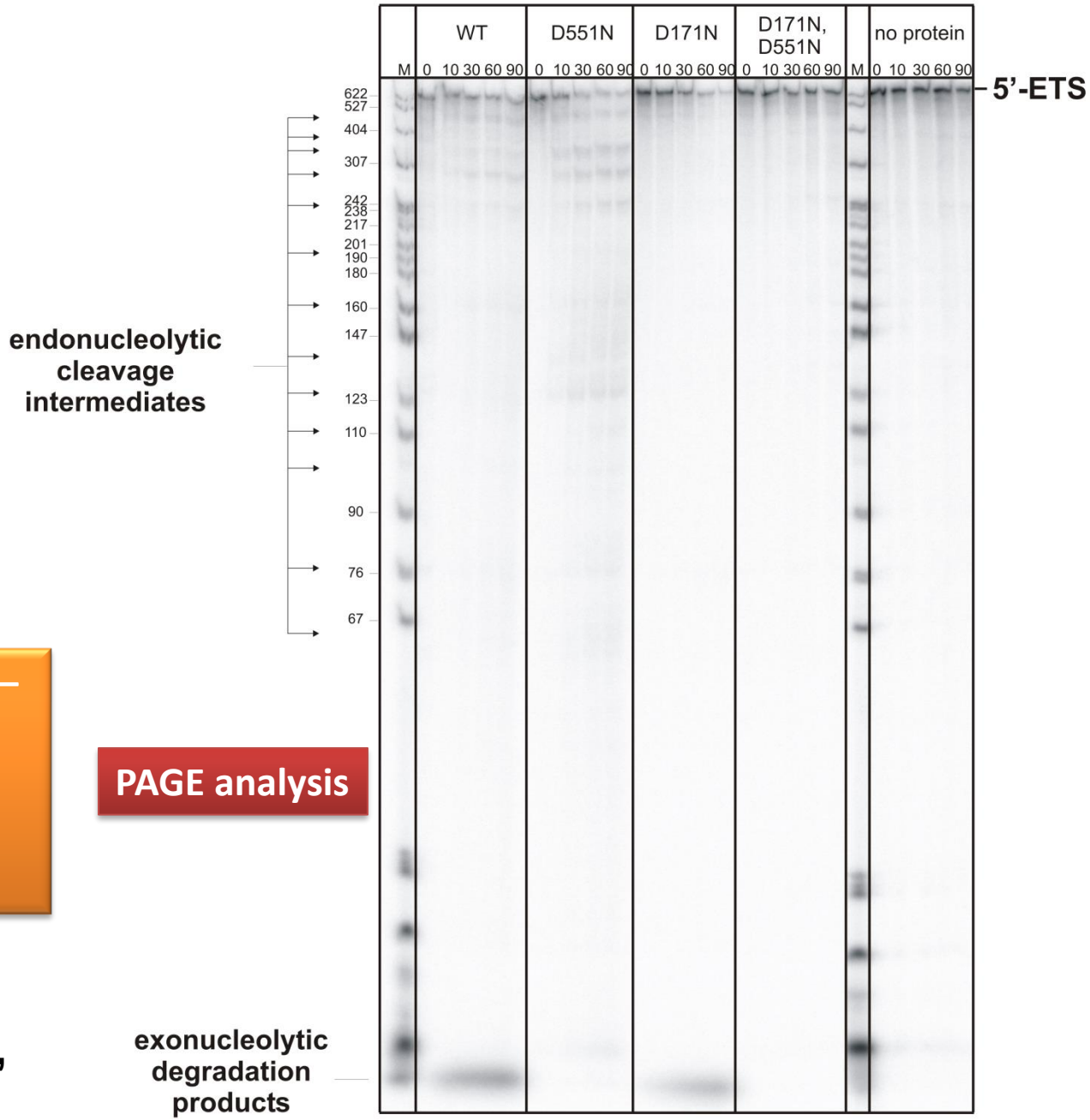
Impact of Dis3 mutations on the exosome subtrate – 5’-ETS – *in vivo*



northern-blot hybridization

Lebreton, Tomecki et al., *Nature* 2008

5’-ETS cleavage by Dis3 PIN was confirmed *in vitro*



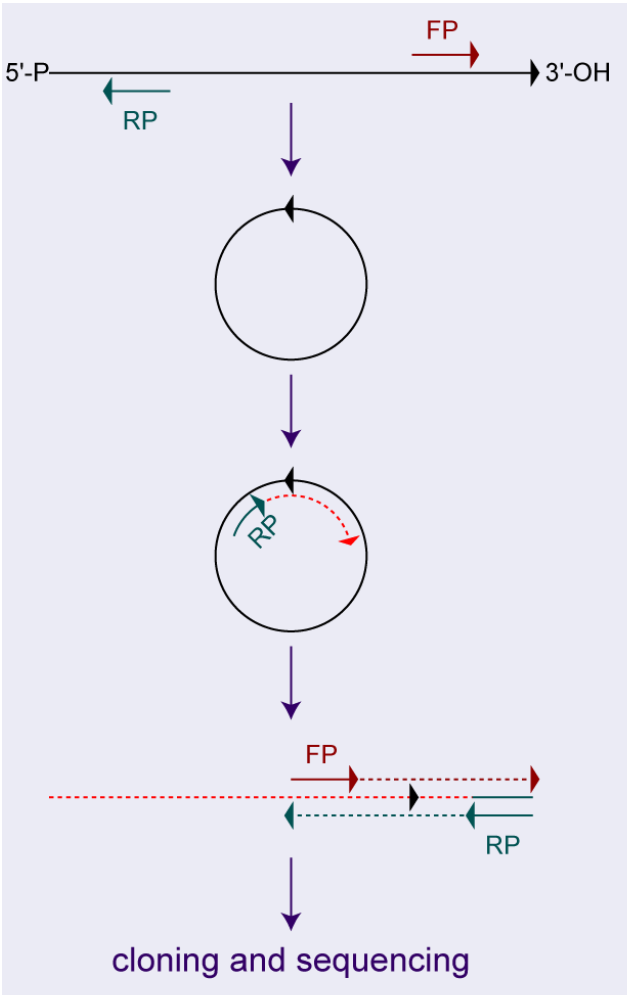
5’-ETS obtained by *in vitro* transcription in the presence of [α-^{32}P]UTP

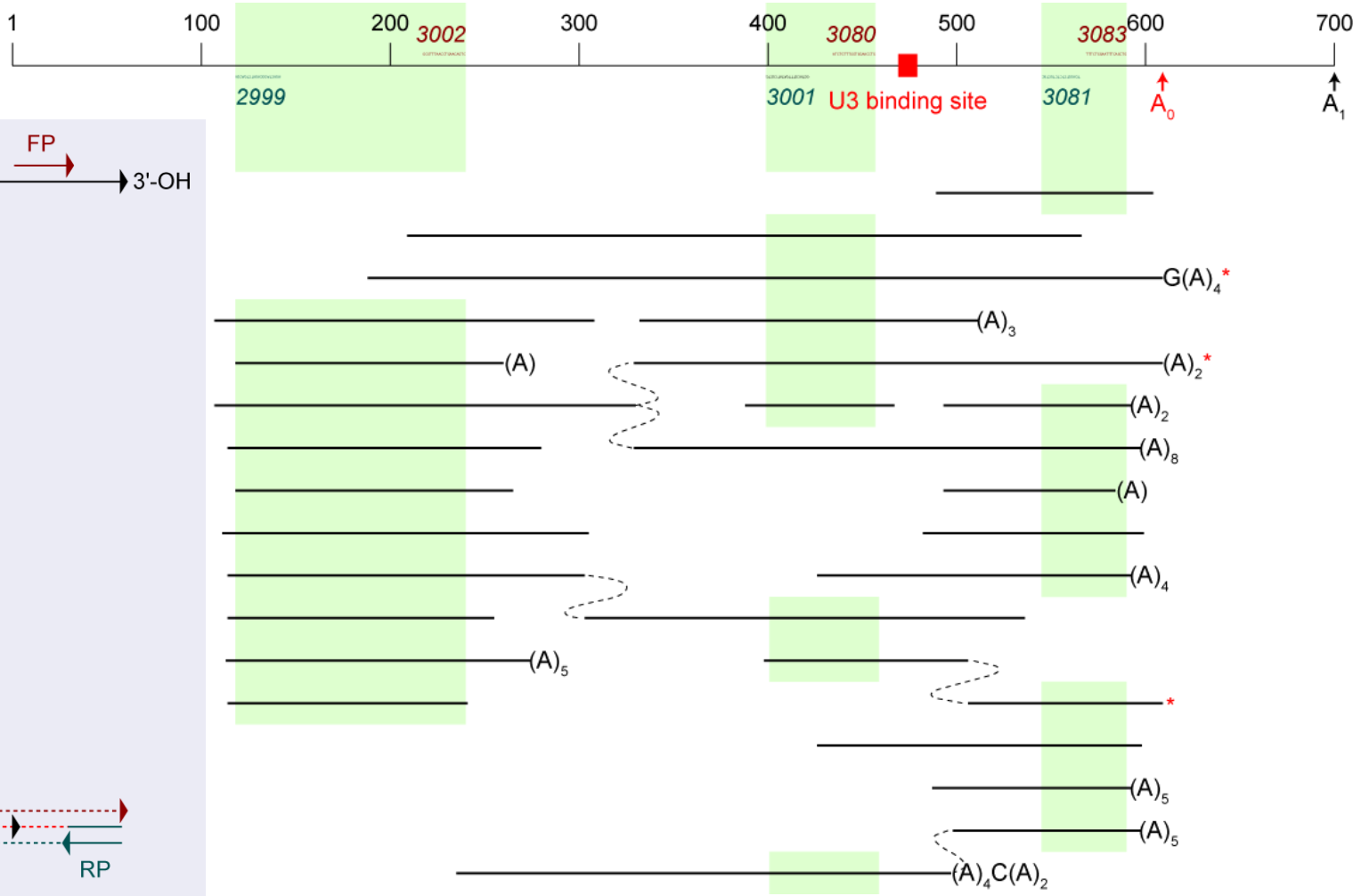
molecular weight marker– pBR322/*Msp*I 5’ end-labeled using T4 PNK and [γ-^{32}P]ATP

PAGE analysis

Lebreton, Tomecki et al., *Nature* 2008

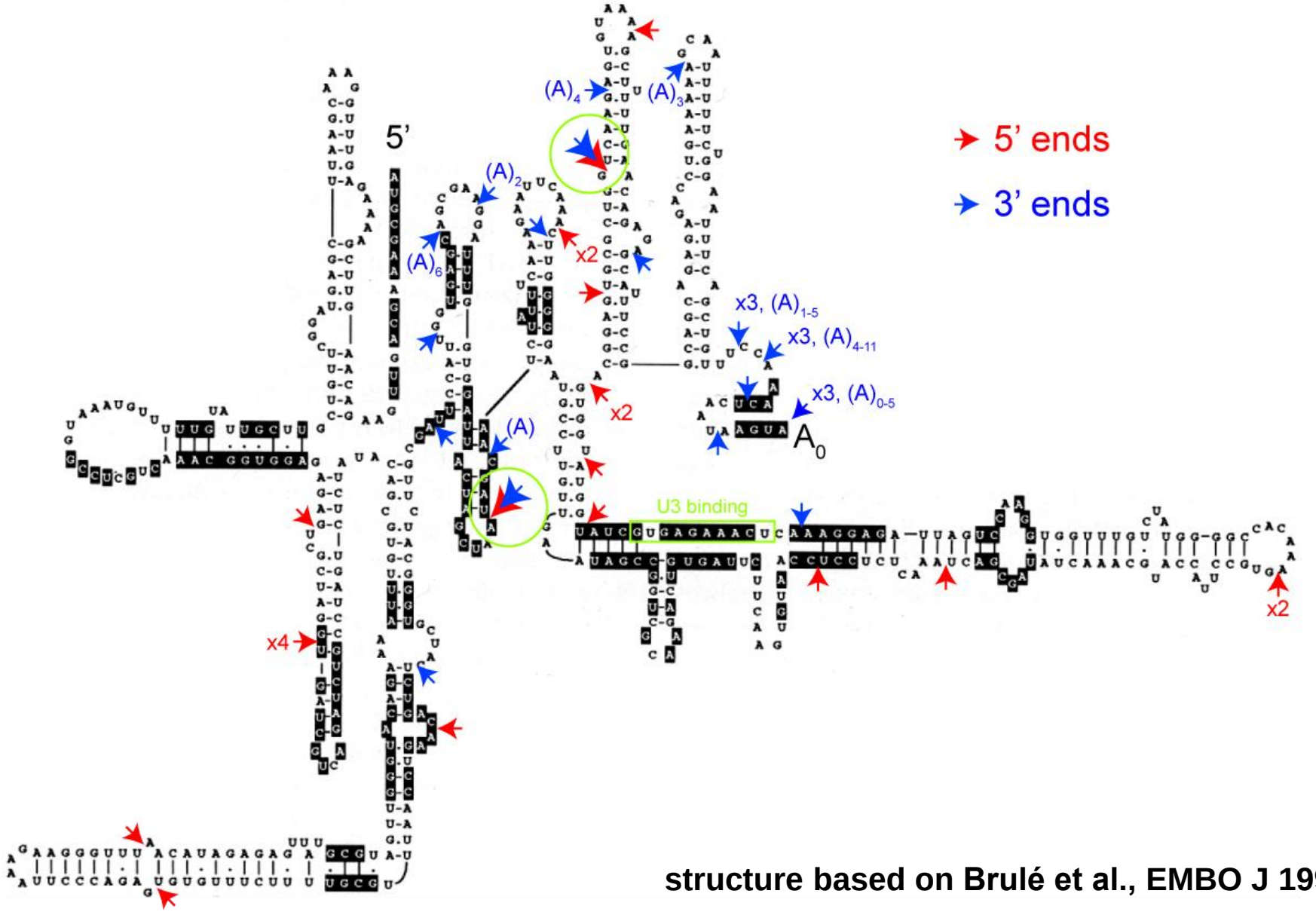
Mapping of degradation intermediates by cRT-PCR supports the endonucleolytic cleavage *in vivo*





Lebreton, Tomecki et al., *Nature* 2008

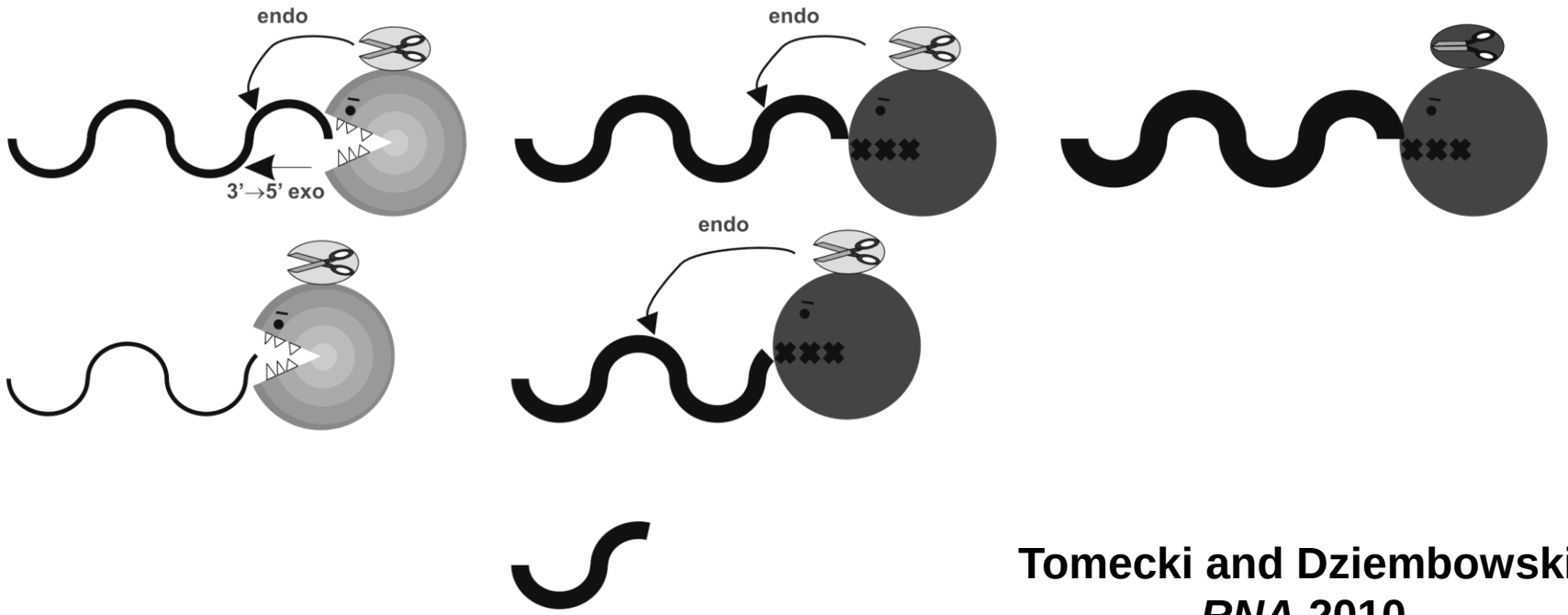
$Dis3_{PIN}$ introduces cuts preferentially within the single-stranded loops



structure based on Brulé et al., EMBO J 1996

How both Dis3 activities collaborate with one another? – a model

Hypothesis based on data from biochemical assays and *in vivo* experiments *in vivo*



Tomecki and Dziembowski, *RNA* 2010

Dis3p WT (exo+ endo+)

Dis3p D551N (exo- endo+)

Dis3p D171N D551N (exo- endo-)

The third activity of the exosome (in addition to Dis3 and Rrp6 exoribonucleases)

$Dis3_{PIN}$ is an endoribonuclease specific towards ssRNA

- *In vitro*, cleaves both linear and circular RNA substrates
- *In vivo,* participates in the decay of known natural exosome substrates
- PIN domain catalytic mutations cause synergystic phenotypes in combination with mutations of exonucleolytic activities

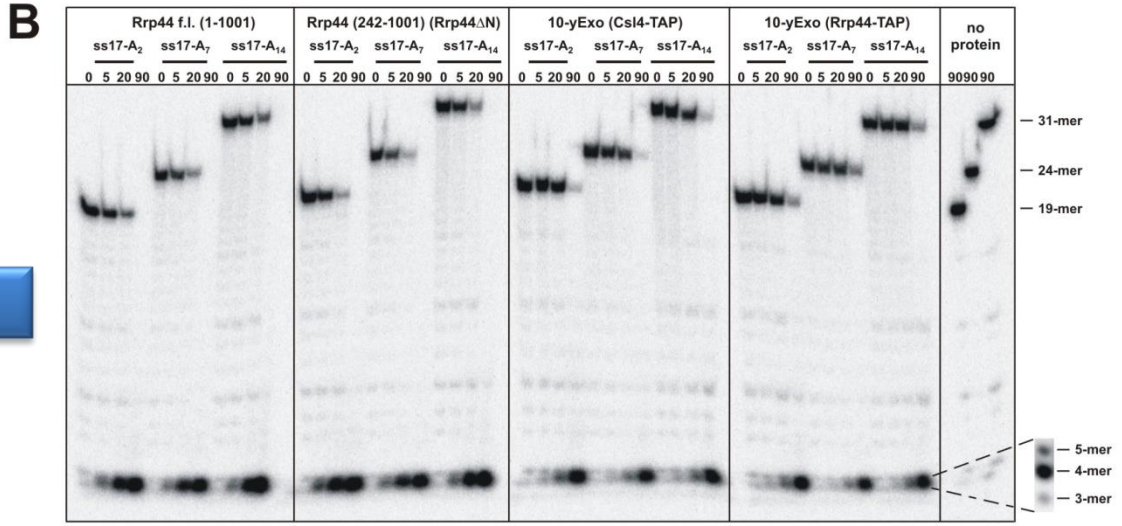
PIN domain endonucleolytic activity may assist exonucleases

- by providing alternative sites of degradation initiation when the exosome path is blocked by the presence of secondary structures within the RNA substrate

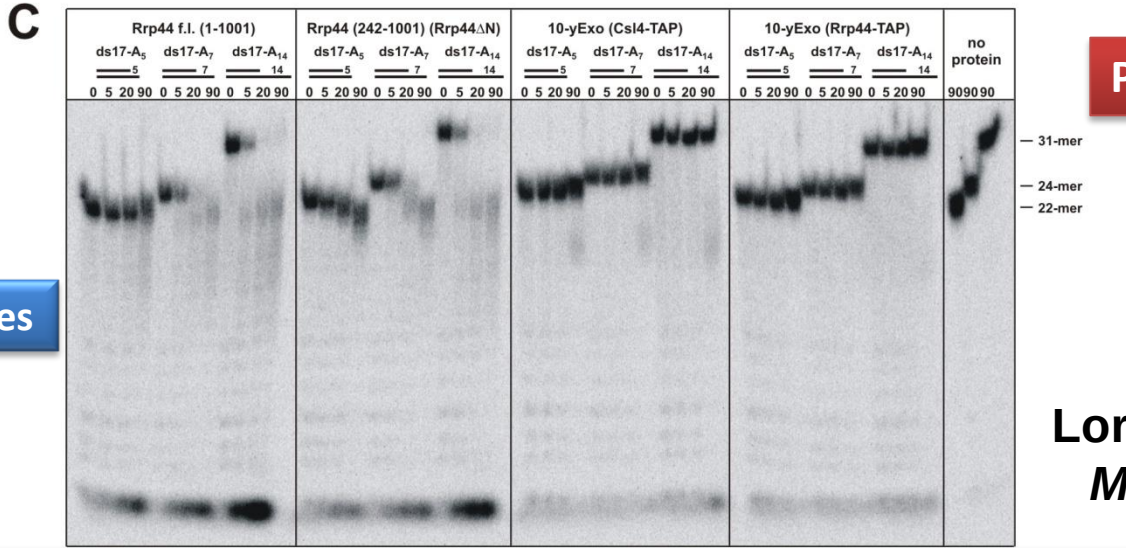
Activity of Dis3 and activity of the entire exosome – what can such comparisons tell us?

… On that it is worth examining the degradation pattern of substrates with different structures

single-stranded substrates



double-stranded substrates



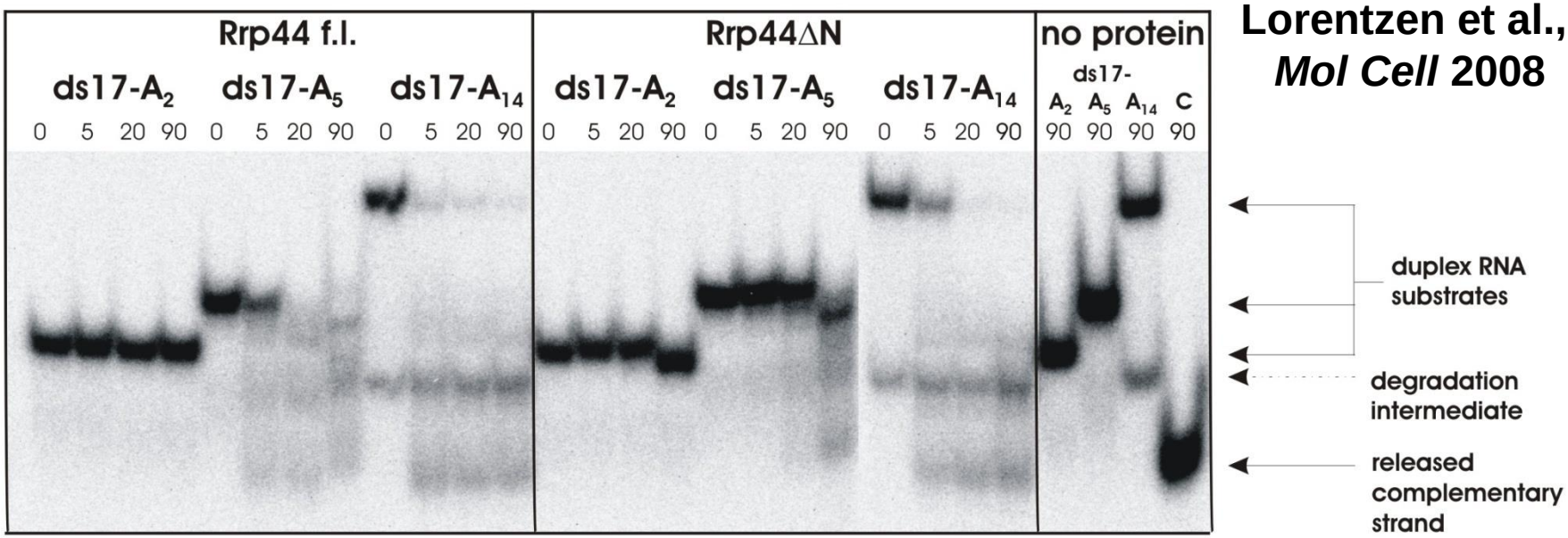
PAGE analysis

Lorentzen et al., *Mol Cell* 2008

Some properties of the exosome make the degradation of double-stranded substrates less efficient than this observed for Dis3 alone

Does Dis3p display RNA helicase properties?

double-stranded substrates



Lorentzen et al., *Mol Cell* 2008

electrophoresis of degradation products in native polyacrylamide gel

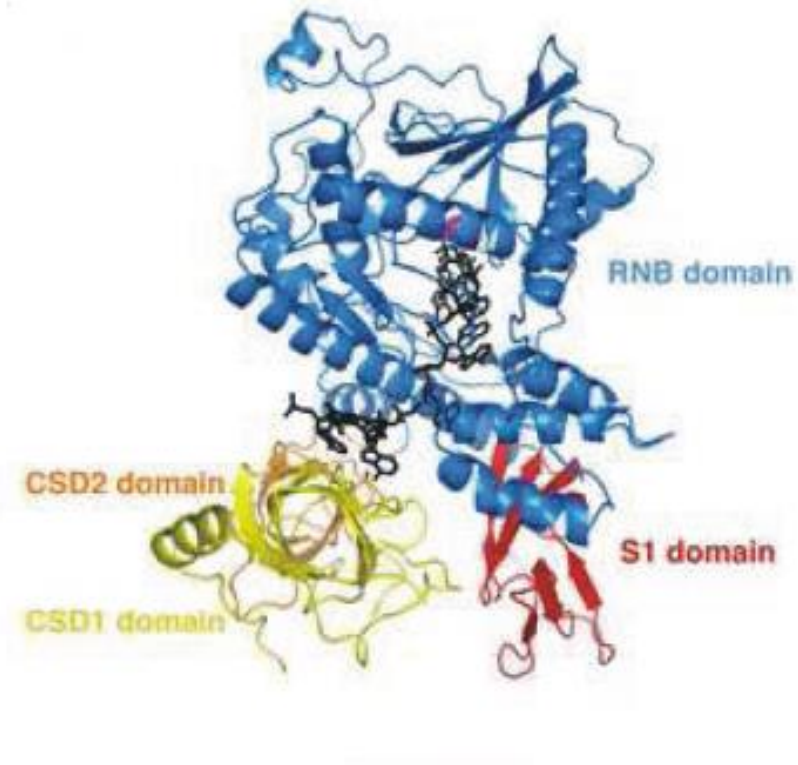
Dis3p is able to unwind double-stranded RNA substrates provided that the single-stranded extension of appropriate length is present at the 3' end of one of the strands

Lack of PIN domain decreases the efficiency of unwinding of double-stranded substrates with single-stranded fragment of „intermediate" length

The differences in the biochemical properties of Dis3 and RNase II arise from the different spatial location of the RNA-binding domains

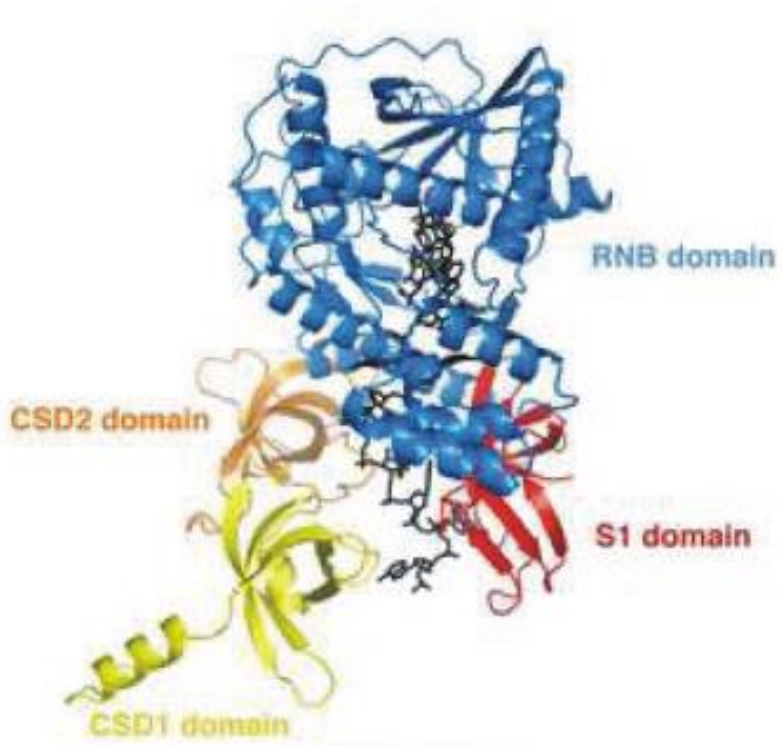
…. on how important is combining biochemical data with structural information

Dis3p



degrades dsRNA

RNase II

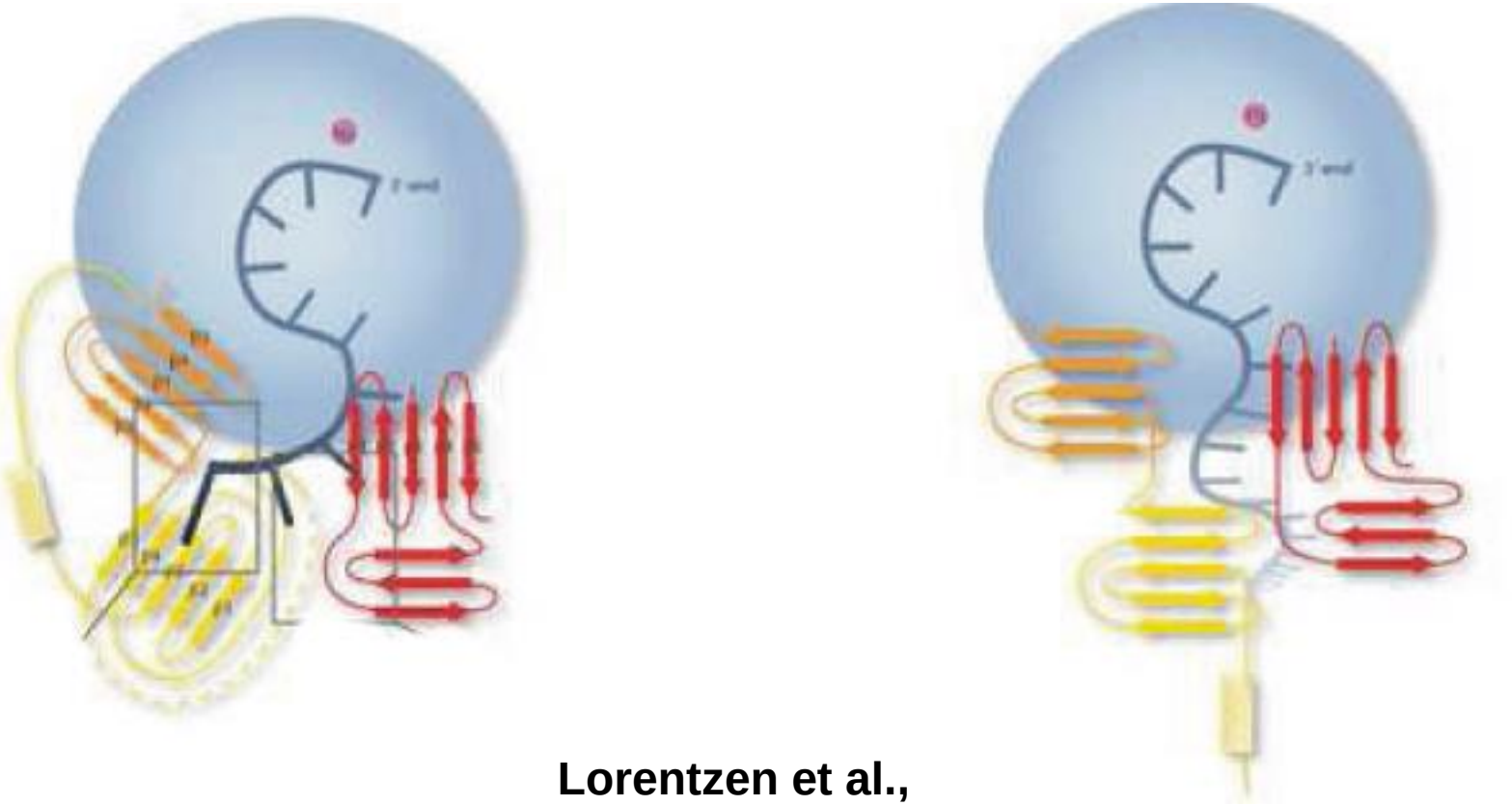


stalls upon encountering secondary structure

Lorentzen et al., *Mol Cell* 2008

Structural data explain the ability of Dis3 to unwind double-stranded RNA substrates in the course of their degradation

Dis3p **RNase II**

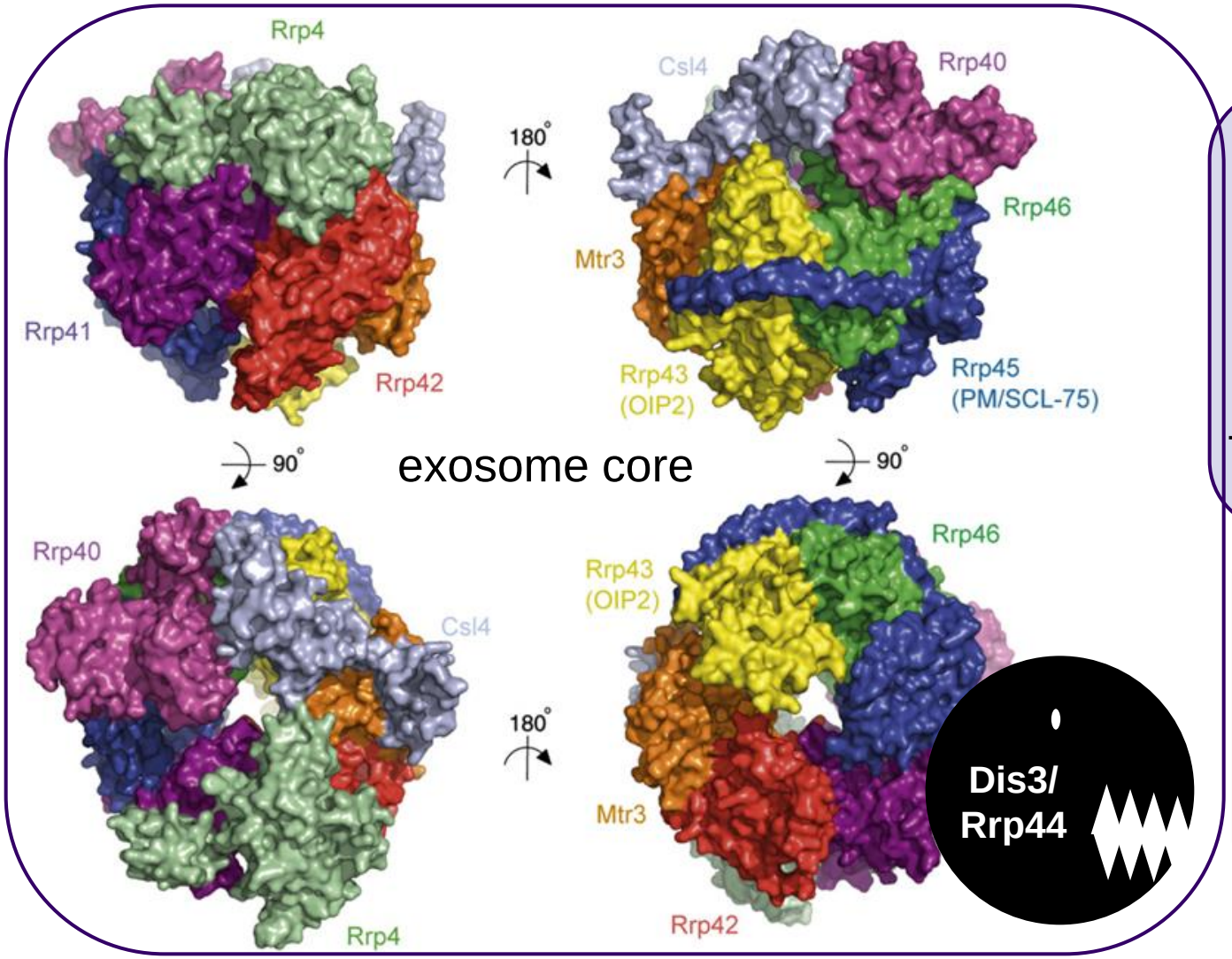


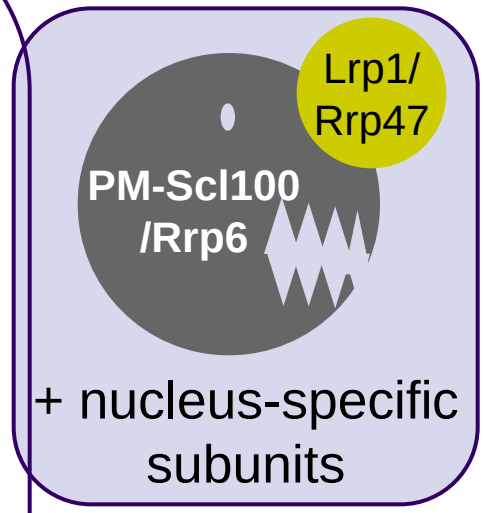
Lorentzen et al.,
***Mol Cell* 2008**

RNA hydrolysis leads to the rotation of the RNA chain, which provides energy allowing the strands to be separated during subsequent rounds of catalysis

Does the mystery of the differences between the activities of Dis3 and the exosome lie in the structure of the complex?

Where is Dis3 localized with respect to the ring?



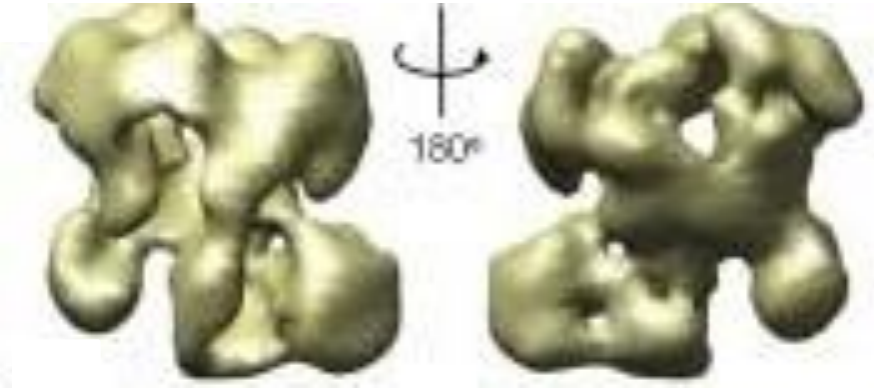


crystallization of the reconstituted 9-subunit ring of the human exosome

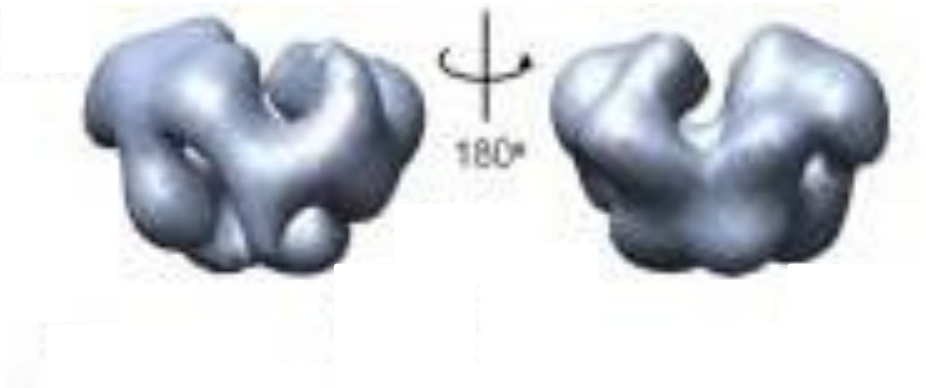
Liu et al., *Cell* 2006; Hernandez et al., *EMBO Rep* 2006; Dziembowski et al., *NSMB* 2007

Dis3 is localized underneath the exosome ring (*i.e.* opposite the site of KH/S1 cap subunits location)

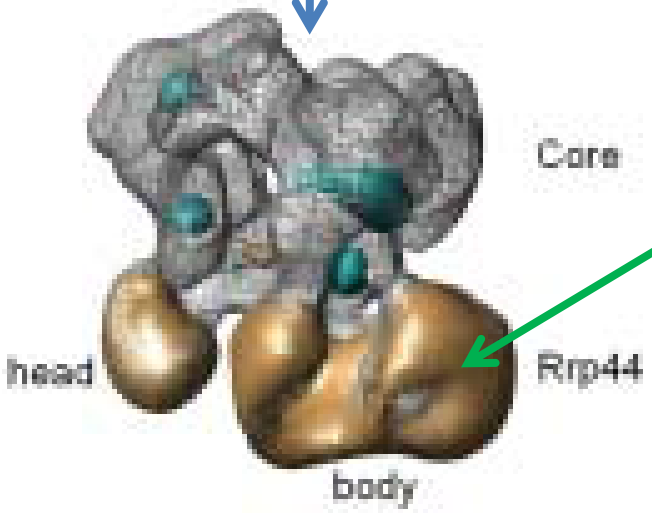
core with Dis3



core without Dis3



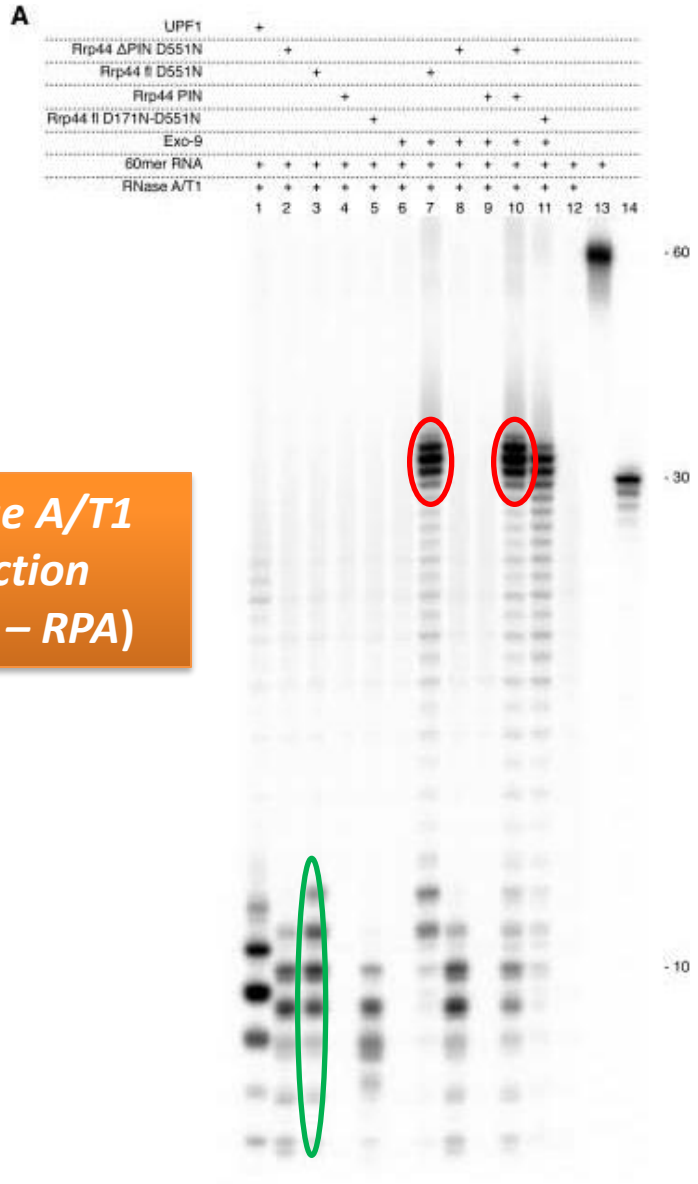
structure of the exosome core (9-subunit ring + Dis3) solved using electron microscopy (*negative staining*)



Wang et al., *PNAS* 2007

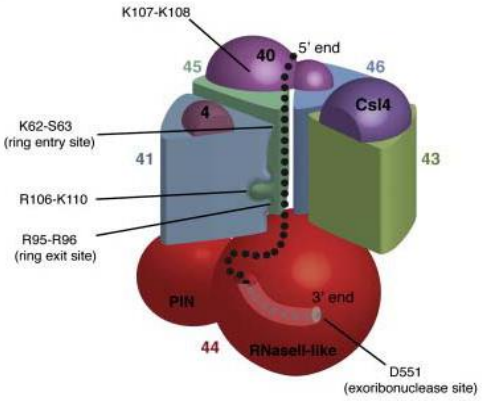
Dis3 alone binds single-stranded RNA fragments of significantly different length than when working in the context of the exosome core

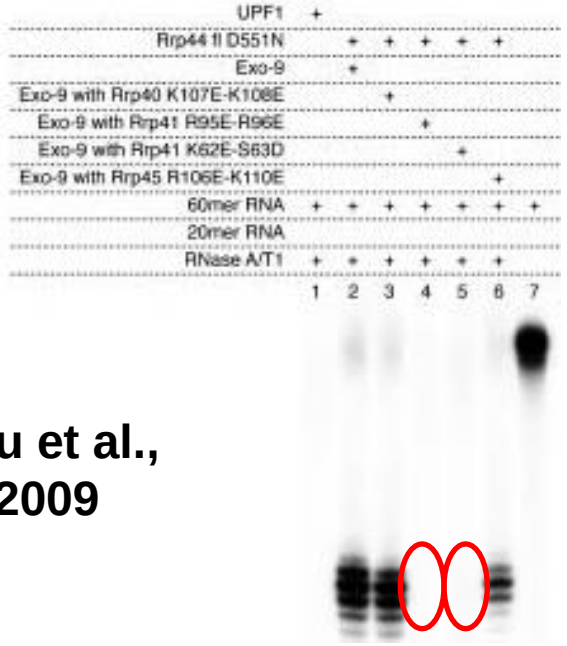
(*RNase A/T1 protection assay – RPA*)



channel entrance

channel exit



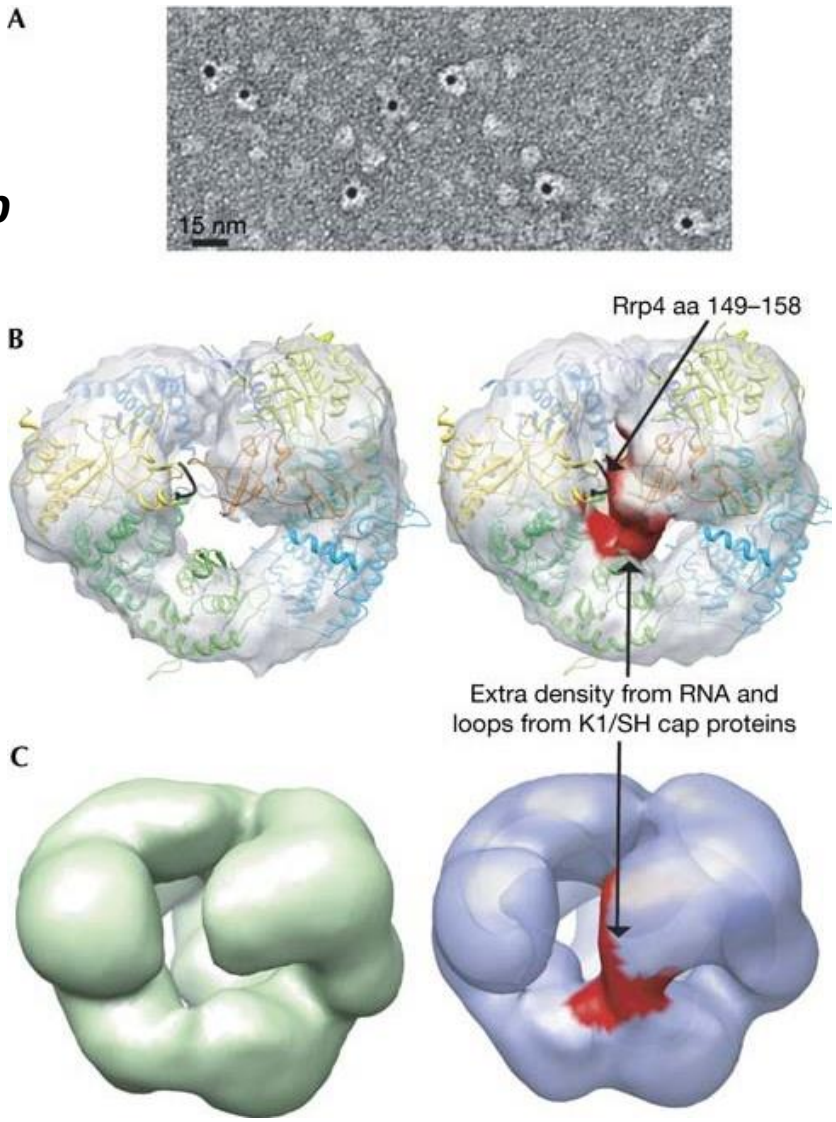


Bonneau et al., *Cell* 2009

CONCLUSION: RNA traverses the channel before reaching Dis3 active site

The substrate path through the ring channel is indeed evolutionary conserved

Malet et al., *EMBO Rep* 2010



RNA oligonucleotide (partially double-stranded, with ~35 nt single-stranded stretch), biotinylated at the 5' end, conjugated with colloidal gold (5 nm)-labeled streptavidin – BLACK DOTS

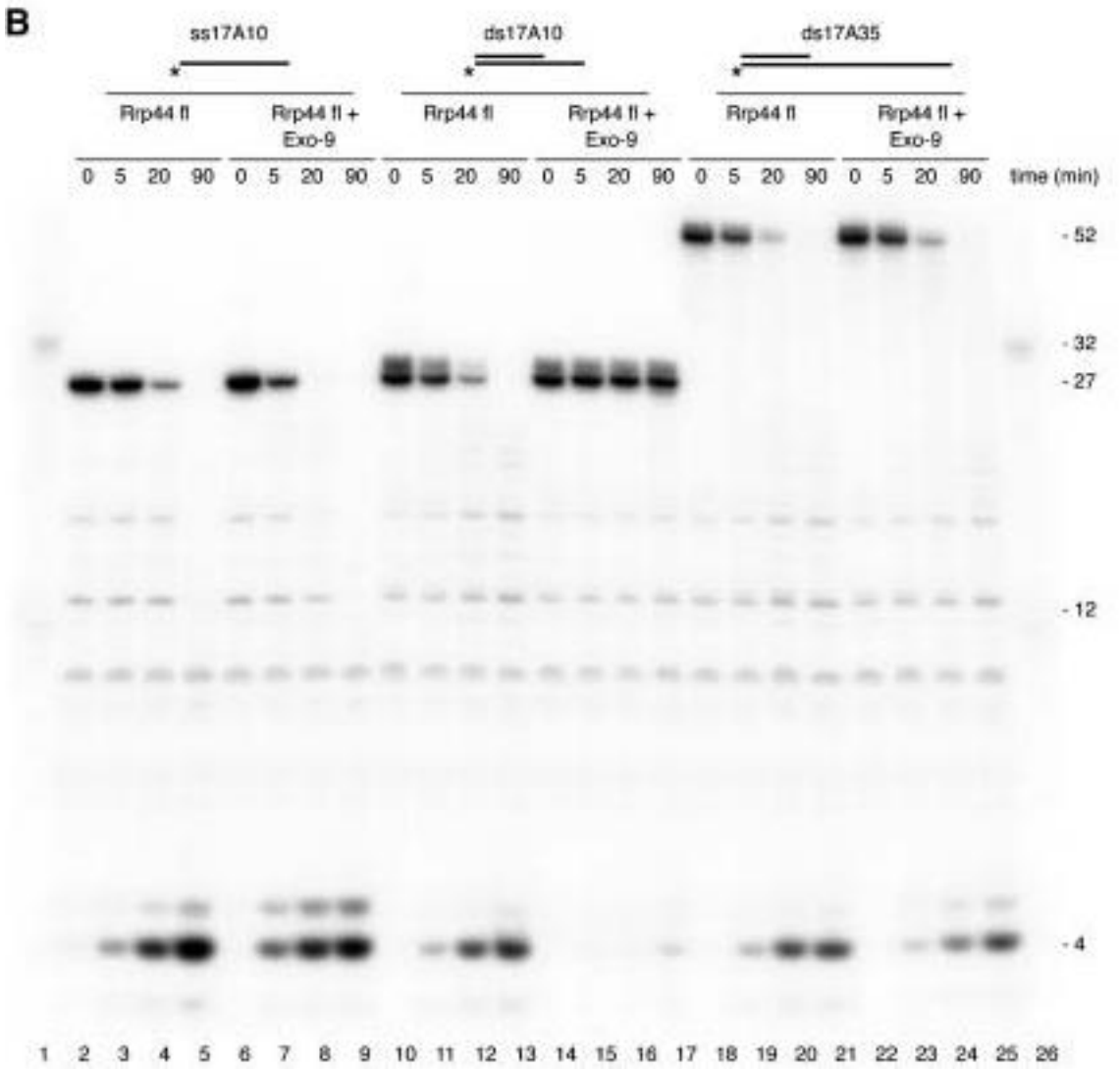
structure of the exosome core solved by cryo-electron microscopy

top-views

structure of the exosome core solved using electron microscopy (*negative staining*)

„apo" structure **structure with RNA**

Double-stranded RNA substrates are degraded by RNB domain in the context of exosome core provided that the single-stranded 3’ extension of appropriate length is present

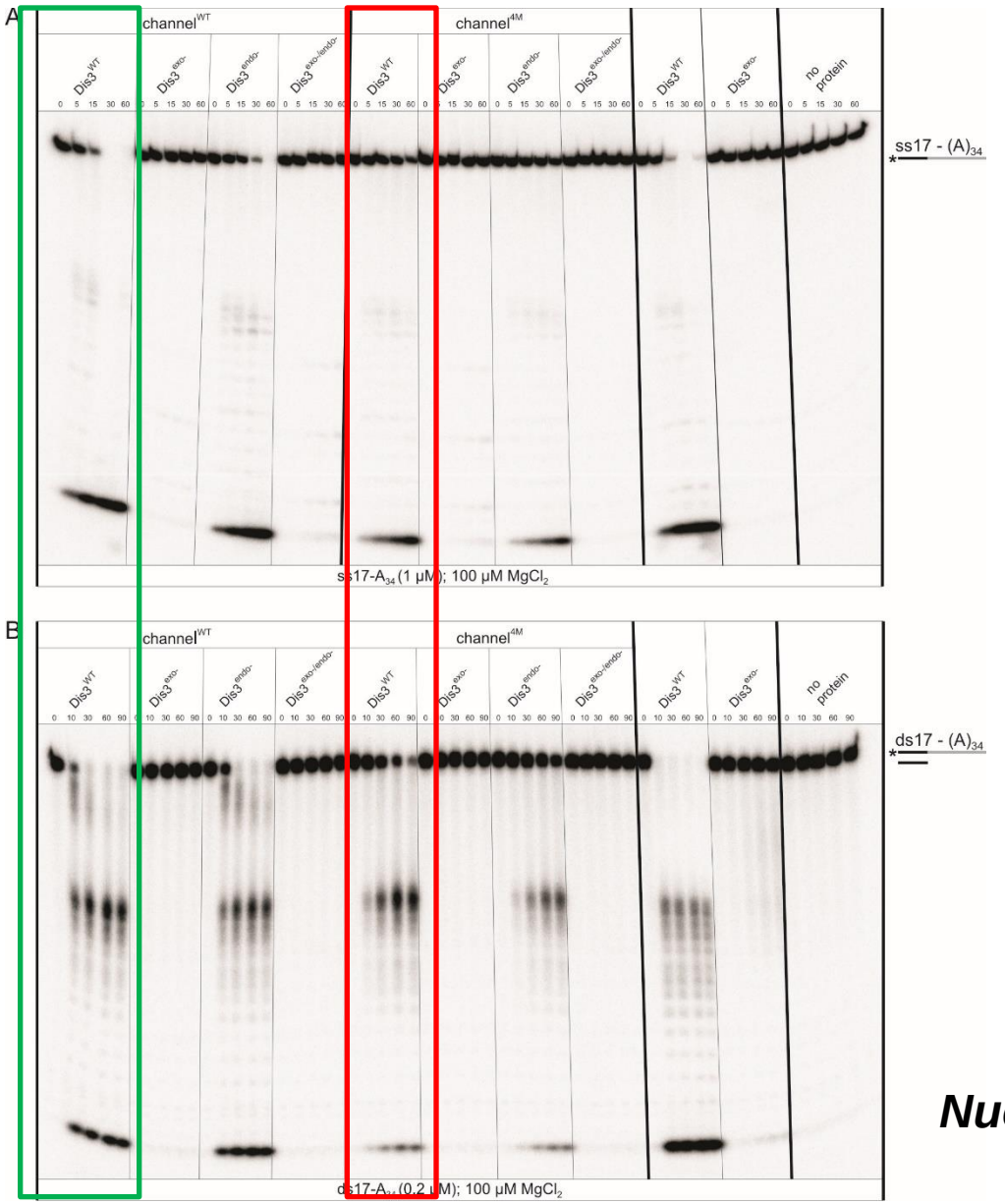


PAGE analysis

Bonneau et al., *Cell* 2009

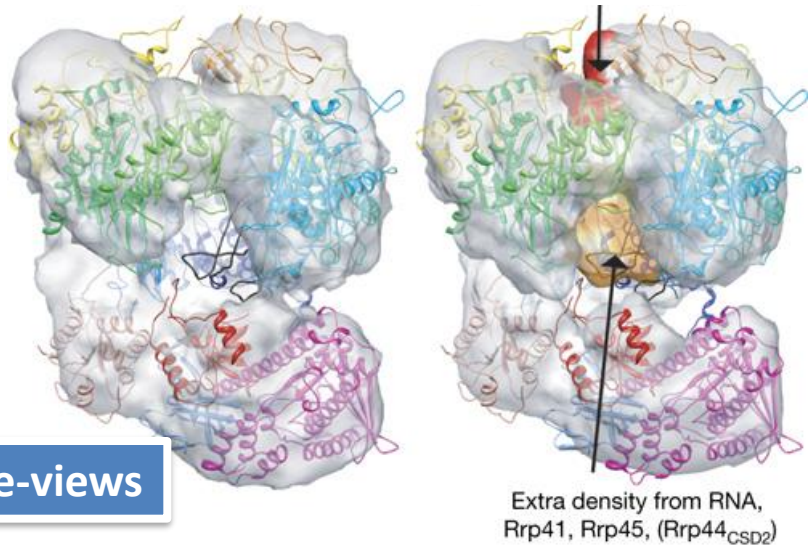
Blocking the channel decreases degradation efficiency of RNA substrates – both single- and double-stranded

PAGE analyses

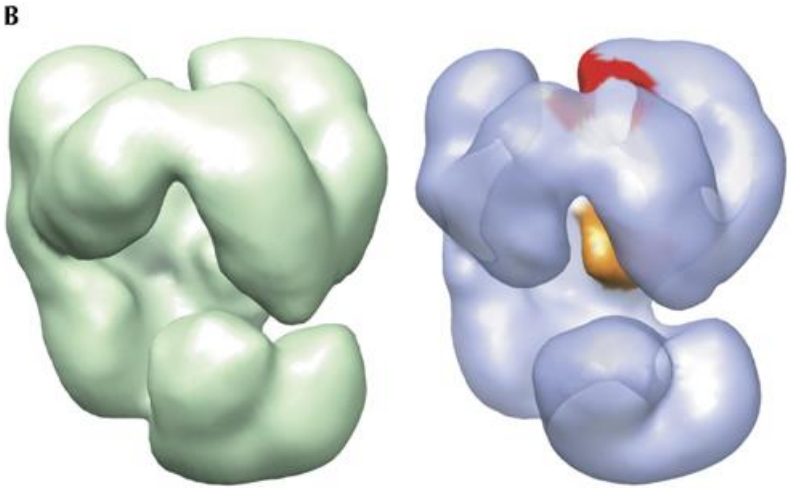


Drążkowska et al., *Nucleic Acids Res* 2013

Is this route utilized both for directing the substrate to the RNB domain as well as to the PIN domain?



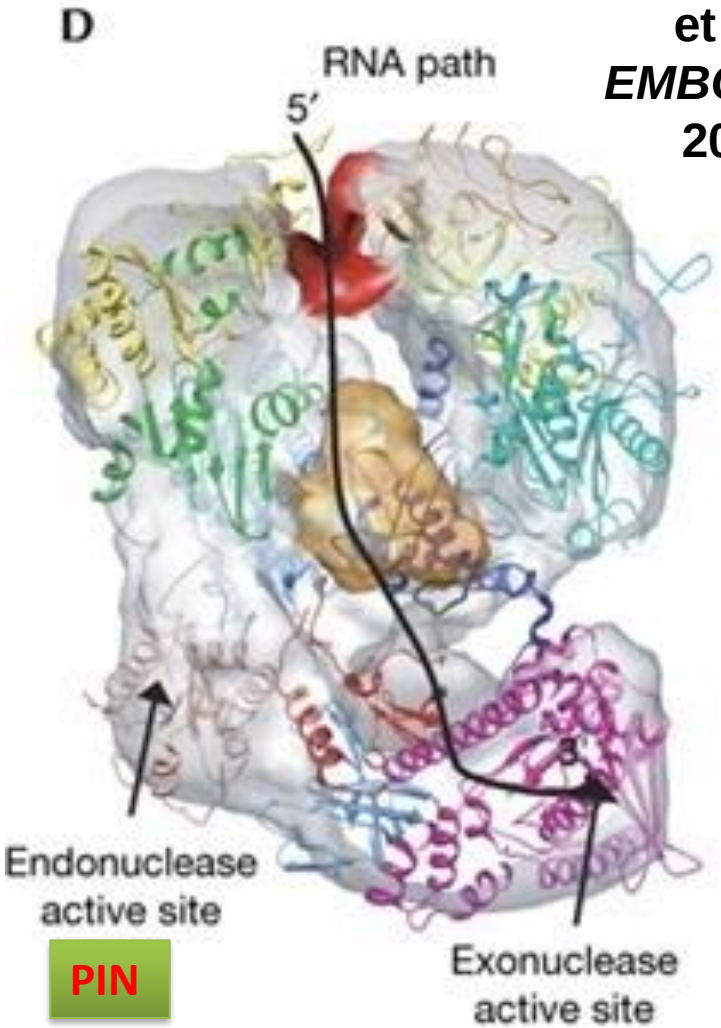
side-views



„apo" structure

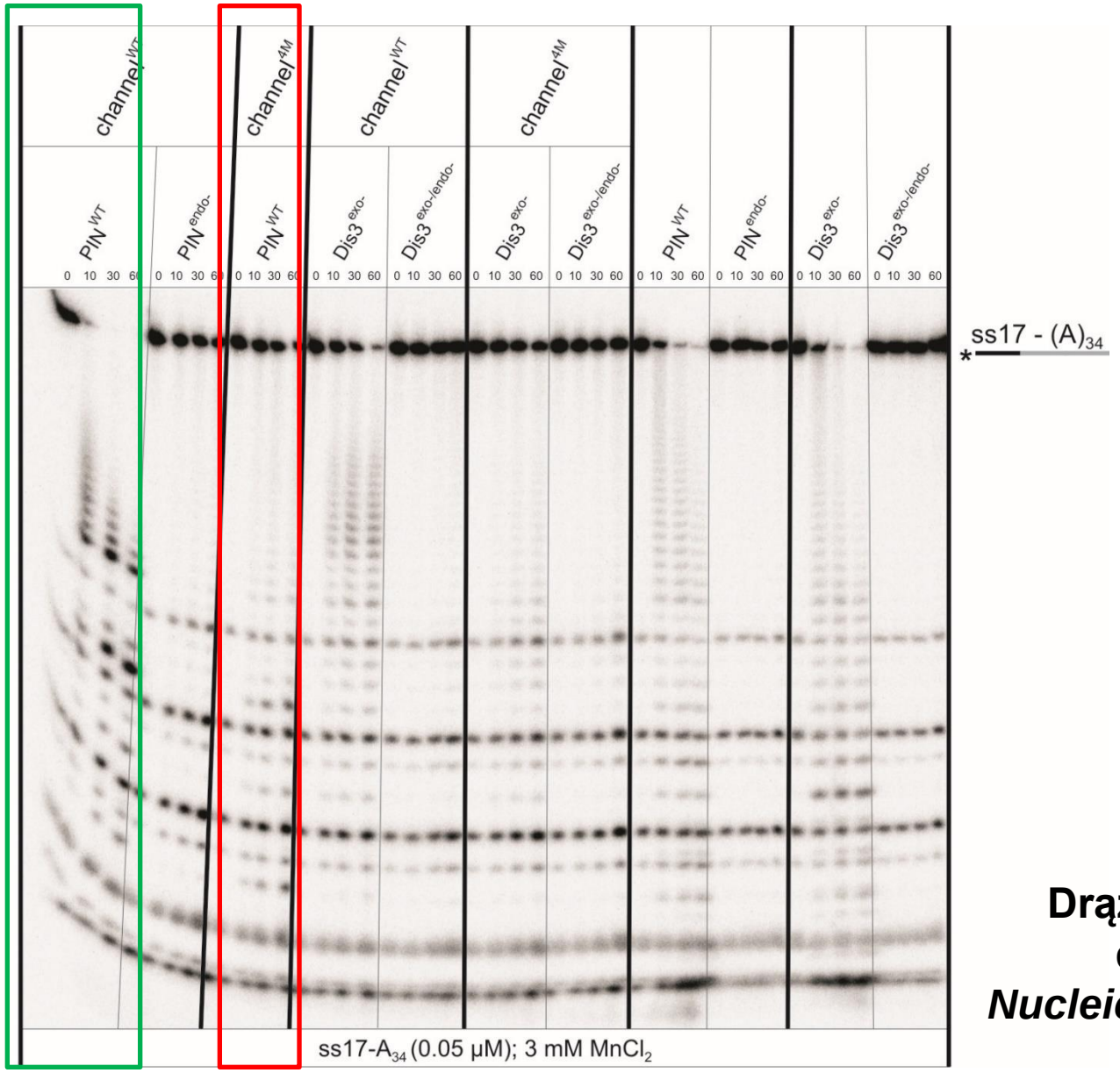
structure with RNA

Malet et al., *EMBO Rep* 2010



Occluding the channel inhibits PIN domain endonucleolytic activity too!

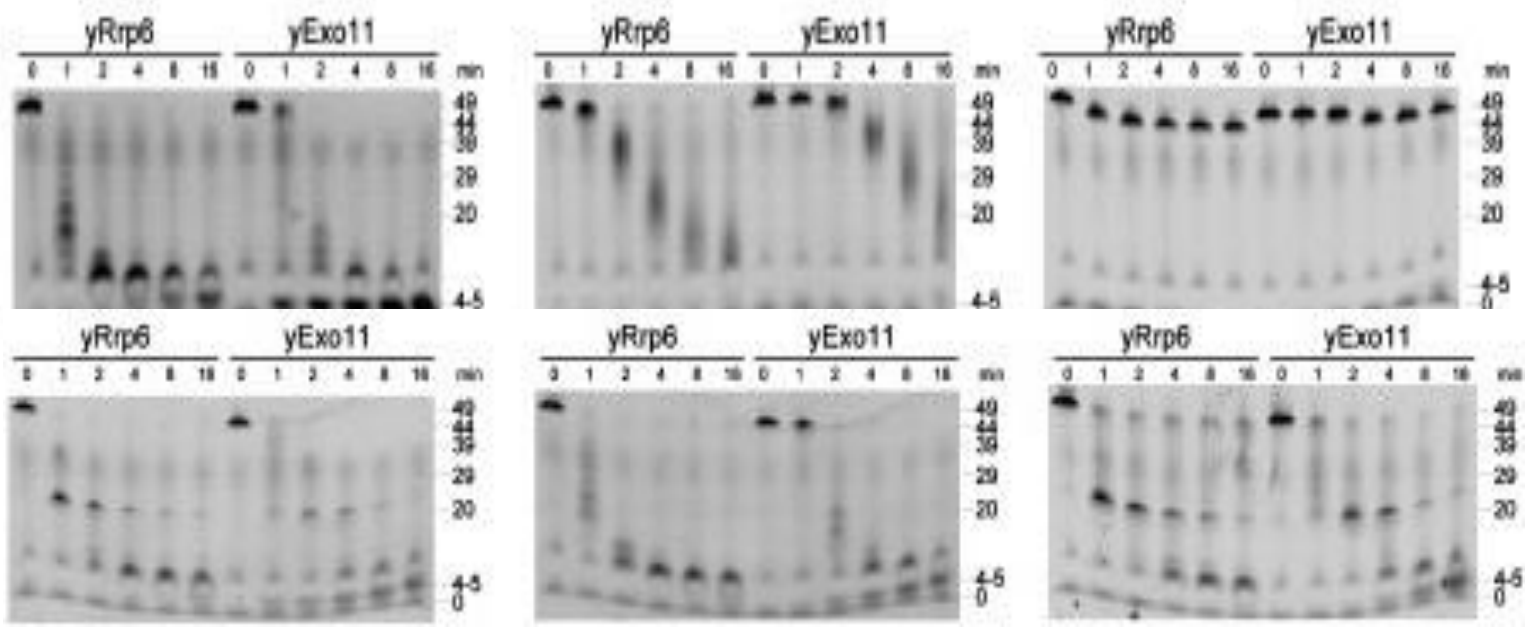
PAGE analysis



Drążkowska et al., *Nucleic Acids Res* 2013

It appears that the central channel rather does not partake in the regulation of Rrp6 activity

PAGE analyses



Liu et al., *Cell* 2006

Summary – what you should remember

1. Always start with a detailed analysis of the sequence of the protein being examined and available information about homologs

2. Test as many reaction conditions as possible (different substrates, cofactors, various buffers) and remember about all possible controls (both negative ones – particularly mutations in the putative catalytic sites, and positive)

3. Compare activities of individual proteins and entire complexes or sub-complexes – this can sometimes unveil interesting information

4. Strive to obtain the structure of the tested protein/complex, because only then proper interpretation of the „wet lab" experiments would be possible, but …

5. … the structure on its own will not tell us much without biochemical data

6. Attempt to verify structural and biochemical data through experiments in a living system – whether what we discovered in the test tube actually works similarly in the cell and has biological significance?