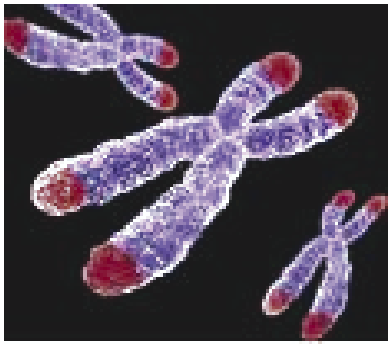
Toward Saccharomyces cerevisiae telomere architecture: an integrated structural biology study.

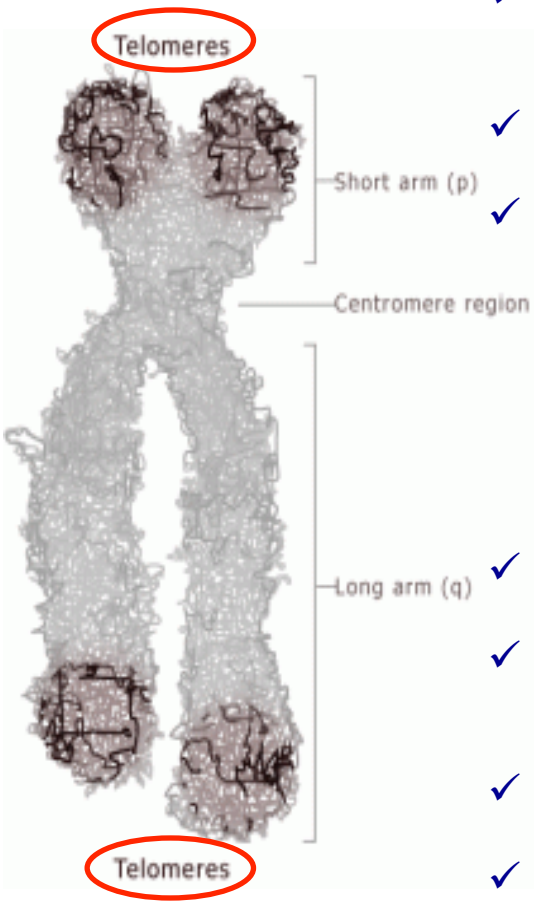


M-H Le Du

Laboratoire de Biologie Structurale et Radiobiologie (CEA/Saclay)

Oléron, 2015/06/05

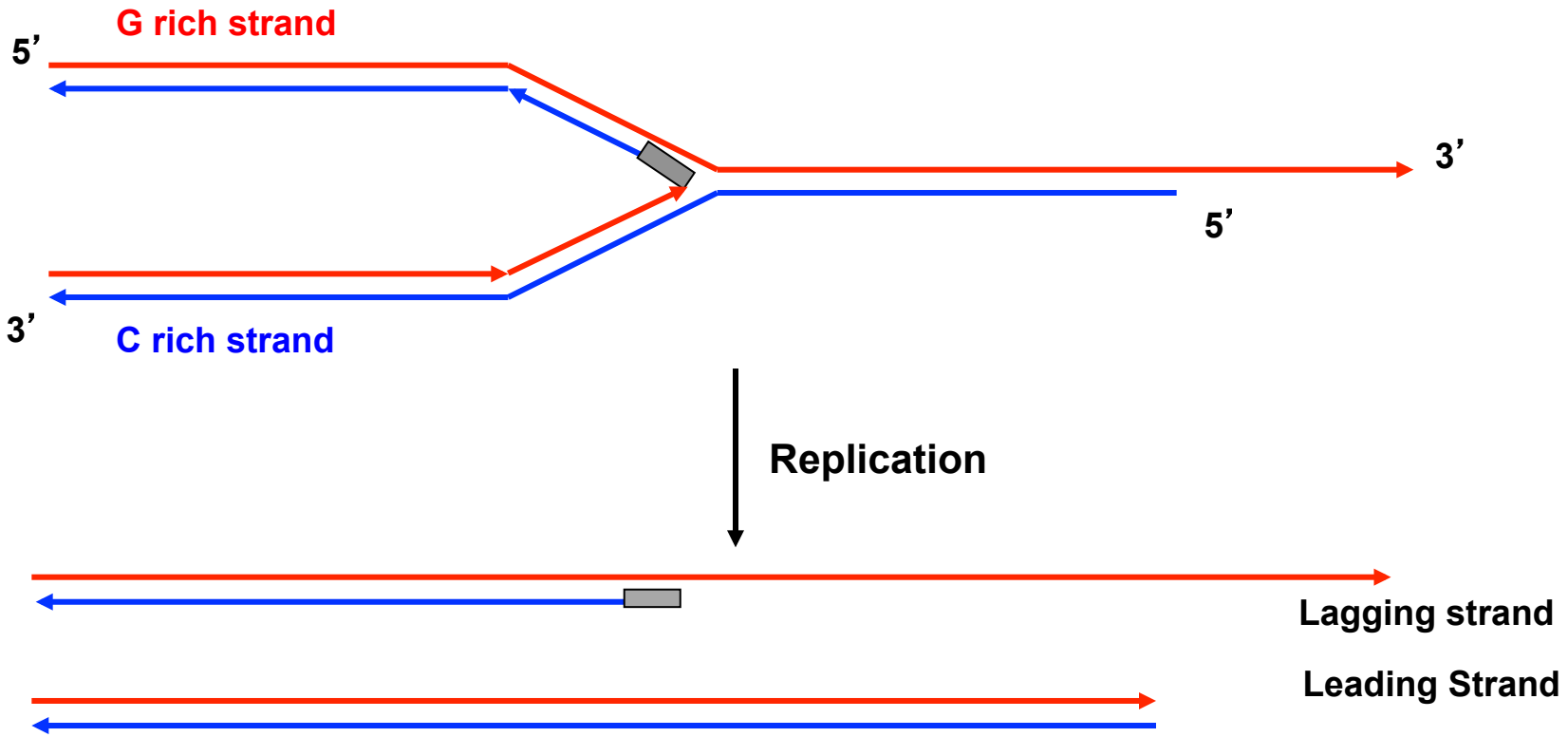
Telomeres :



- ✓ **Terminal protein-DNA complexes of linear eukaryotic chromosomes**
- ✓ **Does NOT code for any genetic information**
- ✓ **Protects the chromosomal ends from:**
  - **Recombination**
  - **End-to-end fusion (NHEJ)**
  - **Recognition as damaged DNA**
- ✓ **Controls the terminal replication of chromosomal DNA**
- ✓ **Contributes to the functional organization of chromosomes in the nucleus**
- ✓ **Participates in regulation of gene expression**
- ✓ **Serves as "mitotic clock": shortens with each cell division**

Replication problem:

Natural shortening upon cell divisions



(based on Gilson E. & Géli V., 2007)

Answer to telomere shortening: molecular counting mechanism

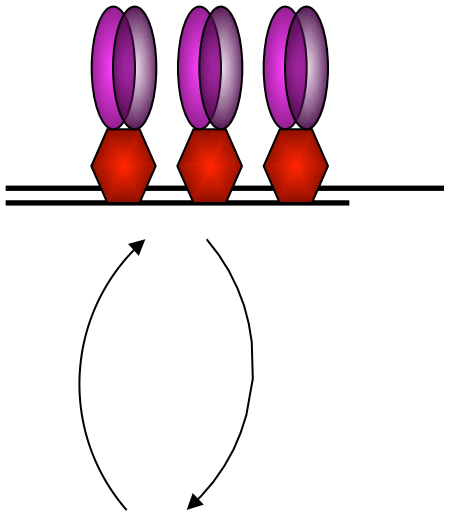
Preferential elongation of short telomeres

Natural shortening

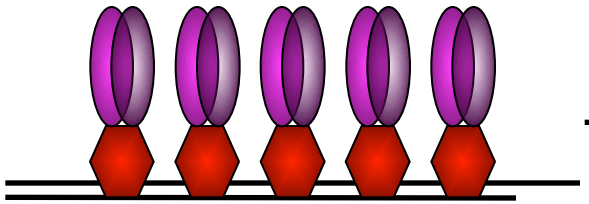
Molecular counting mechanism

Lengthening by telomerase at the end of S phase

Short telomeres: « **extendible** » state

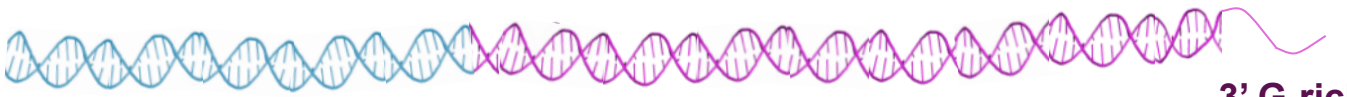


Long telomeres: « **non-extendible** » state



Marcand S & al (1997),
Teixeira M.T. & al. (2004)

Telomeres in *Saccharomyces cerevisiae*



3' G-rich overhang

40-200 base pairs

Subtelomeric area

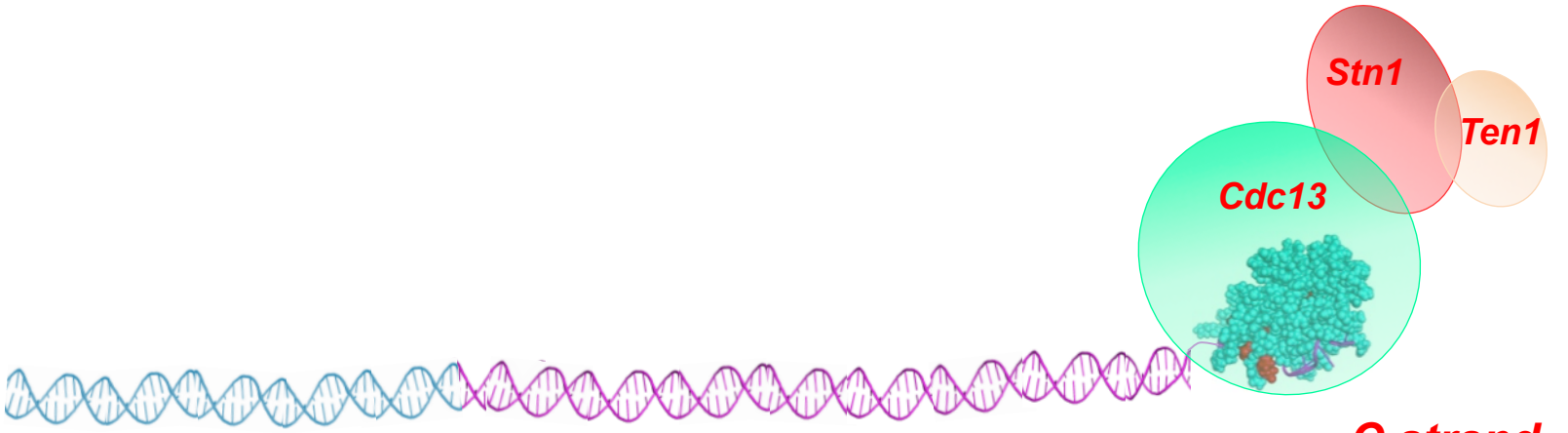
TG_{1-3} repeats

Adapted from Giraud-Panis M-J, Pisano S, Poulet A, Le Du M-H, and Gilson E. FEBS Lett 2010, 584(17):3785-3799.

Telomeres in *Saccharomyces cerevisiae*

Telomerase activation

End protection



C strand protection

Subtelomeric area

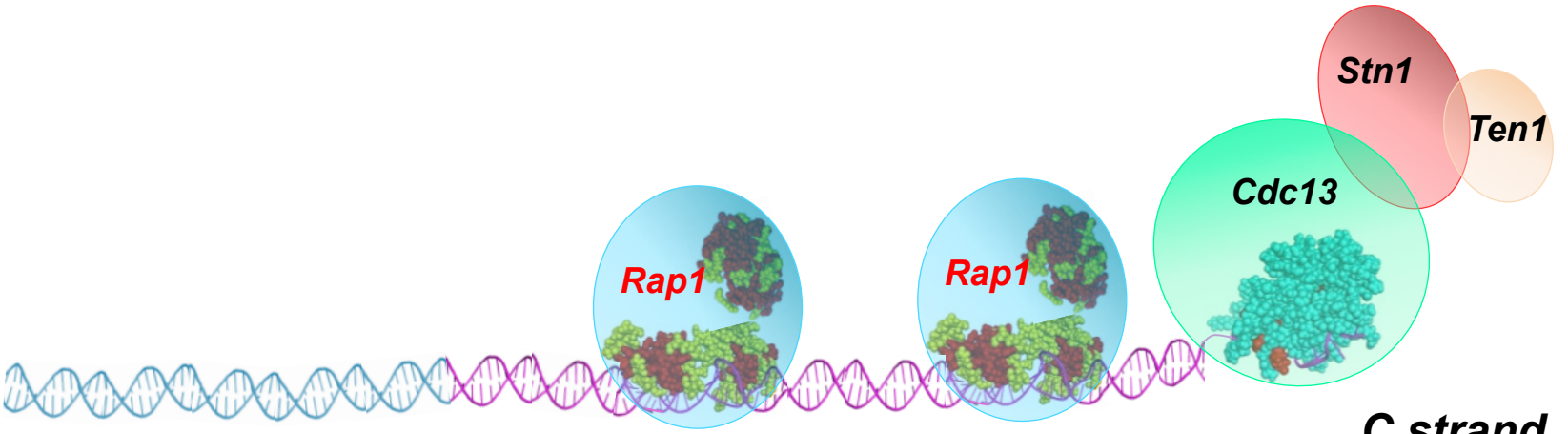
TG_{1-3} repeats

Adapted from Giraud-Panis M-J, Pisano S, Poulet A, Le Du M-H, and Gilson E. FEBS Lett 2010, 584(17):3785-3799.

Telomeres in *Saccharomyces cerevisiae*

Telomerase activation

End protection



C strand protection

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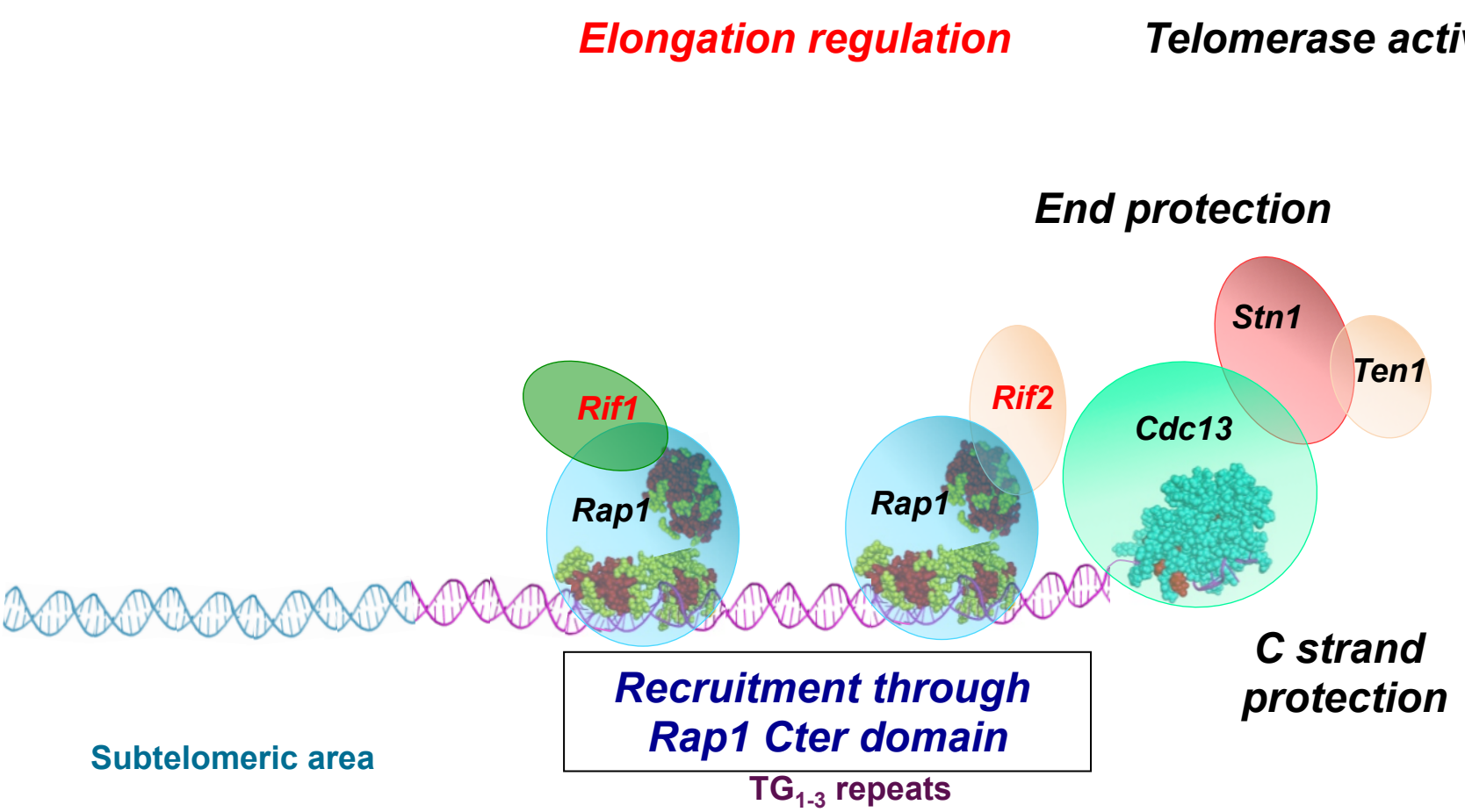
Telomeres in *Saccharomyces cerevisiae*

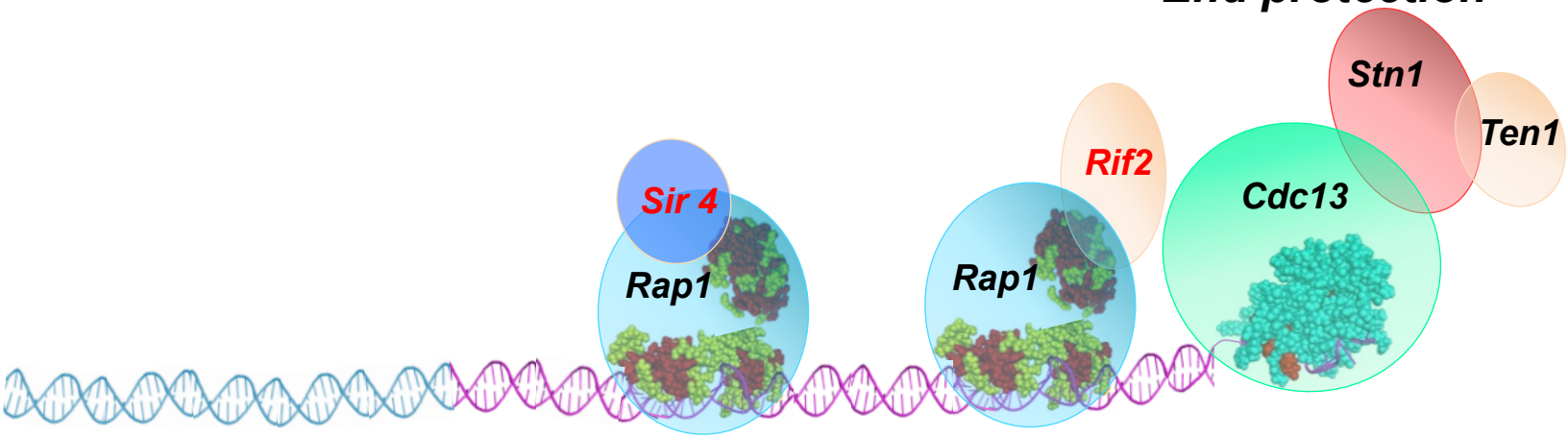


Adapted from Giraud-Panis M-J, Pisano S, Poulet A, Le Du M-H, and Gilson E. FEBS Lett 2010, 584(17):3785-3799.

Telomeres in *Saccharomyces cerevisiae*

Elongation regulation ***Telomerase activation***

End protection



C strand protection

Subtelomeric area

TG_{1-3} repeats

DNA repair inhibition

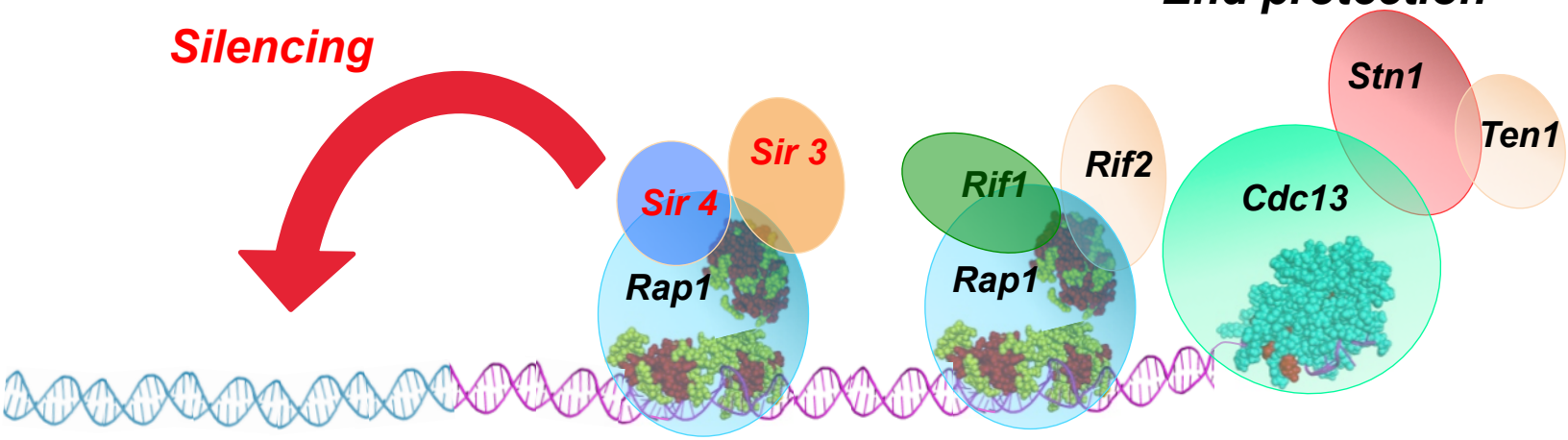
Adapted from Giraud-Panis M-J, Pisano S, Poulet A, Le Du M-H, and Gilson E. FEBS Lett 2010, 584(17):3785-3799.

Telomeres in *Saccharomyces cerevisiae*

Elongation regulation ***Telomerase activation***

End protection

Silencing



C strand protection

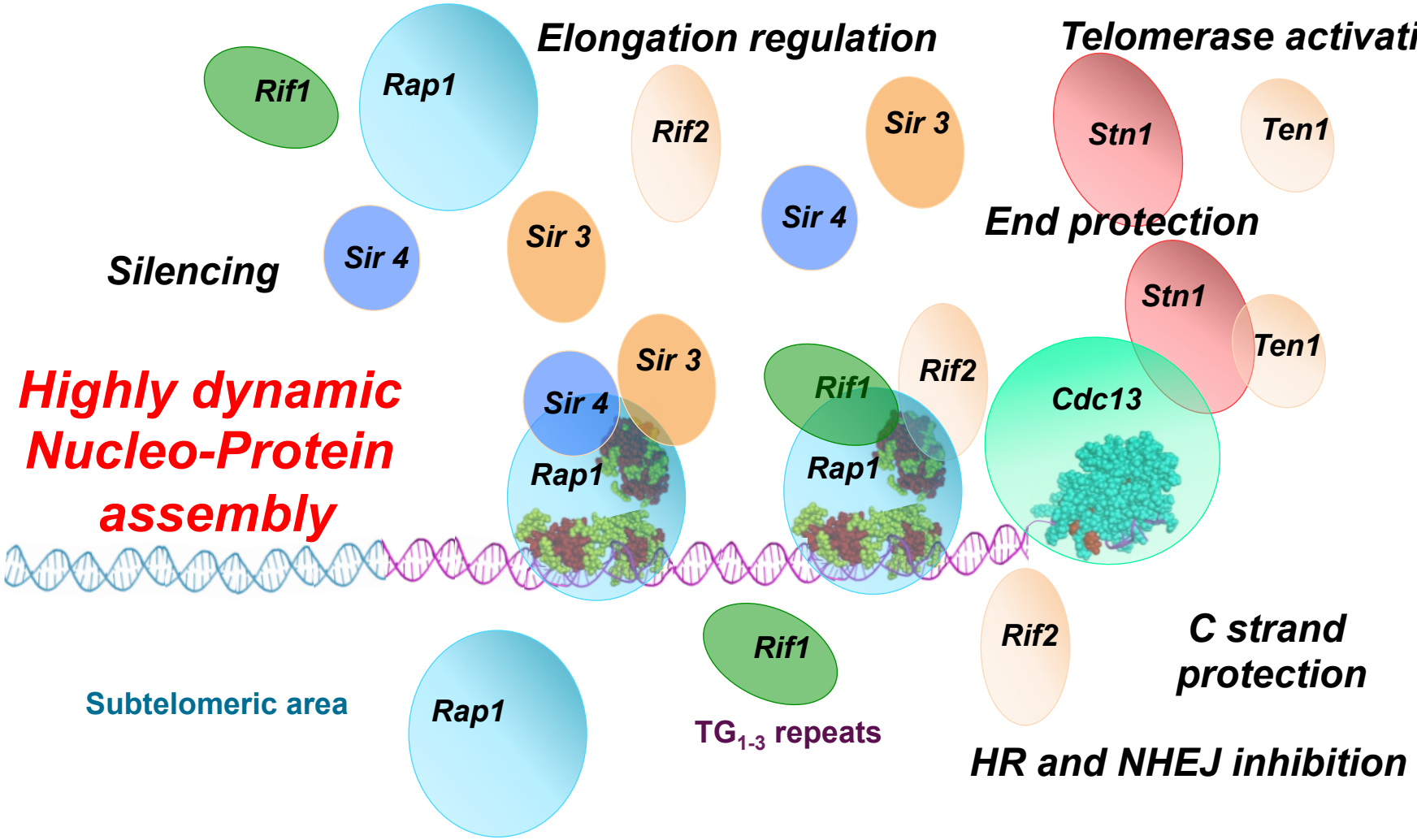
Subtelomeric area

TG_{1-3} repeats

HR and NHEJ inhibition

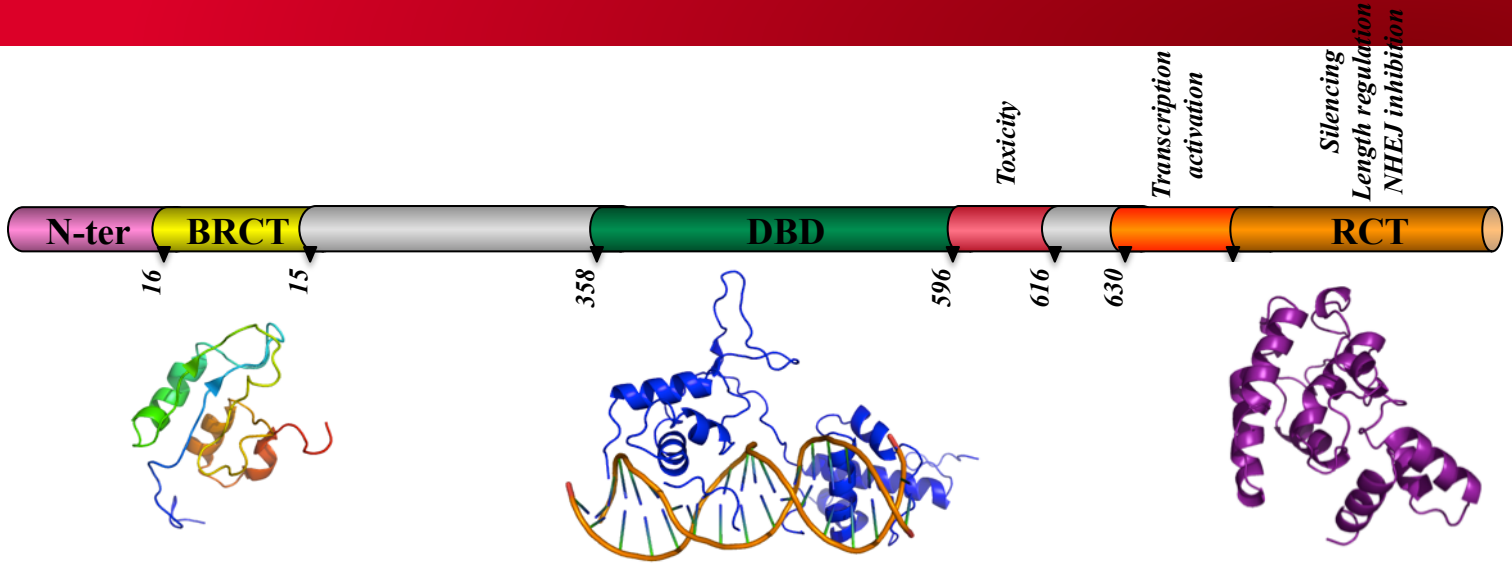
Adapted from Giraud-Panis M-J, Pisano S, Poulet A, Le Du M-H, and Gilson E. FEBS Lett 2010, 584(17):3785-3799.

Telomeres in *Saccharomyces cerevisiae*



Adapted from Giraud-Panis M-J, Pisano S, Poulet A, Le Du M-H, and Gilson E. FEBS Lett 2010, 584(17):3785-3799.

Rap1 (Repressor Activator Protein 1)



NMR structure
(Zhang et al., 2011)

Crystal structure
(König P. et al., Cell, 1996)

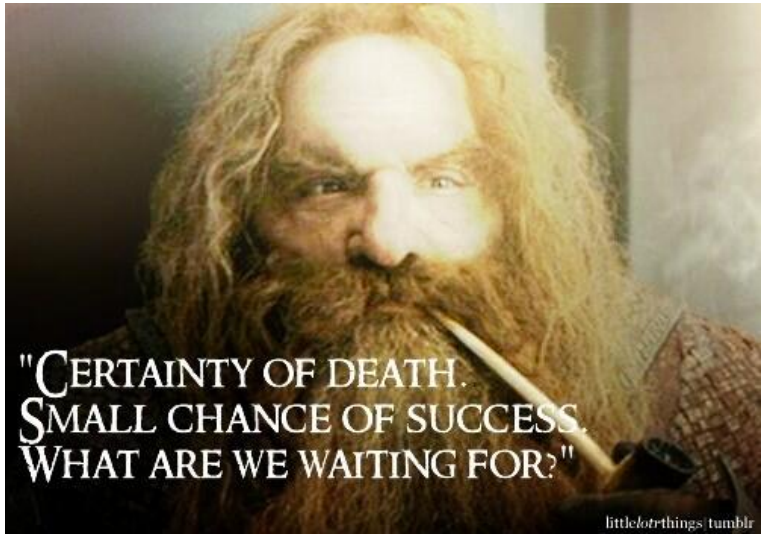
Crystal structure
(Feeser & Wolberger, J Mol Biol, 2008)

- 3 domains (BRCT, DBD, RCT)

- ≈ 40 % of the sequence predicted as unstructured

Highly flexible molecule

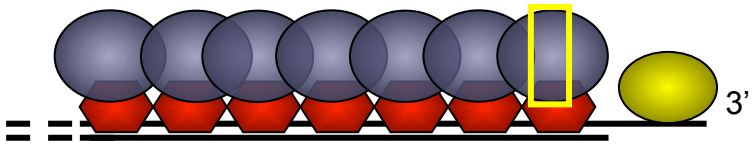
Telomere is a highly dynamic nucleo-protein assembly, centered around a highly flexible protein



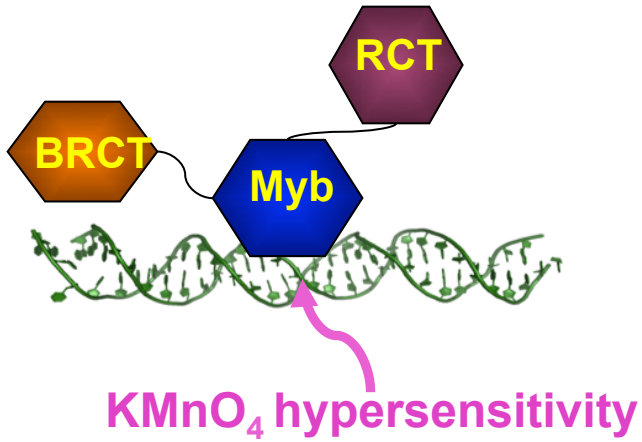
Our hypothesis :

Rap1 flexibility allows conformational adjustments upon DNA binding which optimize its ability to form specific functional quaternary structures.

Rap1/DNA interaction:

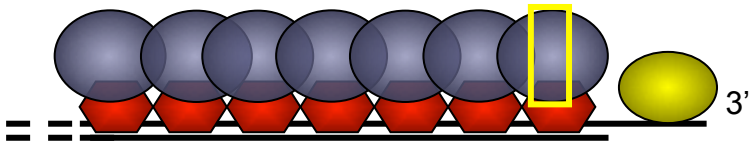


***1 Rap1 every 18 pb** (Gilson et al., 1993)*

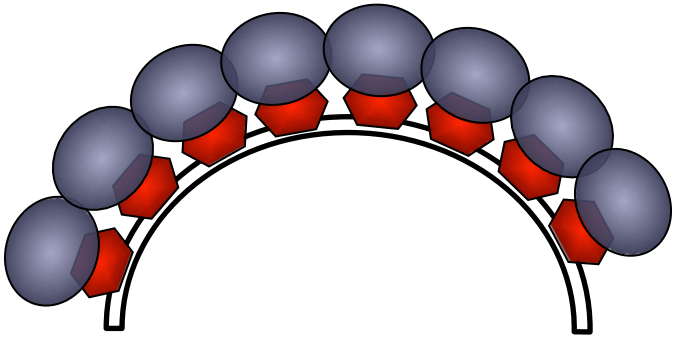


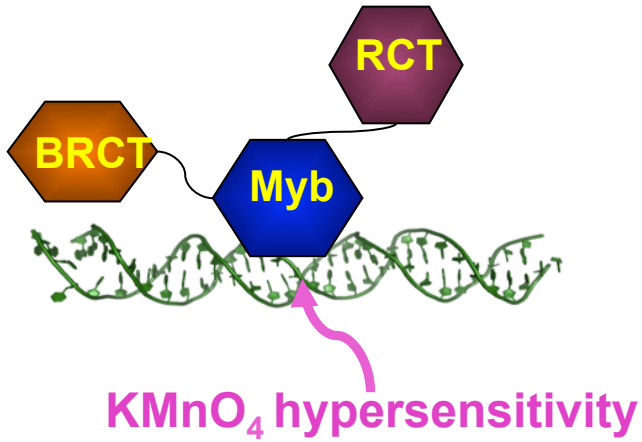
Signature of DNA melting

Rap1/DNA interaction:



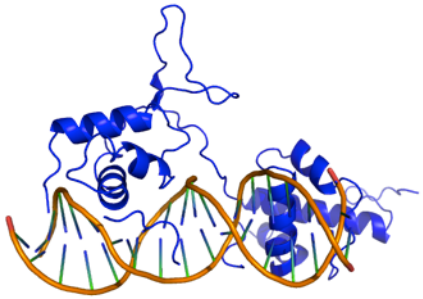
1 Rap1 every 18 pb (Gilson et al., 1993)





Signature of DNA melting

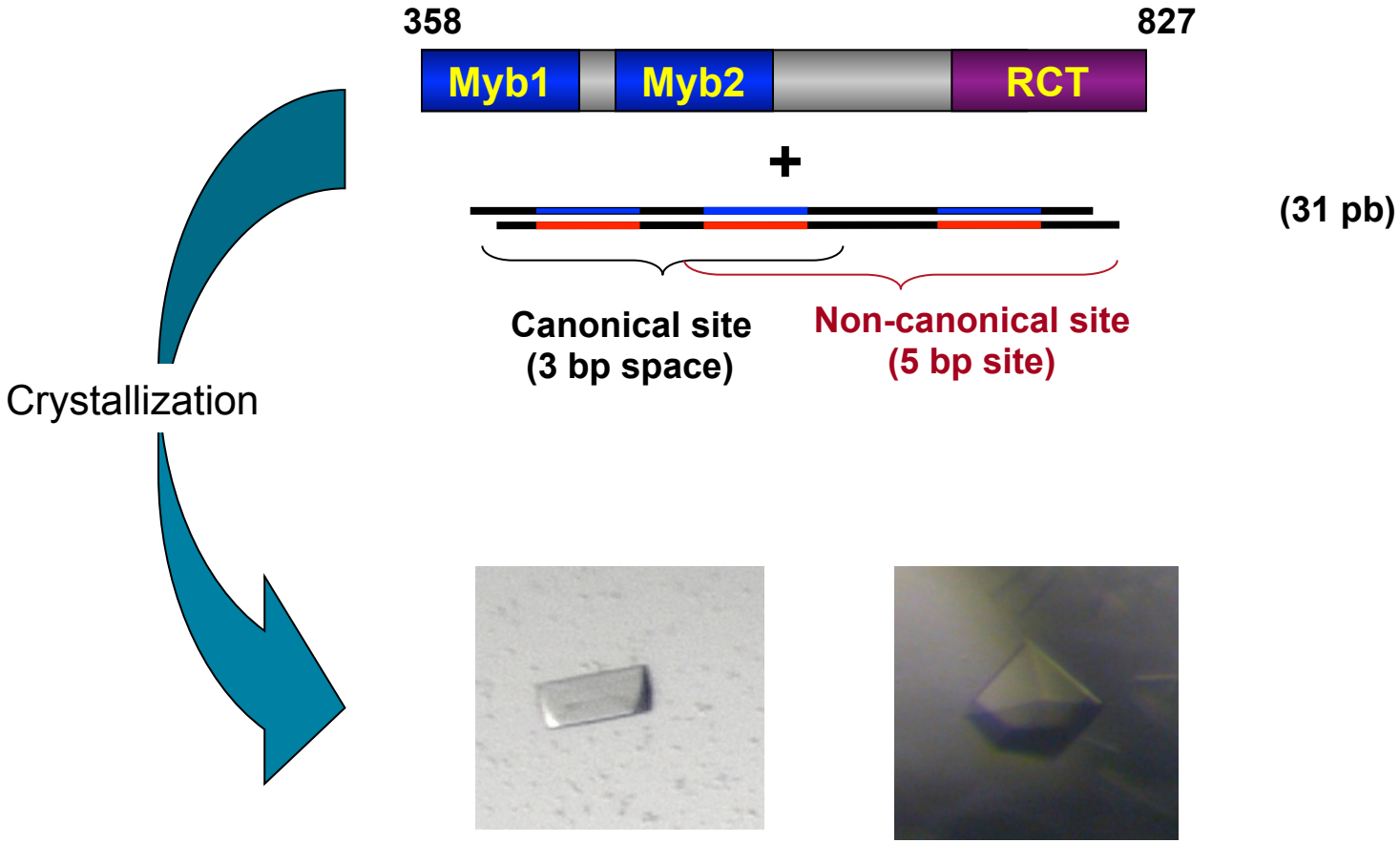
Presence of Rap1 is associated to DNA bending of ≈ 50°
(Gilson et al., 1993)



Do not agree with DBD/DNA crystal structure

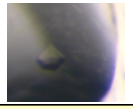
Crystallization

Crystallization trials:



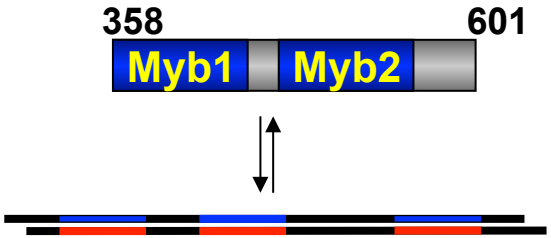
2 crystal forms

Crystal structure analysis

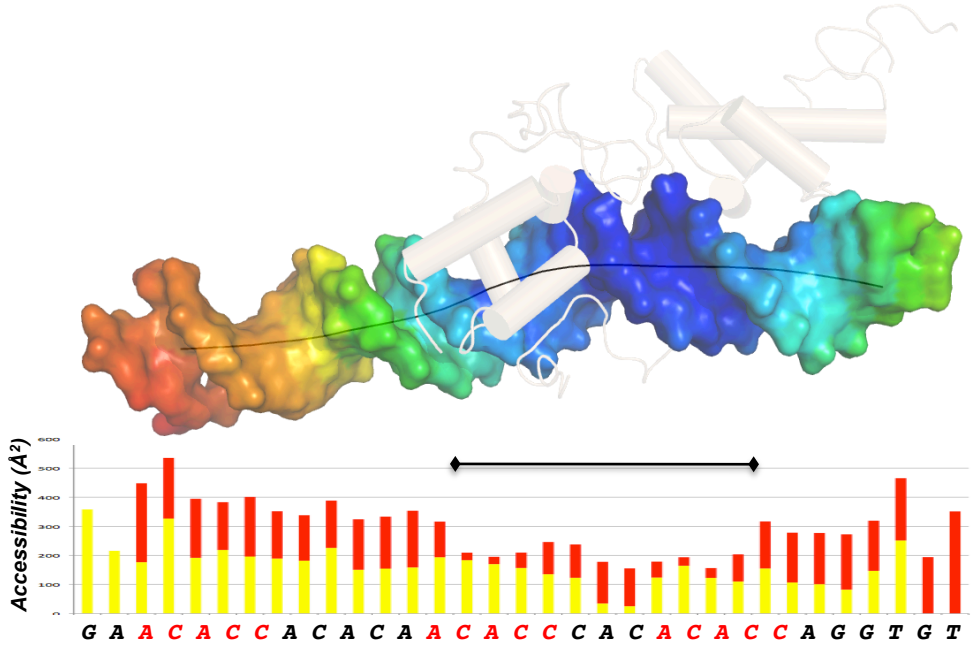
PROXIMA-1	PF6 	PF6 
Collection de données	**$P2_12_12_1$**	**$I2_12_12_1$**
Résolution	**2,95 Å (3,13 Å – 2,95 Å)**	**2,99 Å (3,17 Å – 2,99 Å)**
Paramètres de maille	40,6 x 102,9 x 116,8 90 x 90 x 90	63,8 x 122,6 x 149,4 90 x 90 x 90
R_{merge}	**0,138 (0,688)**	**0,121 (0,701)**
Nombre de réflections uniques	9831	11279
I/σ	14,32 (3,39)	16,61 (3,09)
Complétude	**90,1 (68,8)**	**92,4 (78,7)**
Remplacement moléculaire	**Phaser**	**Phaser**
	1 solution unique (LLG = 1305,76)	1 solution unique (LLG = 905)
Affinement	**Buster5**	**Buster5**
Résolution	**2,95 Å**	**2,99 Å**
R_{factor}	**0,1923**	**0,2044**
R_{free}	**0,2650**	**0,2690**
Figure de mérite	0,869	0,873
Rmsd Bond	0,0010	0,0010
Rmsd angle	1,48	1,40
Nombre de molécules d'eau	**6**	**12**
Nombre de résidus	**223**	**218**
Nombre de bases	**62**	**60**

Crystal structure analysis

Crystals content:



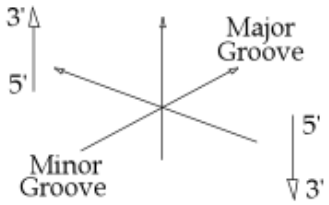
Protein degradation during crystallization process.



$KMnO_4$ hypersensitive cytosine

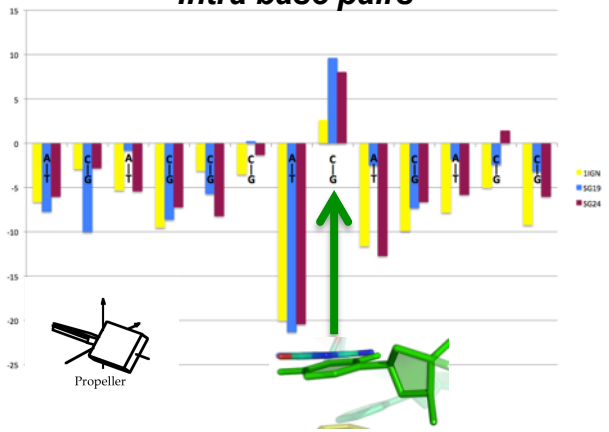
Signature of DNA melting

DNA distortion analysis.

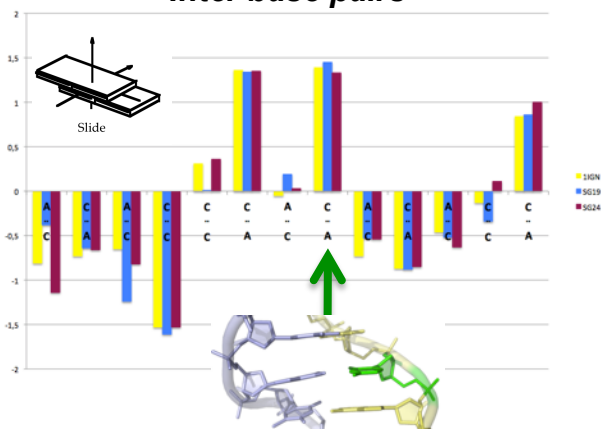


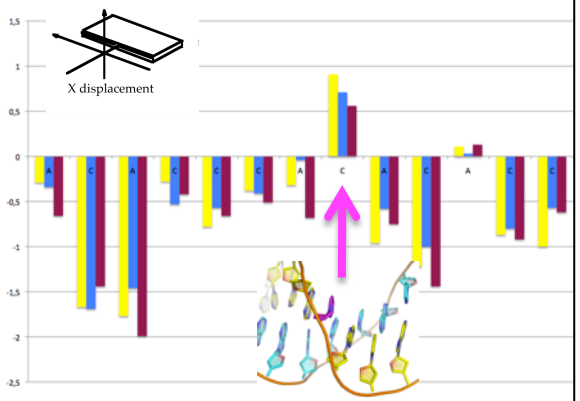
Distortion parameters of DNA from our crystal structure as calculated with Curve+ (R Lavery et al., NAR, 2009)

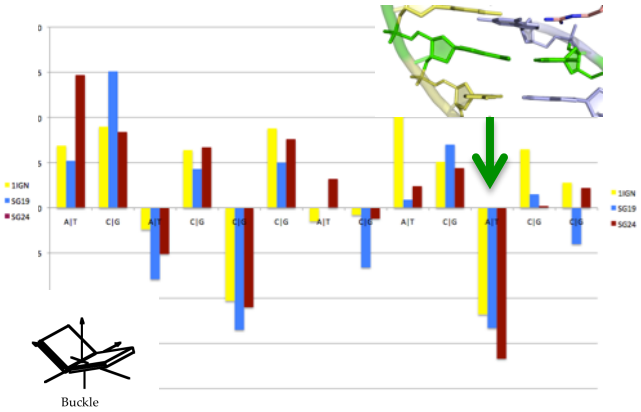
Intra base pairs

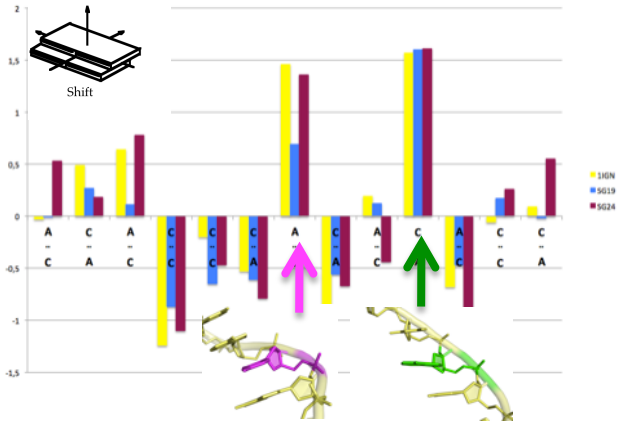


Inter base pairs





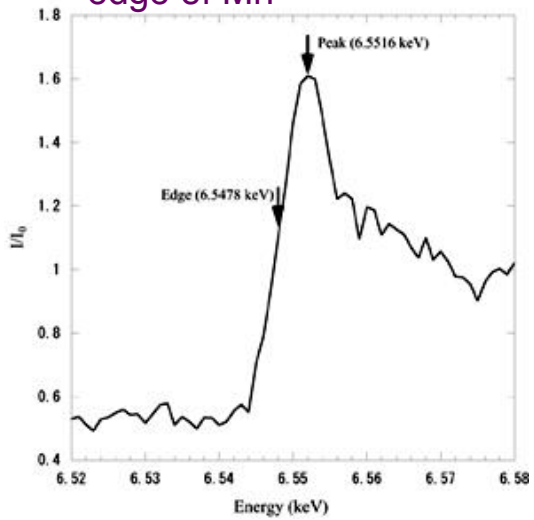


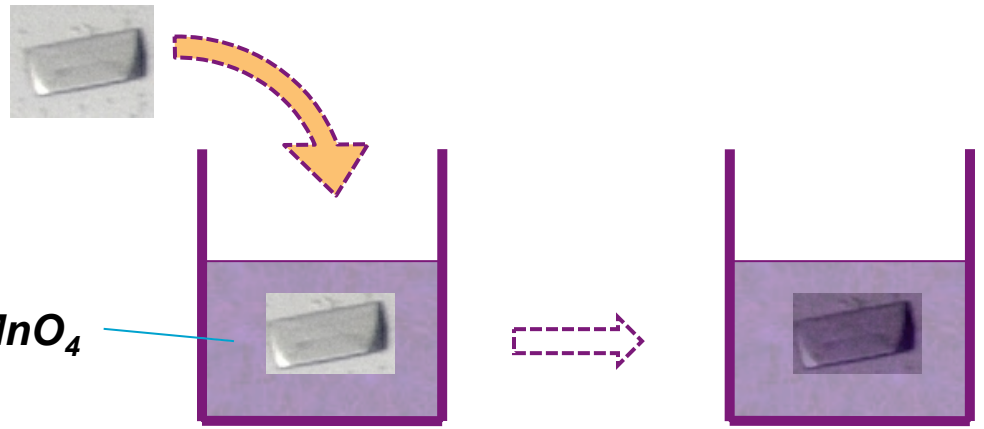


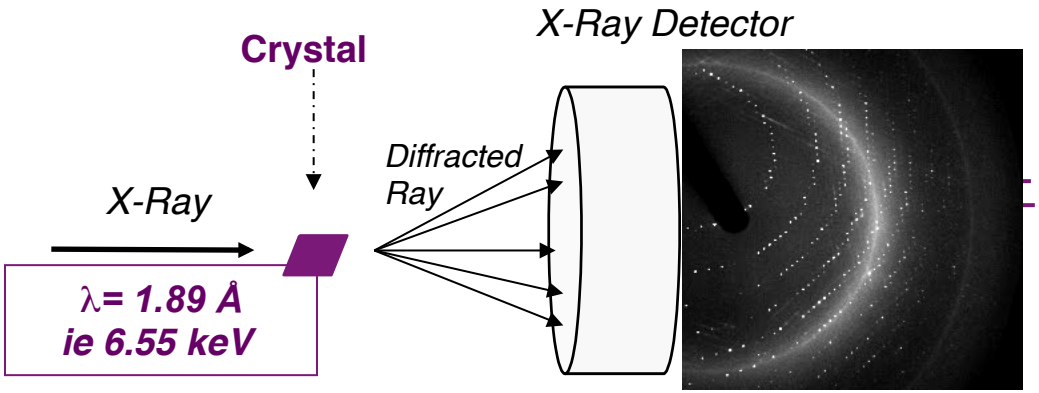
The observed distortion at the hypersensitive Cyt20 is too small to explain the $KMnO_4$ reactivity.

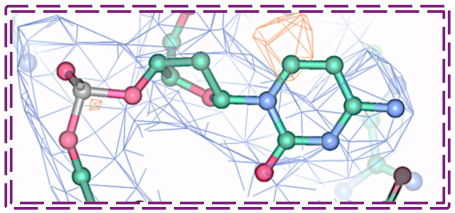
Rap1 induced hypersensitivity to $KMnO_4$

Anomalous absorption edge of Mn







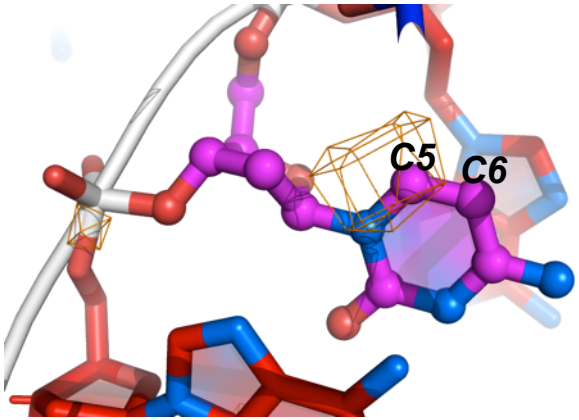


1σ 2Fo-Fc (blue) and 2.8σ anomalous Fourier (orange) at 4.1Å resolution.

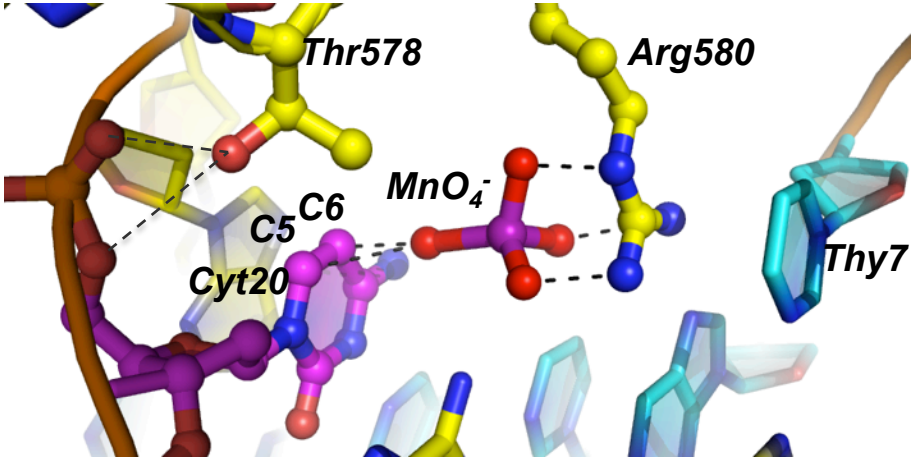
Rap1 induced hypersensitivity to $KMnO_4$

Anomalous signal analysis of RAP1/DNA /$KMnO_4$ crystals

Oligonucleotide	5′-ACCTGGTGTGTGGGTGTTGTGTGGTGTTCAC-3′ 3′-GACCACACACCCACAACACACCACAAGTGTG-5′
Data collection	KMnO4
Wavelength (Å)	1.89
Space-group	$P2_12_12_1$
Diffraction limits (last shell)	4.1 Å (4.32 - 4.10 Å)
Unit cells (axbxcxαxβxγ)	40.6x103.2x115.7 90x90x90
R_{merge}	0.149 (0.376)
Number of unique reflections	4148
I/σ	7.0 (2.8)
Completeness	99.8 (100.0)
R_{work}	0.1973
R_{free}	0.3265
Figure of merit	0.928
RMSD bond	0.011
RMSD angles	1.55

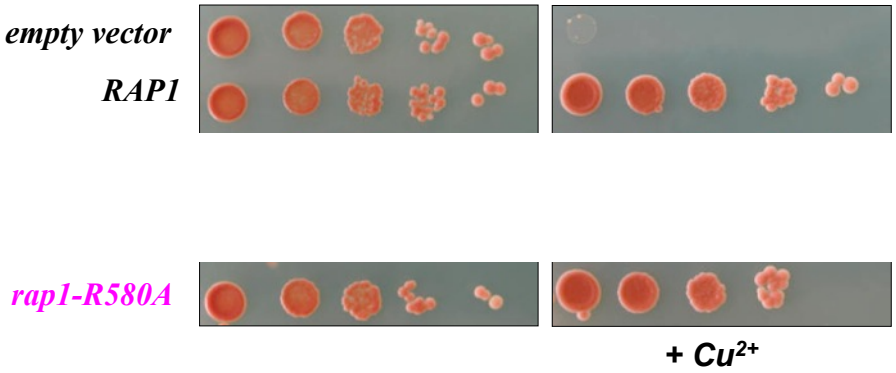


Possible model of interaction

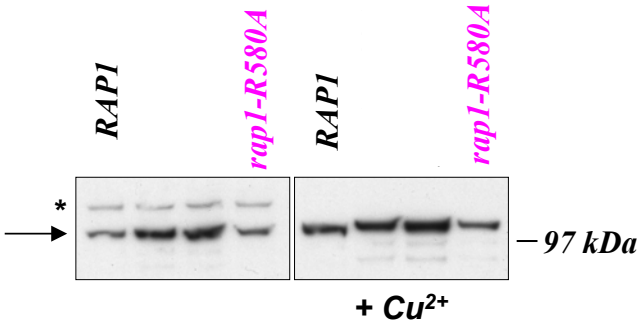


Biological analysis

Ability of the rap1 alleles to complement Rap1 loss in yeast cultures.



Western blot comparing Rap1 amount in the different strains

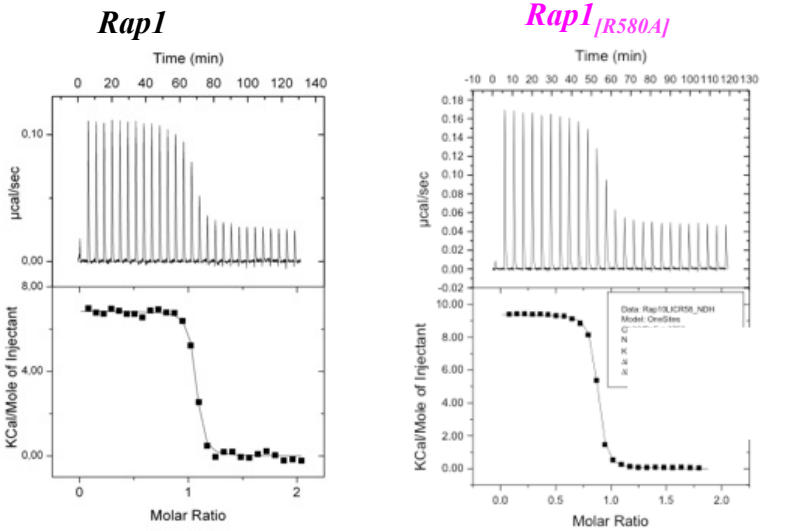


Mutation R580A does not affect cells viability.

Collaboration Rachel Lescasse, Stéphane Marcand, CEA Fontenay

Rap1 induced hypersensitivity to KMnO4

Isothermal calorimetry (ITC) titration of Rap1 or Rap1 [R580A] by DNA.

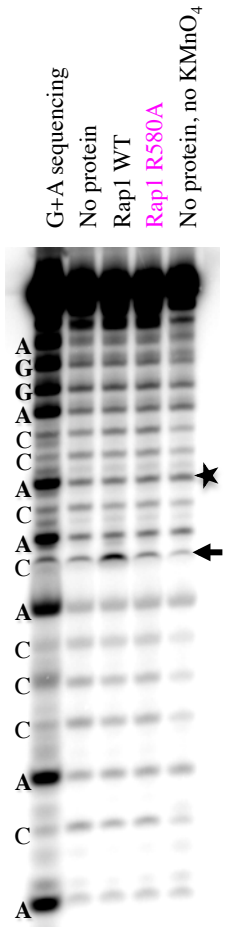


$Ka = 2.5\ 10^8 \pm 4.4\ 10^7\ M^{-1}$
$\Delta H = 6.8 \pm 0.04$ kcal/mol

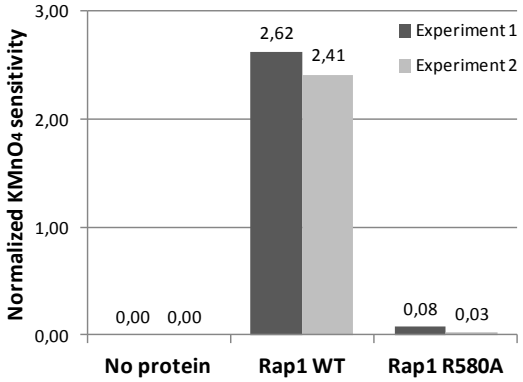
$Ka = 2.02\ 10^8 \pm 1.06\ 10^7\ M^{-1}$
$\Delta H = 9.36 \pm 0.021$ kcal/mol

Mutation R580A does not affects DNA binding.

$KMnO_4$ footprinting showing the role of Arg580 in Rap1 hypersensitivity



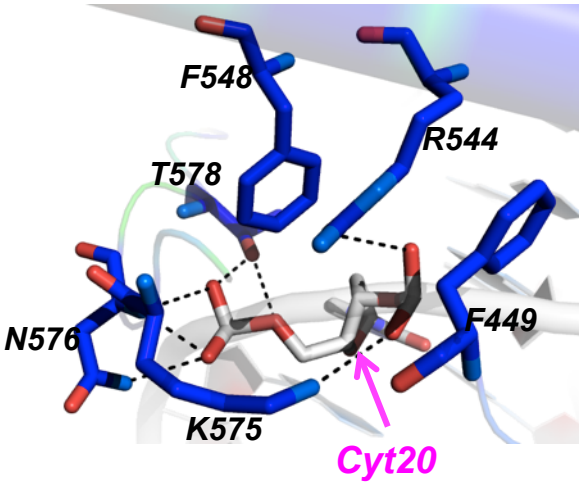
Mutation R580A affect Rap1 induced permanganate potassium hypersensitivity.



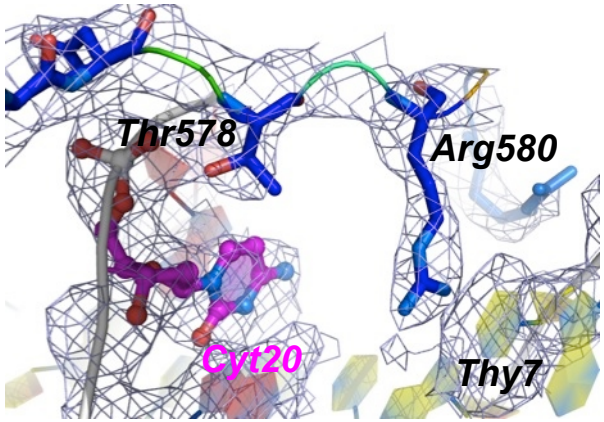
Collaboration Bianca Sclavi, ENS Cachan

Le Bihan Y-V et al., Acta Crystallographica-D, 2013.

Rap1 induced hypersensitivity to KMnO4



Residues involved in Cyt20 distortion are 100% conserved among double-myb containing Rap1 proteins.



Le Bihan Y-V et al., Acta Crystallographica-D, 2013.

Permanganate potassium hypersensitivity induced upon Rap1 DNA binding is driven by **Arg580** that plugs DNA major groove.

Residues involved in Cyt20 distortion as well as Arg580 are **fully conserved** among double-myb containing Rap1.

➔ New questions:

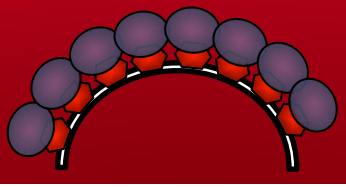
The $KMnO_4$ hypersensitivity is a useful **biochemical artefact.**

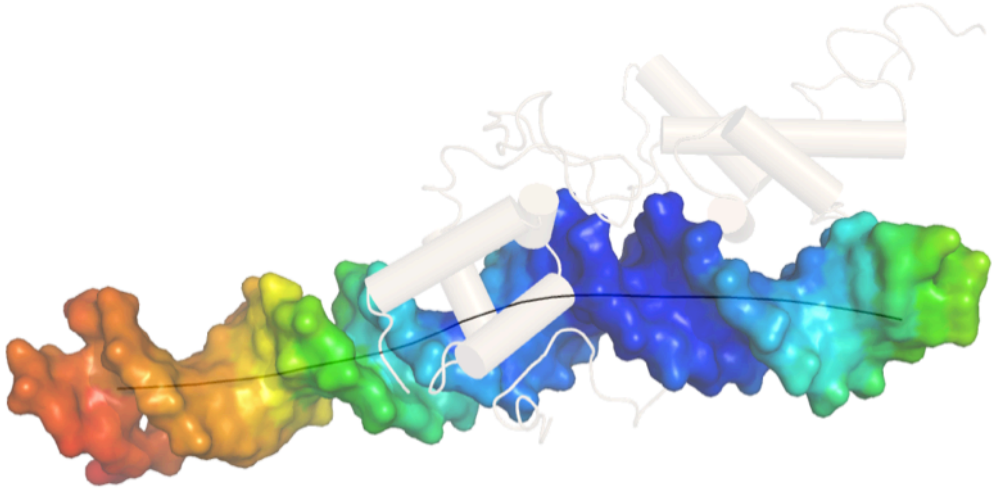
Why the residues involved in the distortion of hypersensitive nucleic acid are they so conserved?

Which function could sign this distortion ?

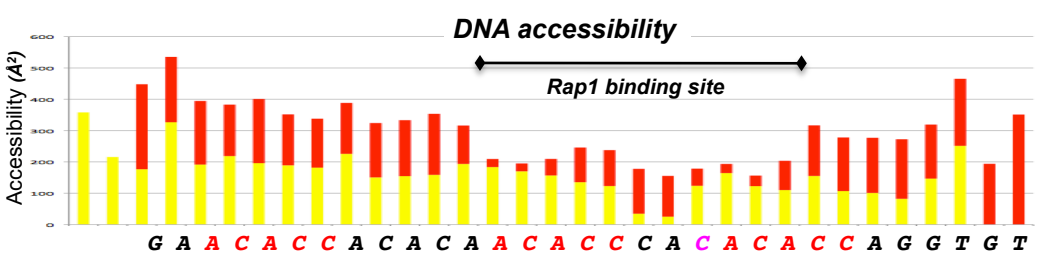
(No methylation of telomere DNA)

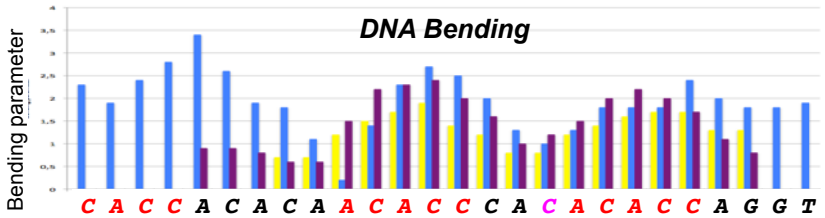
DNA bending analysis.

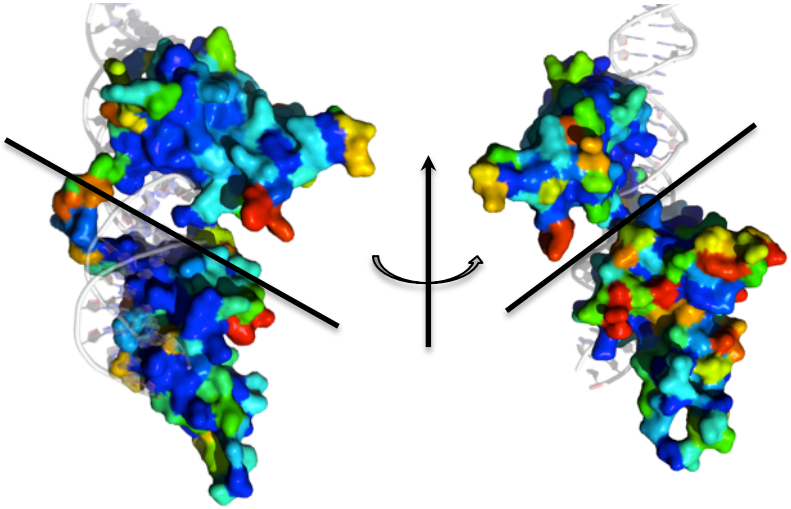




Overall bending of ≈ 20°

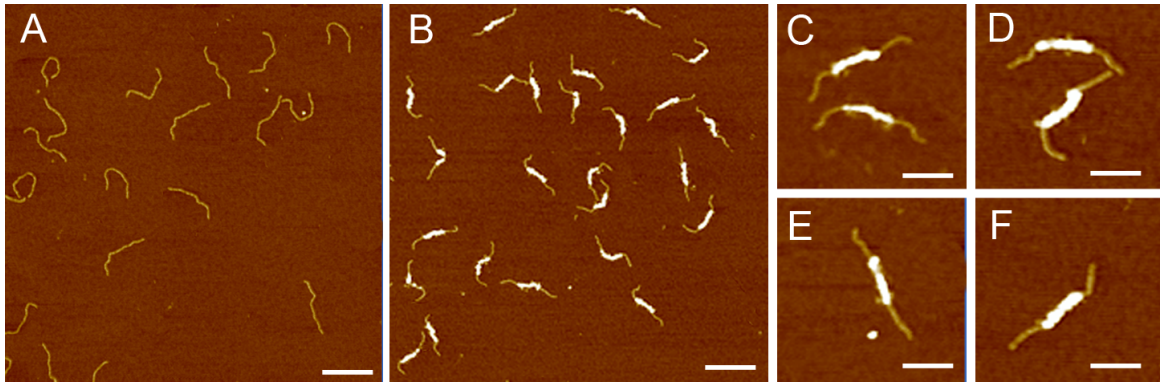




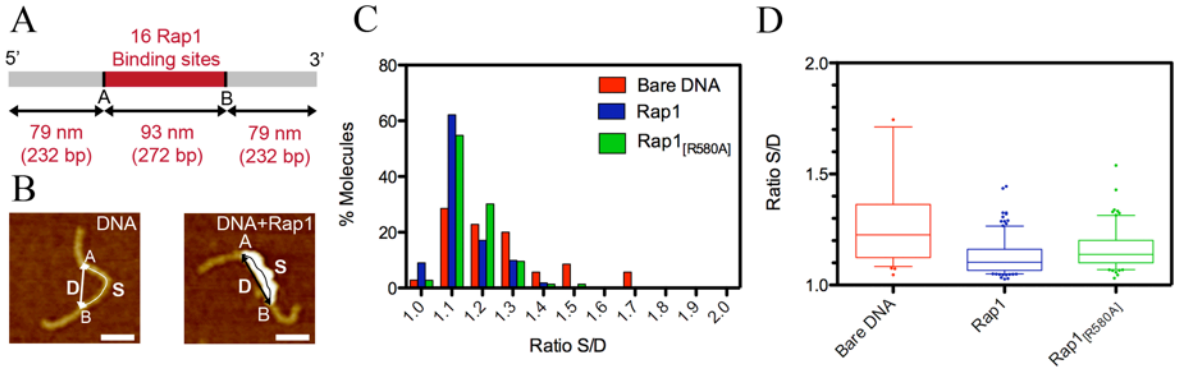


Rap1 binding at telomere fiber.

Atomic Force Microscopy (AFM) experiments. Scale bars corresponds to 200 nm (A, B) and 100 nm (C-F)



Analysis of DNA curvature using the ratio between curvilinear (S) and direct (D) distances.



Binding of Rap1 is associated to local DNA stiffening.

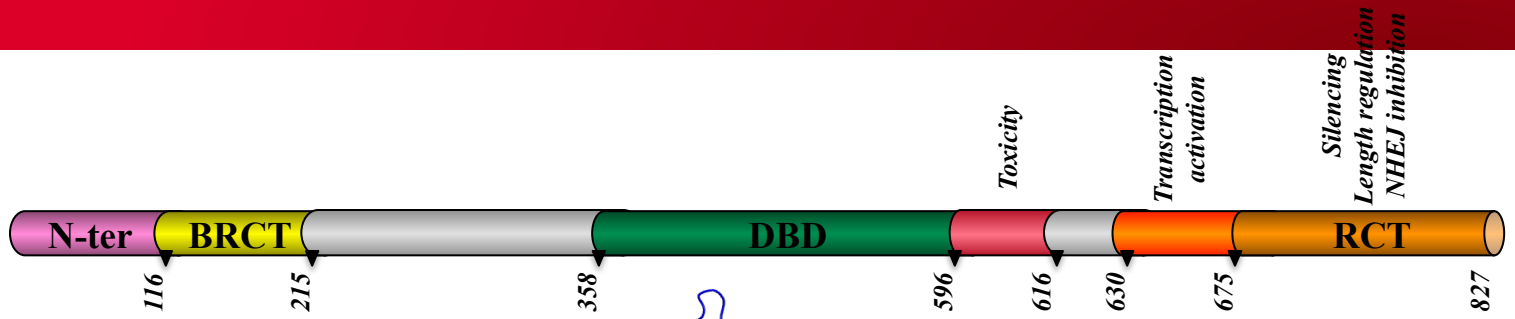
The local bending observed in the crystal structure does not propagate along DNA fiber

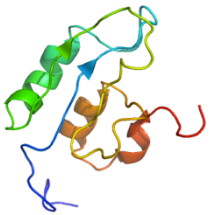
What is the architecture associated to this binding ?

Le Bihan Y-V, et al.,. Acta Crystallographica-D, 2013.

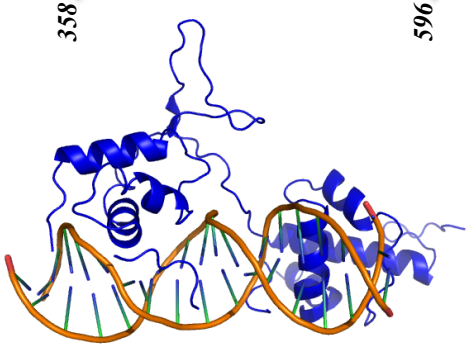
Collaboration Olivier Pietrement, Eric Le Cam, IGR Villejuif

RAP1: available structural data



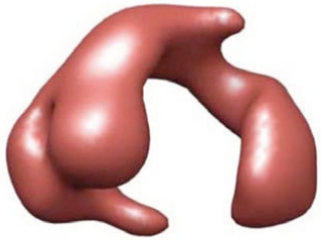


BRCT domain
NMR structure
(Zhang et al., BBRC, 2011)

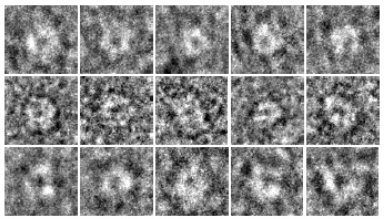


DBD/ DNA
Crystal structure
(König P. et al., Cell, 1996)

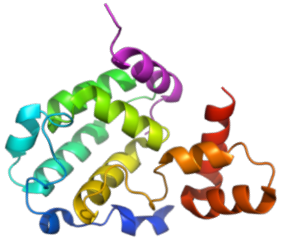
RCT domain
Crystal structure
(Feeser & Wolberger, J Mol Biol, 2008)



Rap1 full length
Electron microscpy
Papai et al., Nature, 2010

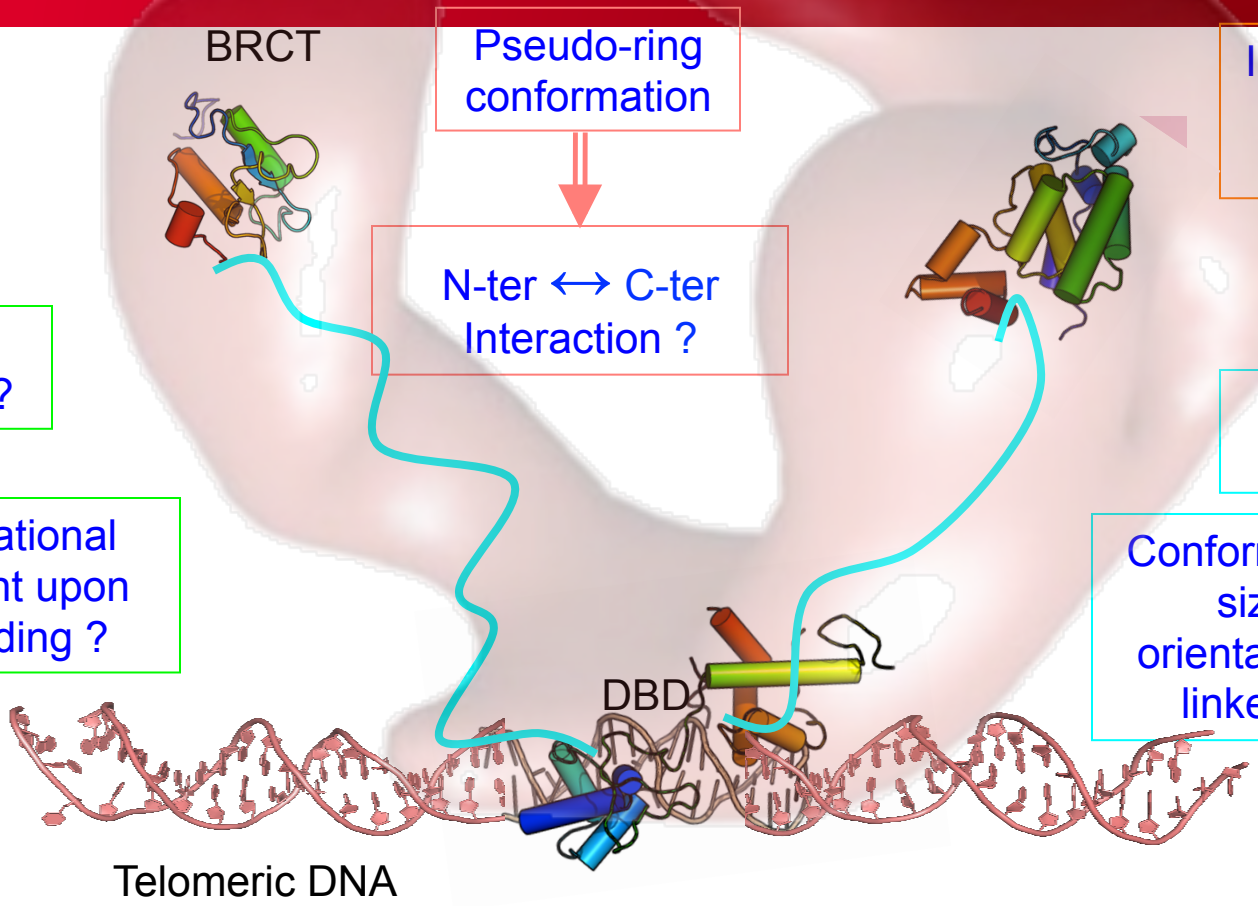


Diameter = 120±13 Å (avg 255 images)
Rap1 full length
Electron microscopy
Williams et al., J Biol Chem, 2010



RCT/Sir3-peptide
Crystal structure
Chen et al., NSMB, 2011

Remaining questions:

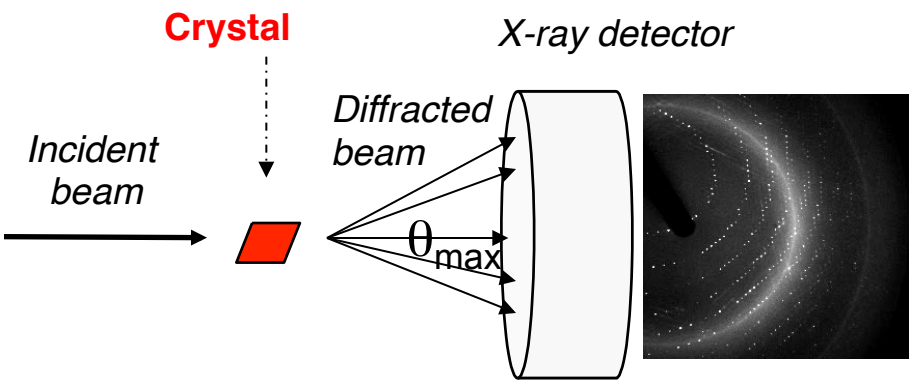


No unique method allows to resolve architecture of a flexible molecule involved in dynamics and transients interaction ⟹ **Integrative structural biology approach**

Structural approaches :

X-ray crystallography

High resolution structure



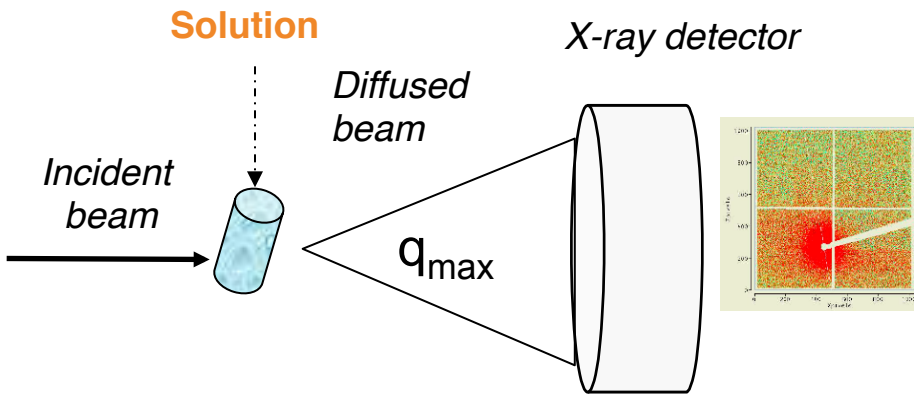
Data analysis

Structure Resolution
Molecular Replacement

3D Structure

Small Angle X-ray Diffusion (SAXS)

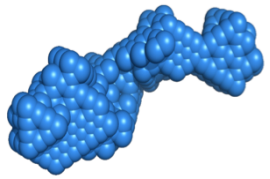
Averaged conformation in solution



Data analysis

Ab initio
Molecular modelisation

3D Model or envelope



Limiting step : Obtaining diffracting crystals

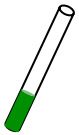
Limiting Factors : aggregation and heterogeneity

Structural approaches :

Nuclear Magnetic Resonance (NMR)

Interaction surface mapping (low affinities)

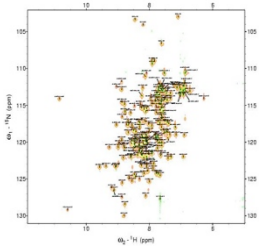
Labelled protein
^{15}N-, ^{15}N,^{13}C-



+/- partners

3D experiments on backbone + side-chains

Fingerprint
^{1}H, ^{15}N- HSQC



Interaction titration
(+/- partner)

Chemical shifts variations

Interaction surface

Limiting factor: solubility, size

Contribution of each method

Crystallography

High resolution structure

SAXS

Averaged overal shape in solution

NMR

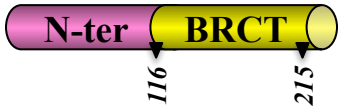
Interaction surface mapping

Our approach:

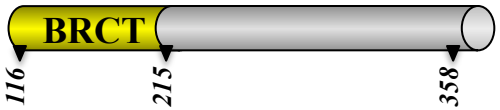
Question	Tool
N-ter ⟷ C-ter interaction ?	NMR ITC
Conformational adjustment upon DNA binding ?	SAXS Analytical ultracentrifugation
Overall architecture ?	
Molecule flexibility ?	
Conformation, size, orientation of linkers ?	
Structural keystones of conformational adjustments ?	Crystal structure of a DBD/DNA complex
Interaction with functional partners ?	NMR mapping

First step: inter-domains interaction analysis

2- NMR titration:

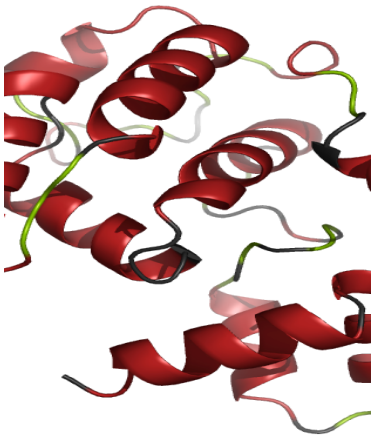


No variation of RCT chemical shifts signals



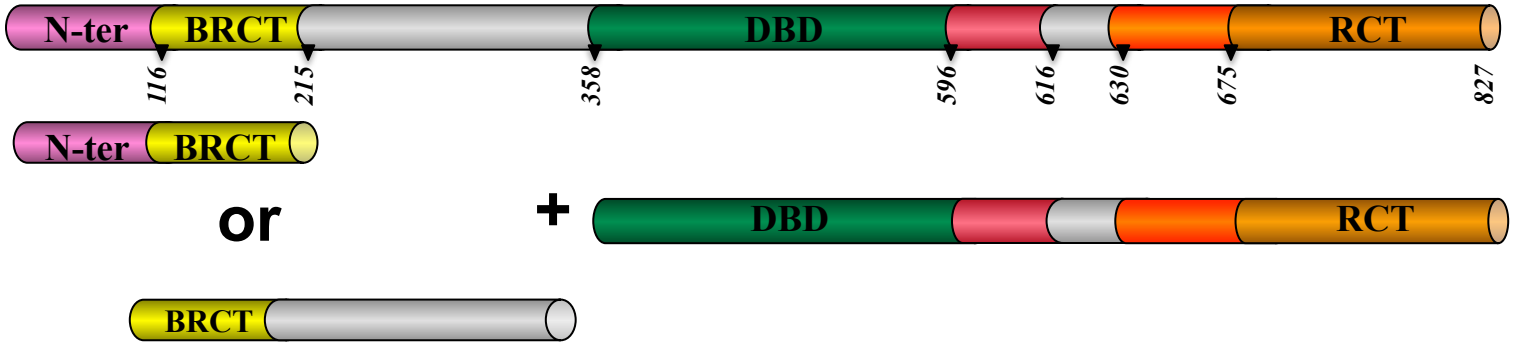
No NMR detectable interaction

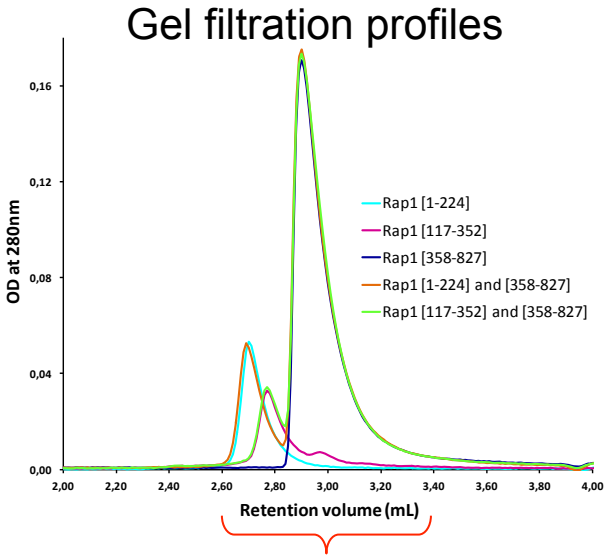
1- Tool for NMR analysis of interaction

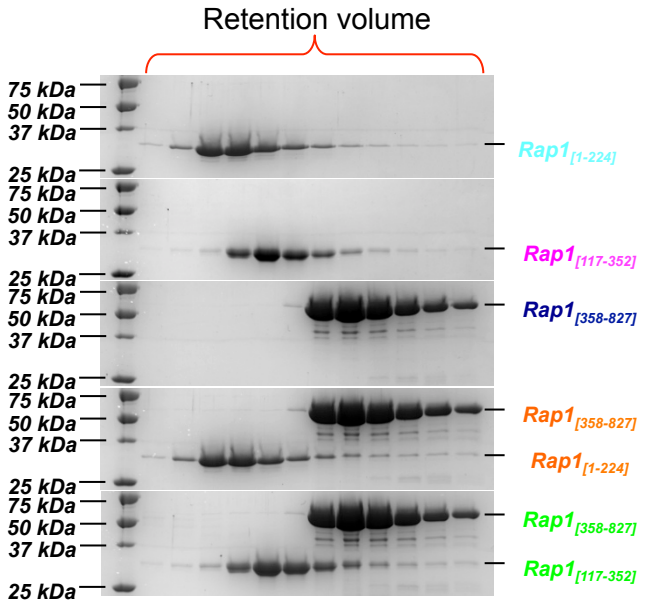


α helices
β sheets
undetermined

First step: inter-domains interaction analysis

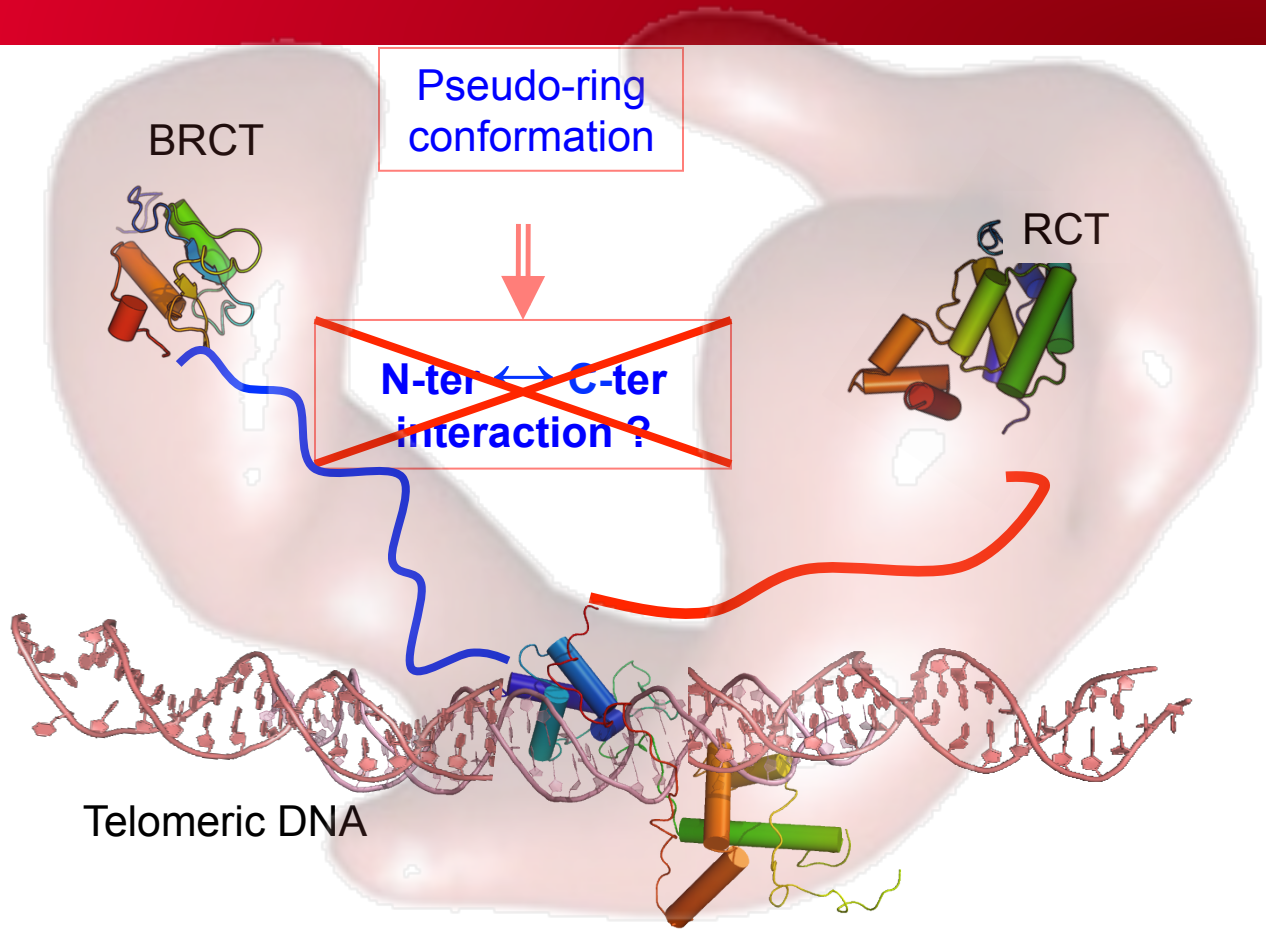






No detectable interaction between N-ter and DBD-Cter moieties

Intermediate information:

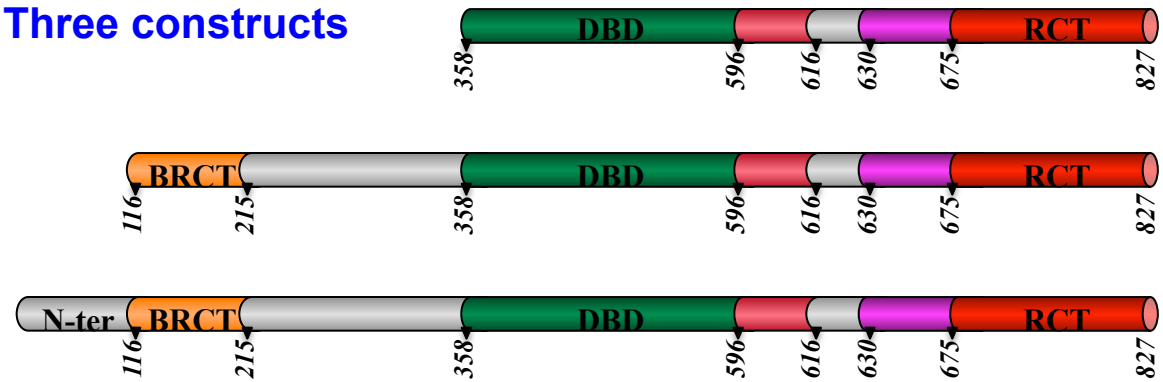


No detectable interaction between RCT and N-ter region
No detectable interaction between N-ter and DBD-Cter moieties

Structural analysis of Rap1 full-length:

Combination of SAXS and AUC

Three constructs



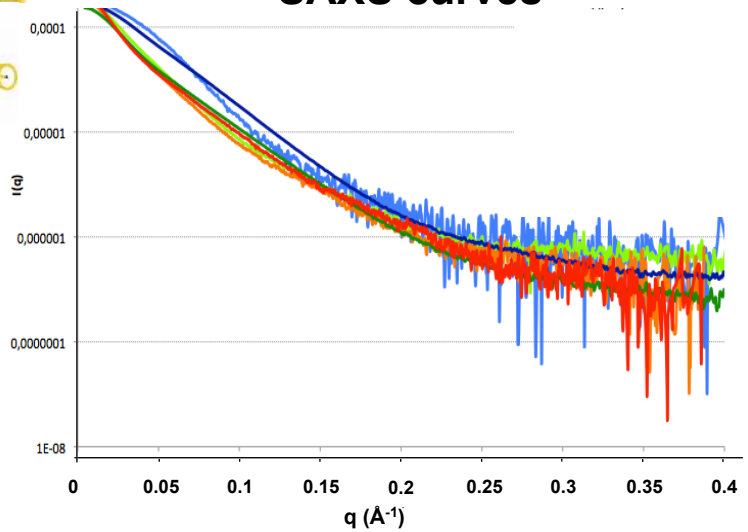
Sufficient for telomeric functions

One telomeric oligonucleotide

5′-TCCTGGTGTGTGGGTGTGCG – 3′
3′- GACCACACACCCACACGCAG – 5′

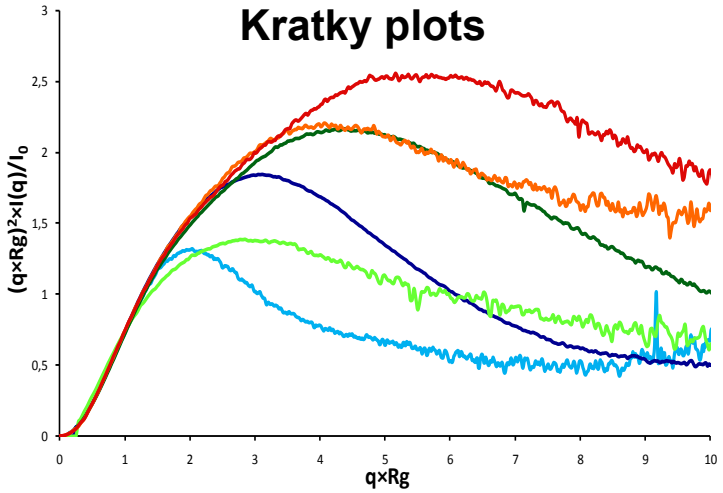
Structural analysis of Rap1 full-length:

SAXS curves

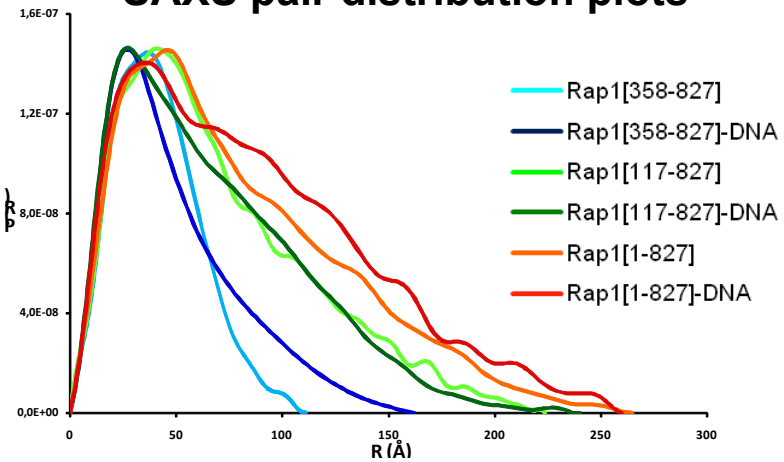


- ✔ Monomers only
- ✔ No complex dissociation
- ✔ Progressive introduction of N-ter region associated to more flexible and elongated molecules
- ✔ DNA/Protein complexes associated to more compact objects

Kratky plots

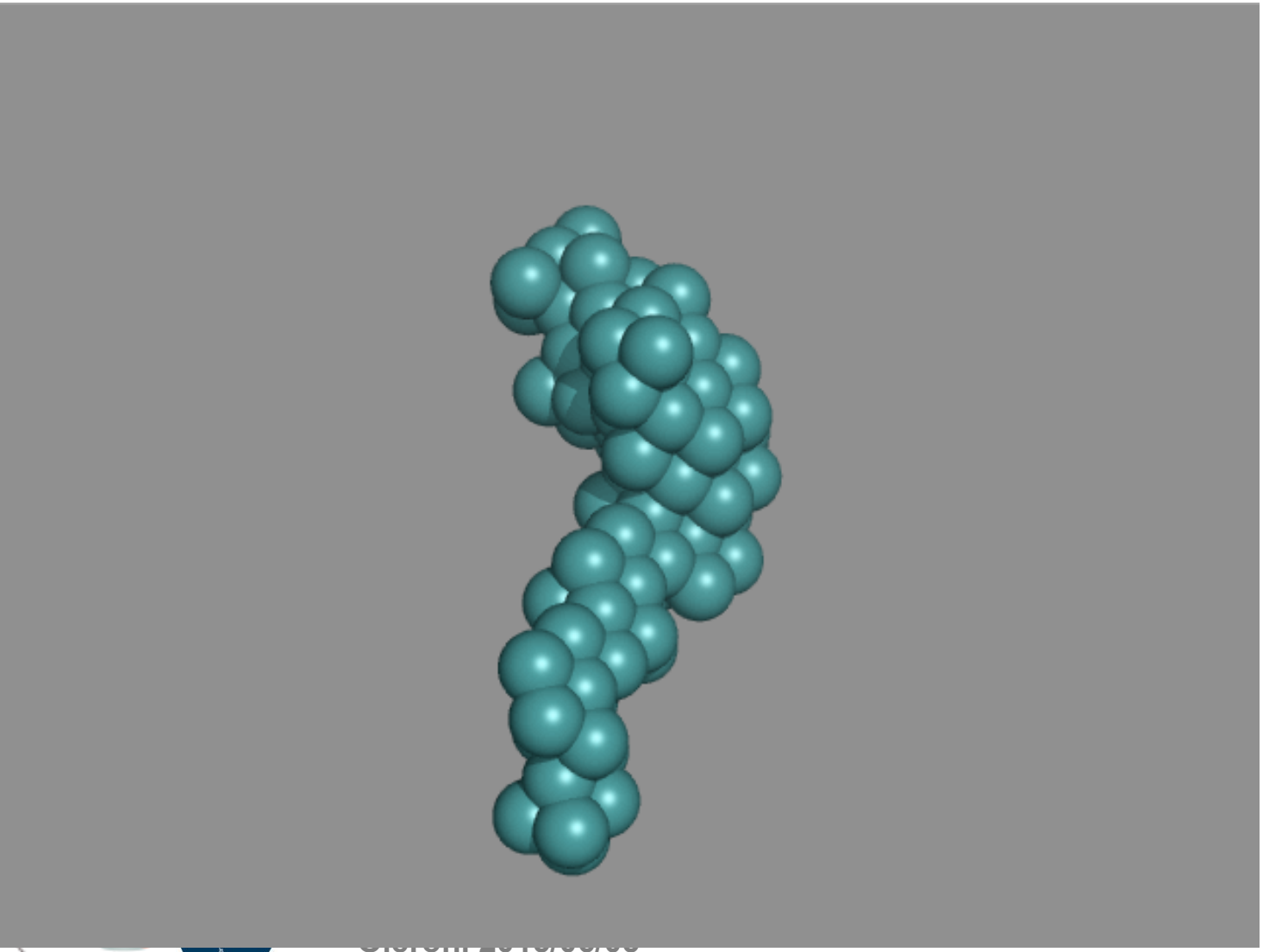


SAXS pair distribution plots



Diffusion aux petits angles : approche *ab initio*

Détermination d'enveloppes ab initio : DAMMIN



Cycle 1 : Rf = 0.8857

Cycle 5 : Rf = 0.8819

Cycle 10 : Rf = 0.8587

Cycle 15 : Rf = 0.0436

Cycle 20 : Rf = 0.0276

Cycle 25 : Rf = 0.0258

Cycle 30 : Rf = 0.0240

Cycle 35 : Rf = 0.0137

Cycle 40 : Rf = 0.0104

Cycle 45 : Rf = 0.0107

Cycle 50 : Rf = 0.0089

Cycle 51 : Rf = 0.0090

Cycle 52 : Rf = 0.0095

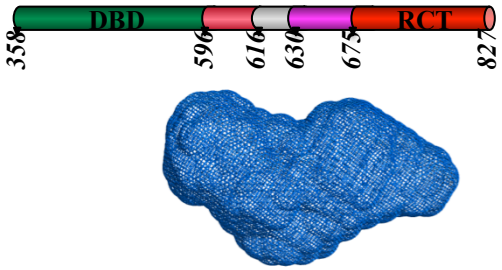
Cycle 53 : Rf = 0.0095

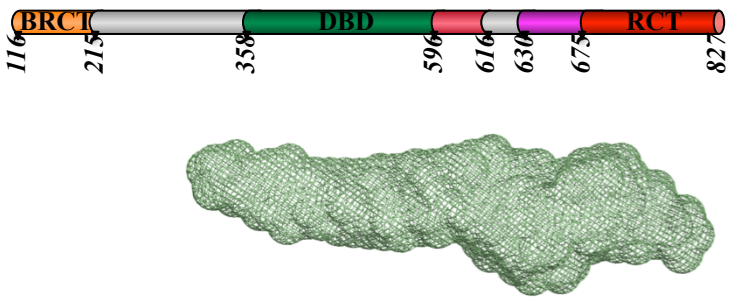
Cycle 54 : Rf = 0.0095

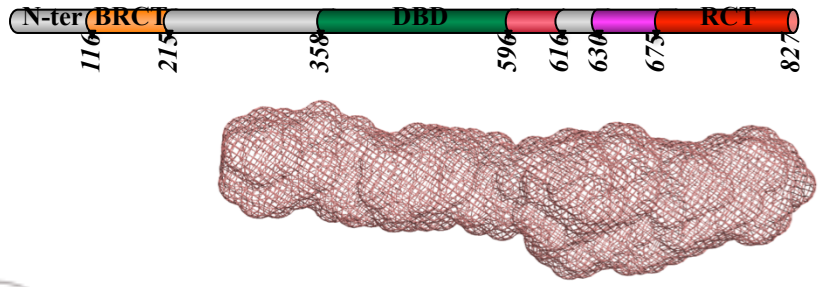
χ2 = 2.06

Progressive construction of Rap1 and Rap1/DNA

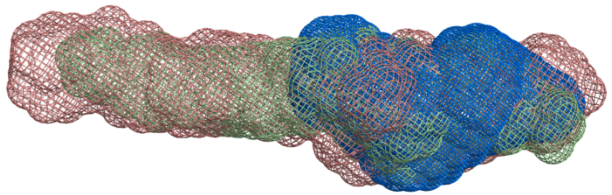
SAXS: *ab initio* approach







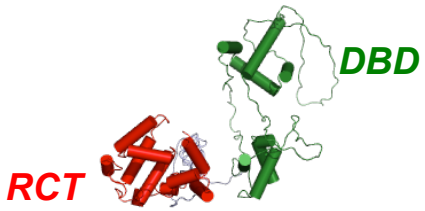
Conserved DBD-RCT region



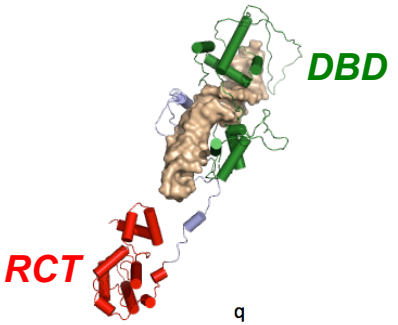
Progressive introduction of N-ter region associated to envelop elongation

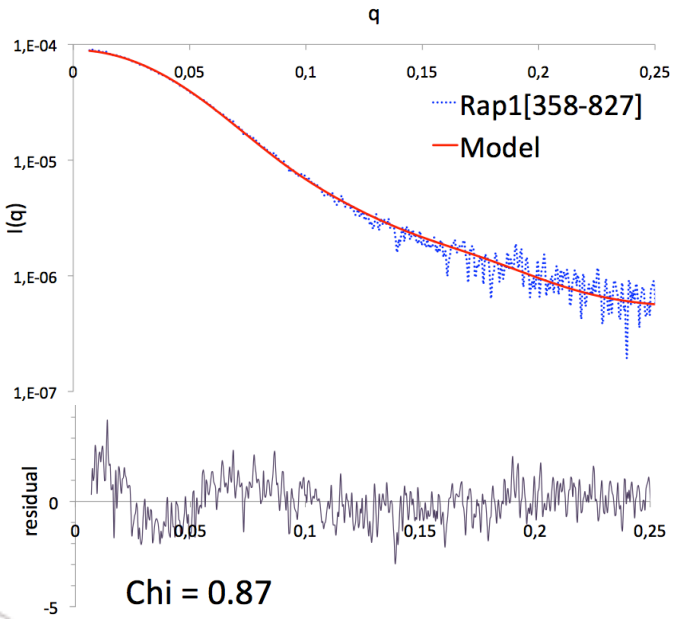
Progressive construction of Rap1 and Rap1/DNA

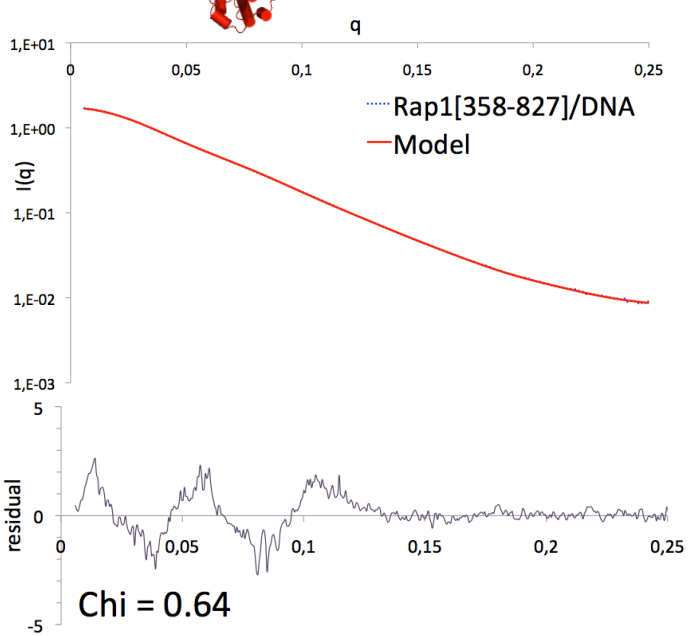
SAXS: *3D models*



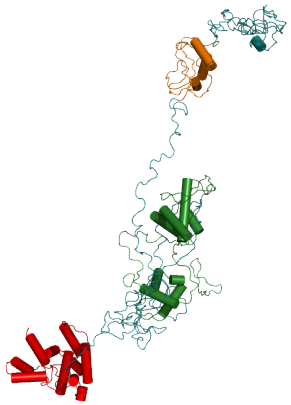
DNA interaction associated with different orientation of RCT





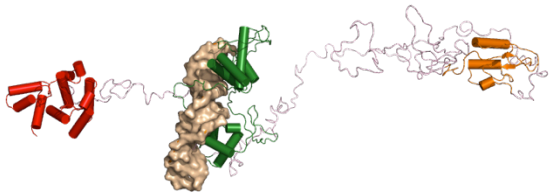


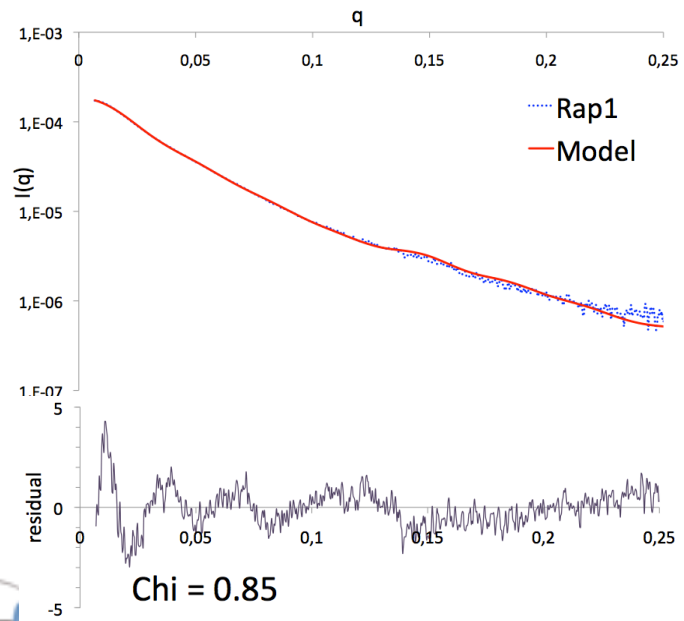
Progressive construction of Rap1 and Rap1/DNA

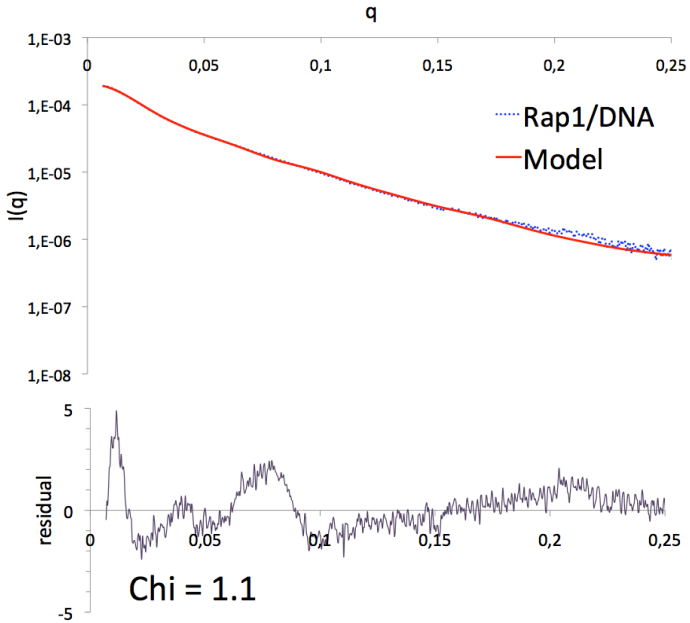


DNA interaction associated with :

- Conformational adjustment
- Increased stability



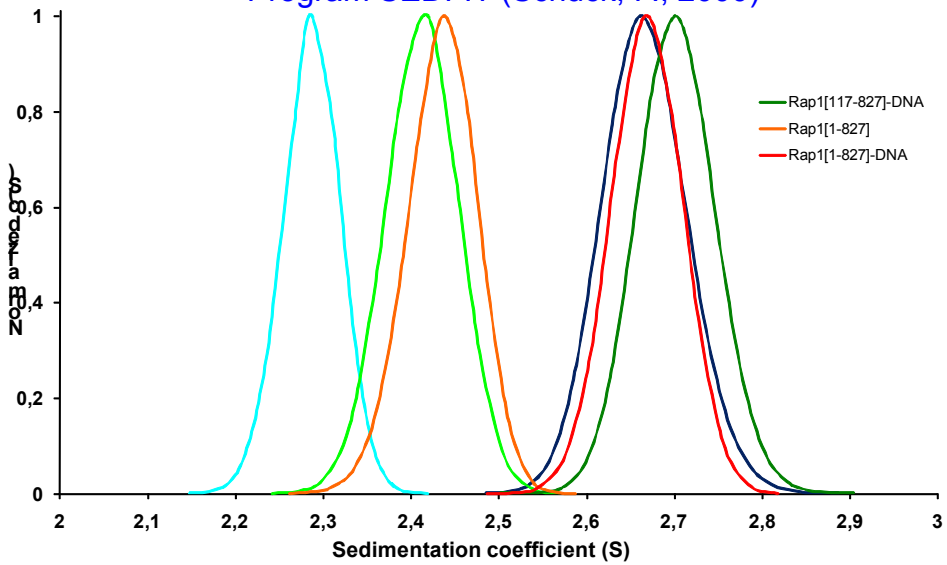




Structural analysis of Rap1 full-length:

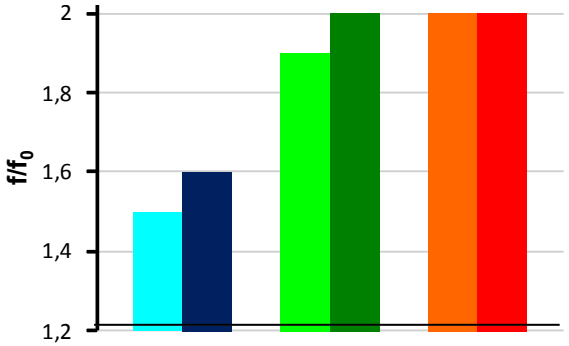
AUC sedimentation profiles

Program SEDFIT (Schuck, P., 2000)



AUC friction coefficients

Program SEDFIT (Schuck, P., 2000)

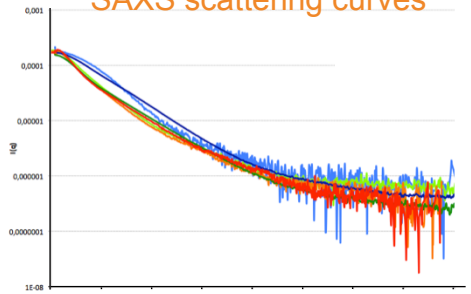


Rap1[358-827]
Rap1[358-827]-DNA
Rap1[117-827]
Rap1[117-827]-DNA
Rap1[1-827]
Rap1[1-827]-DNA

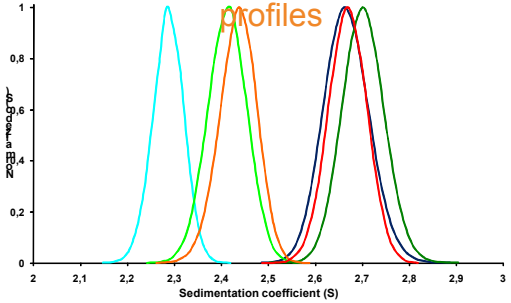
- ✔ **Monomers only**
- ✔ **No complex dissociation**
- ✔ **Progressive introduction of N-ter region associated to higher friction coefficient**

Second step: progressive construction of Rap1 and Rap1/DNA

SAXS scattering curves



AUC sedimentation profiles

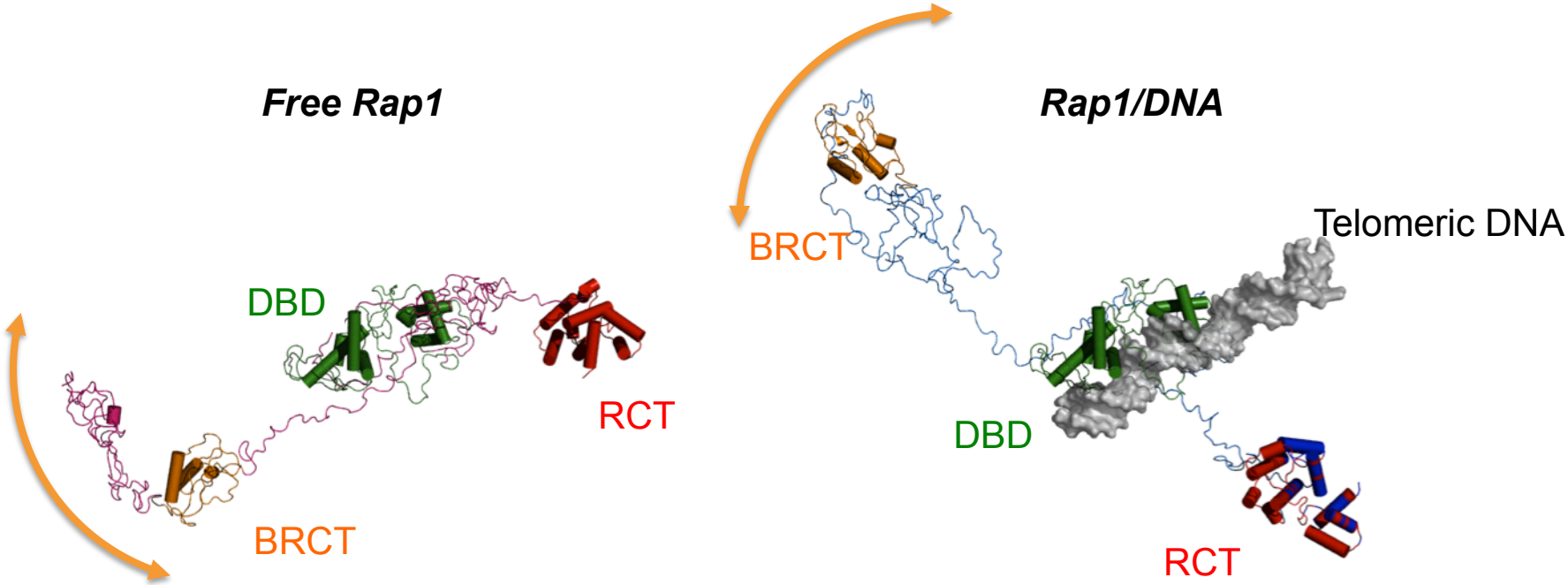


	construct	$Rap1_{[358-827]}$	$Rap1_{[117-827]}$	Rap1	$Rap1_{[358-827]}$/DNA	$Rap1_{[117-827]}$/DNA	Rap1/DNA
	Th. MW (kDa)	55	82	92	67	94	105
	Partial specific volume (ml/g)	0.717	0.712	0.710	0.689	0.693	0.693
AUS	[Sample] (mg/ml)	0.55, 1.1, 2.2	0.82, 1.64, 3.11	0.56, 0.94, 1.4, 1.88, 2.81, 3.74	0.67, 1.35, 2.02	0.56, 0.94, 1.88, 2.81	0.63, 1.06, 2.12, 3.17, 4.23
	MW (kDa)	55	82	94	67	94	106
	Sed coef	3.67	3.76	3.88	4.23	4.29	4.24
SAX	[Sample] (mg/ml)	1, 3.2	1.8, 2.5	1.1[a]	9	0.7, 1.4, 2.8, 4.1	1.2[a]
	Rg (Å)	32.7	58.1	67.4	40.6	71.6	72.4
	Dmax (Å)	120	224	260	162	275	260
	Calc sed coef	3.55	3.75	3.97	4.21	4.31	4.23

Validation of SAXS models by comparison of calculated and experimental sedimentation coefficients.
Convergence between SAXS and AUC analysis increases the reliability of the result.

Structural analysis of Rap1 full-length:

Median of the statistical distribution of the conformations of the molecule in solution, including flexible regions

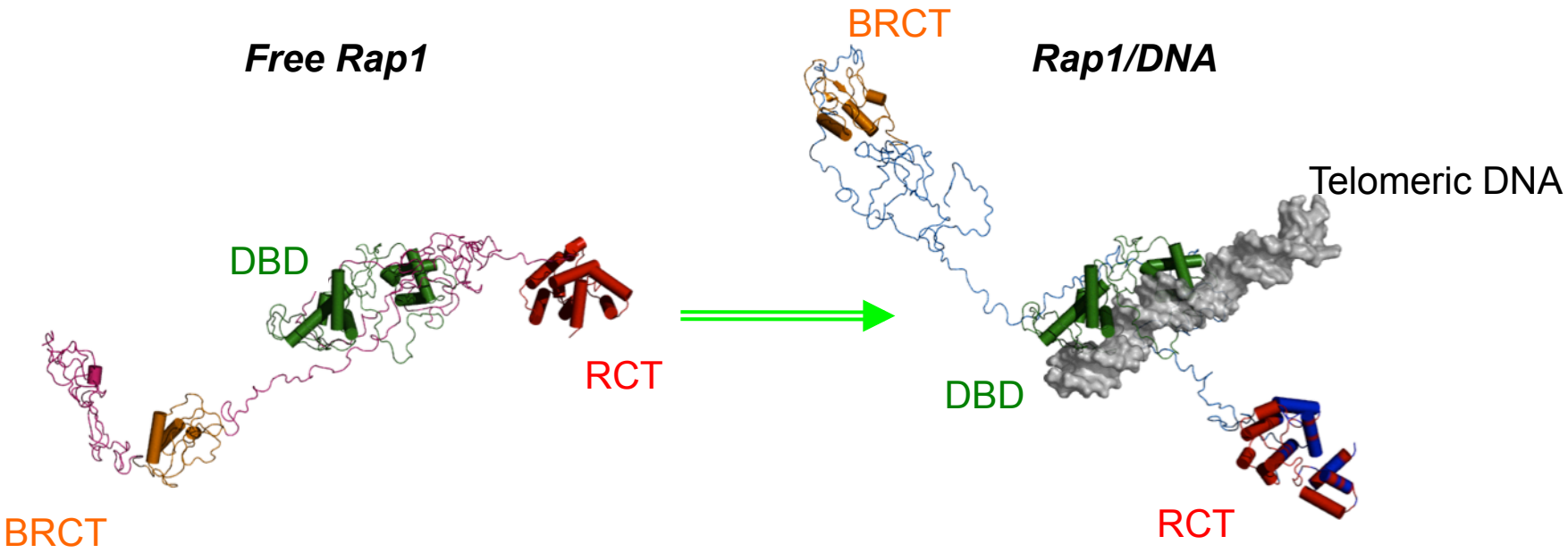


Molecule flexibility ⇒ Associated to N-terminal region

DNA binding ⇒ Stabilization; Conformational adjustment

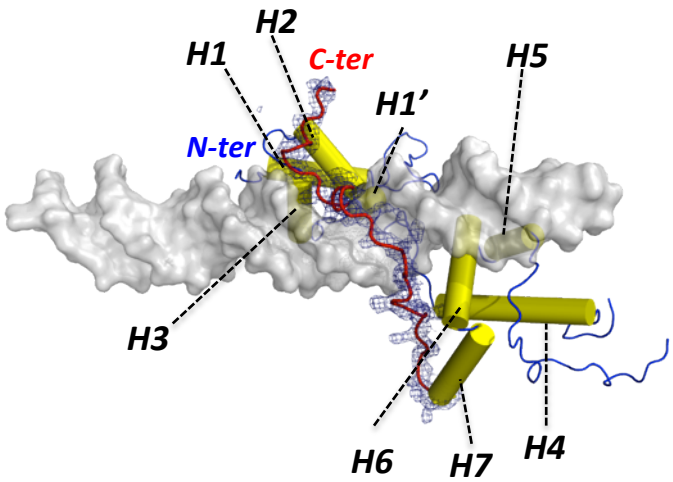
Matot, Le Bihan, et al., Nucl Acid Res, 2012.

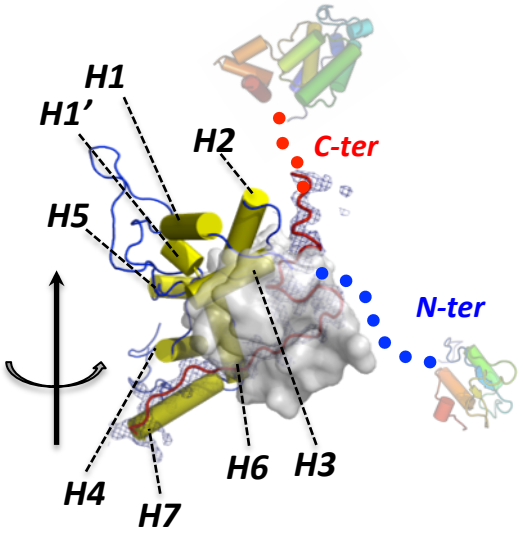
Remaining question:

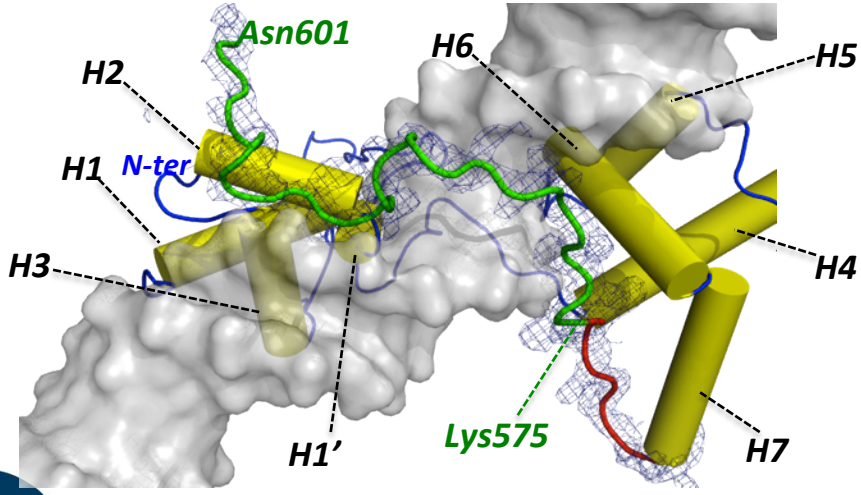


Structural determinants of conformational adjustment upon DNA binding ?

Crystal structure

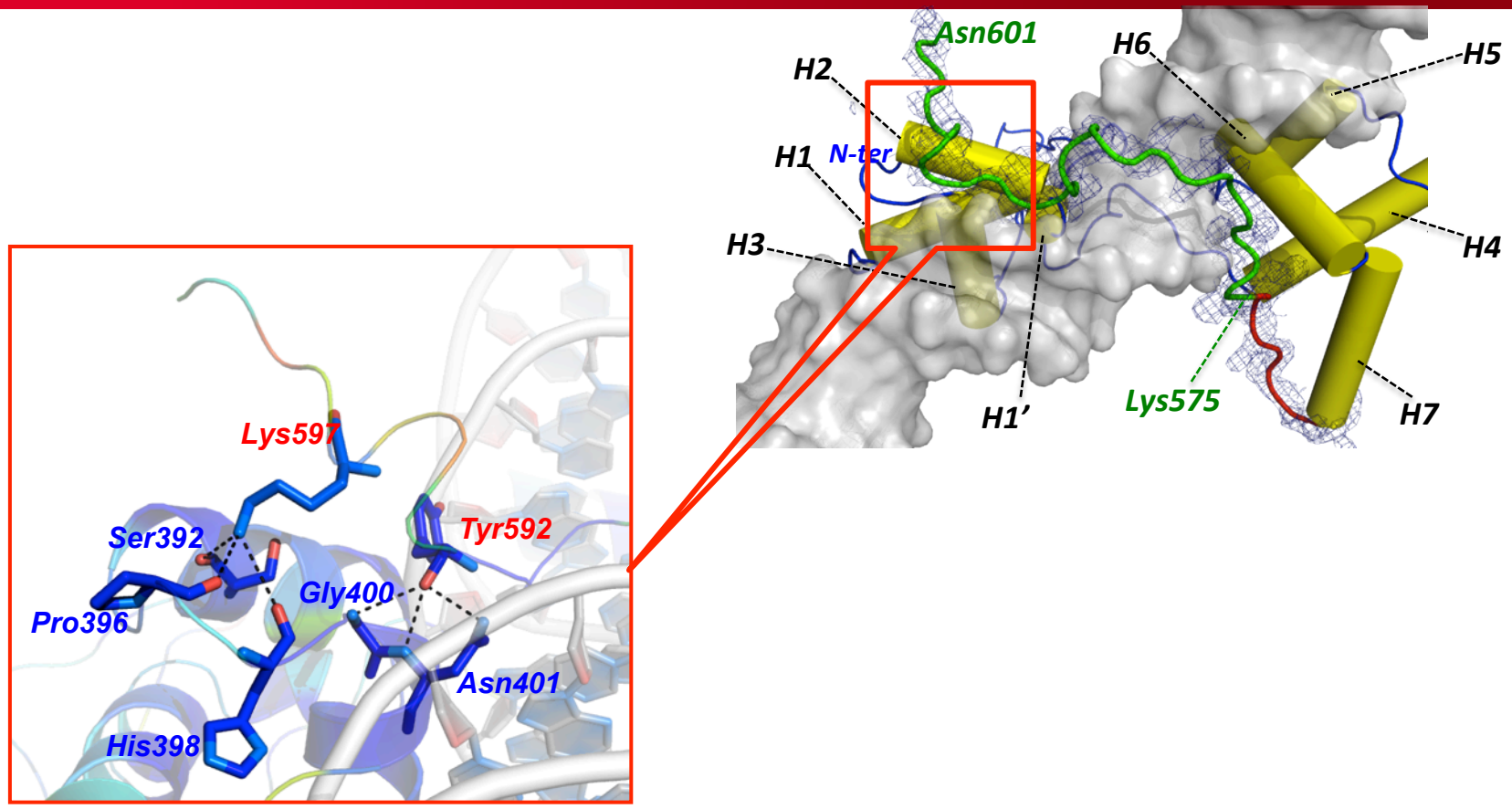






Loop 565-597 wraps DNA and drives N- and C-terminal regions toward opposite directions

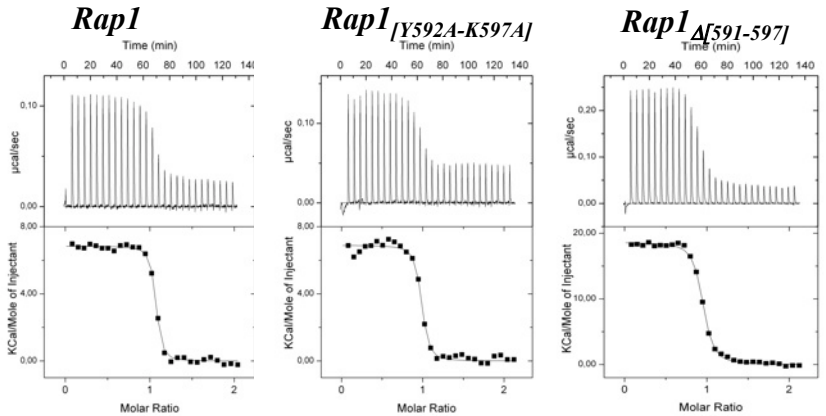
Crystal structure



Segment 591-597 locks Rap1 around DNA.

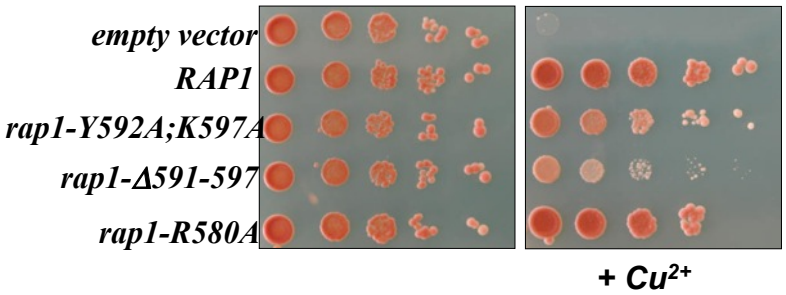
Analysis of Rap1 wrapping determinants

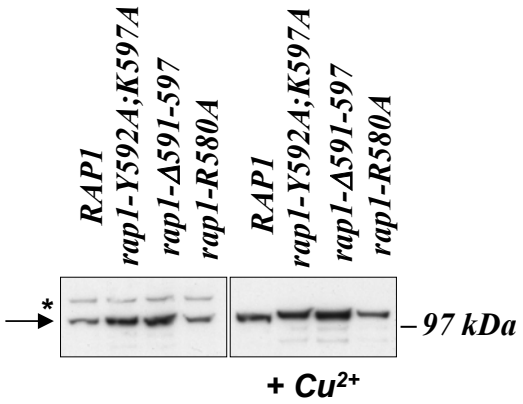
Mutation Y592A-K597A or deletion of segment 591-597 affects DNA binding.



Ka (M^{-1})	2.5 10^8 ± 0.44	1.5 10^8 ± 0.36	6.5 10^7 ± 0.58
Relative Ka	**1**	**0.6**	**0.26**
ΔG	- 10.8	- 10.5	- 10.0
ΔH (kcal/mol)	6.8 ± 0.04	6.8 ± 0.07	18.6 ± 0.1
TΔS	17.6	17.3	28.6

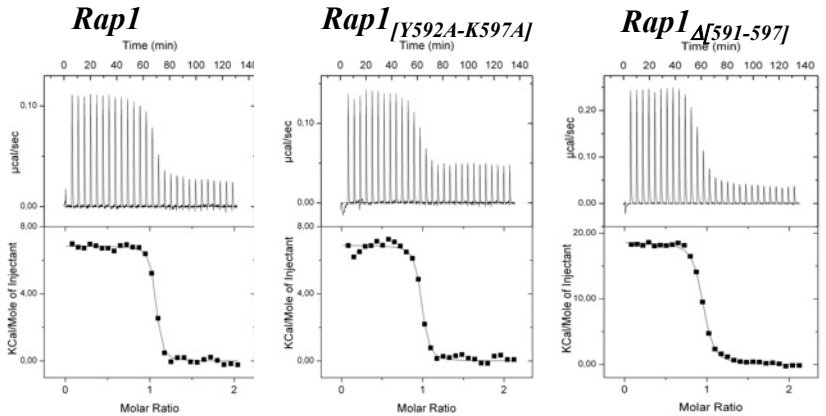
Analysis of Rap1 wrapping determinants





Mutation Y592A-K597A or deletion of segment 591-597 affects cells viability.

Mutation Y592A-K597A or deletion of segment 591-597 affects DNA binding.



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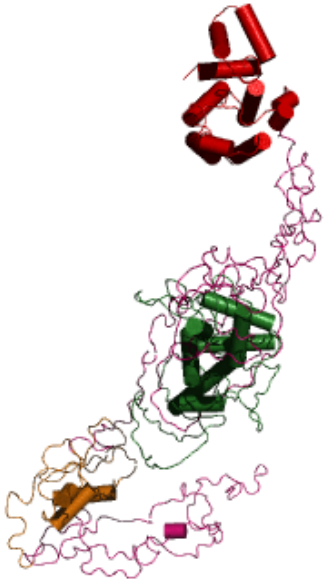
Conclusions :

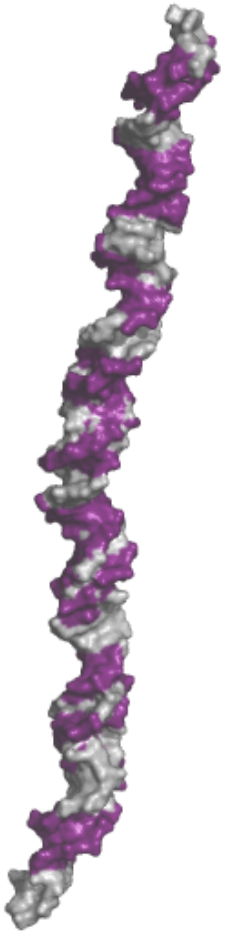
Interaction of Rap1 with DNA induces a complete wrapping of the protein around DNA, and a stiffening of DNA fiber.

Mutation or deletion of region that locks the protein around DNA:
➔ Affects Rap1 affinity for DNA.
➔ Affects Rap1 functional integrity.

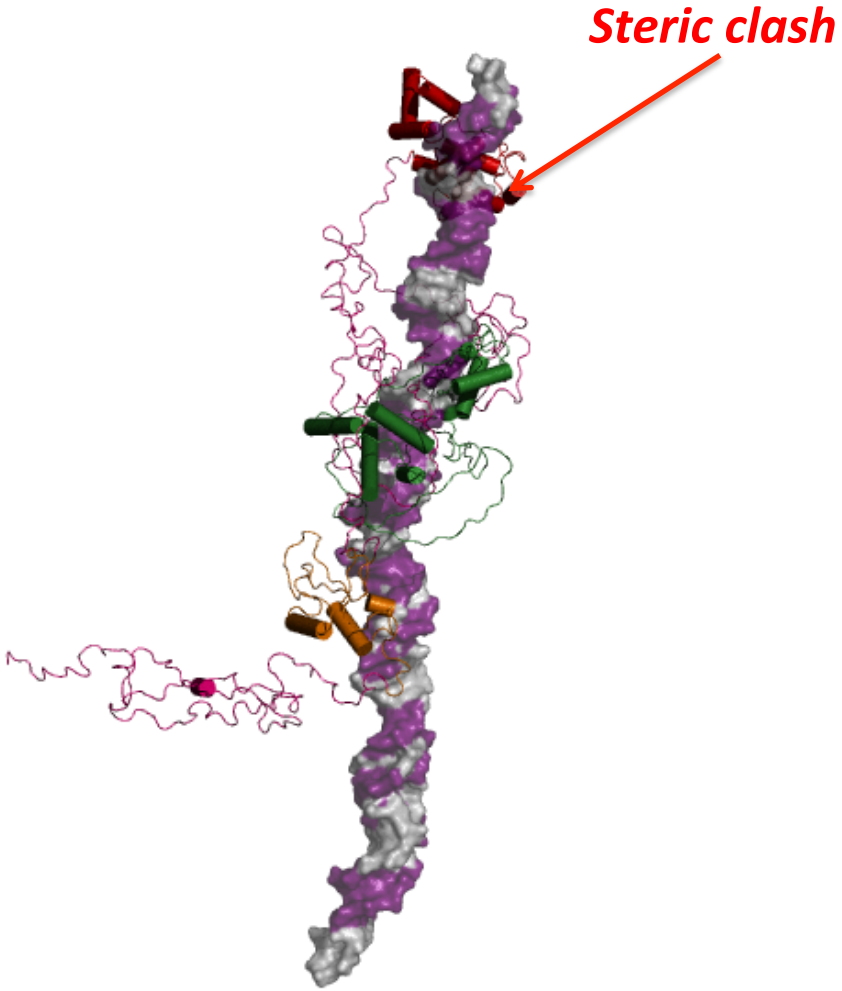
Toward telomeric assembly



Free Rap1

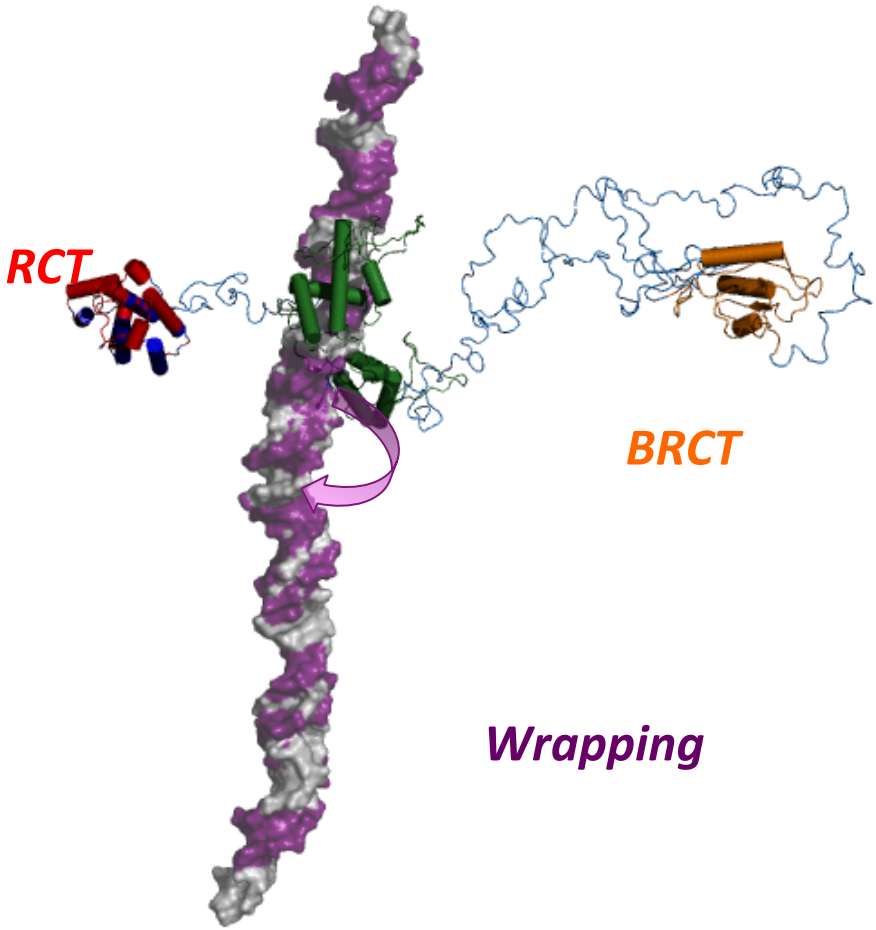


Toward telomeric assembly

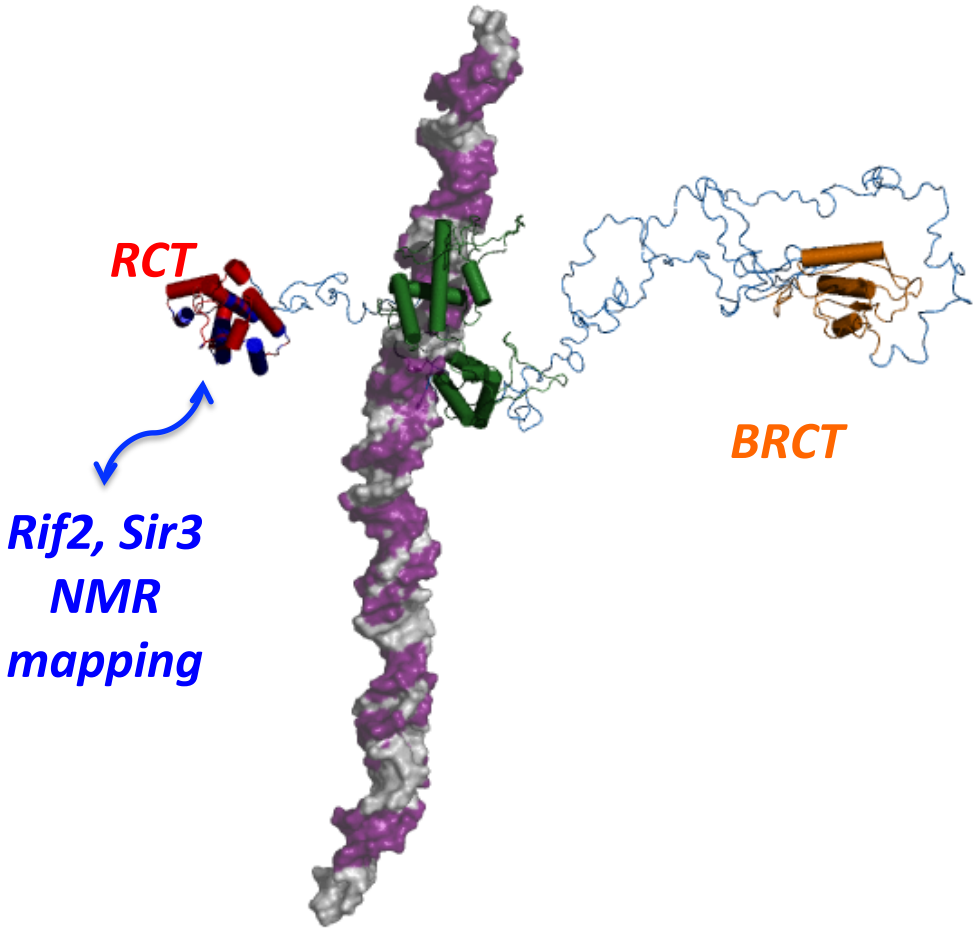


DBD/DNA interaction

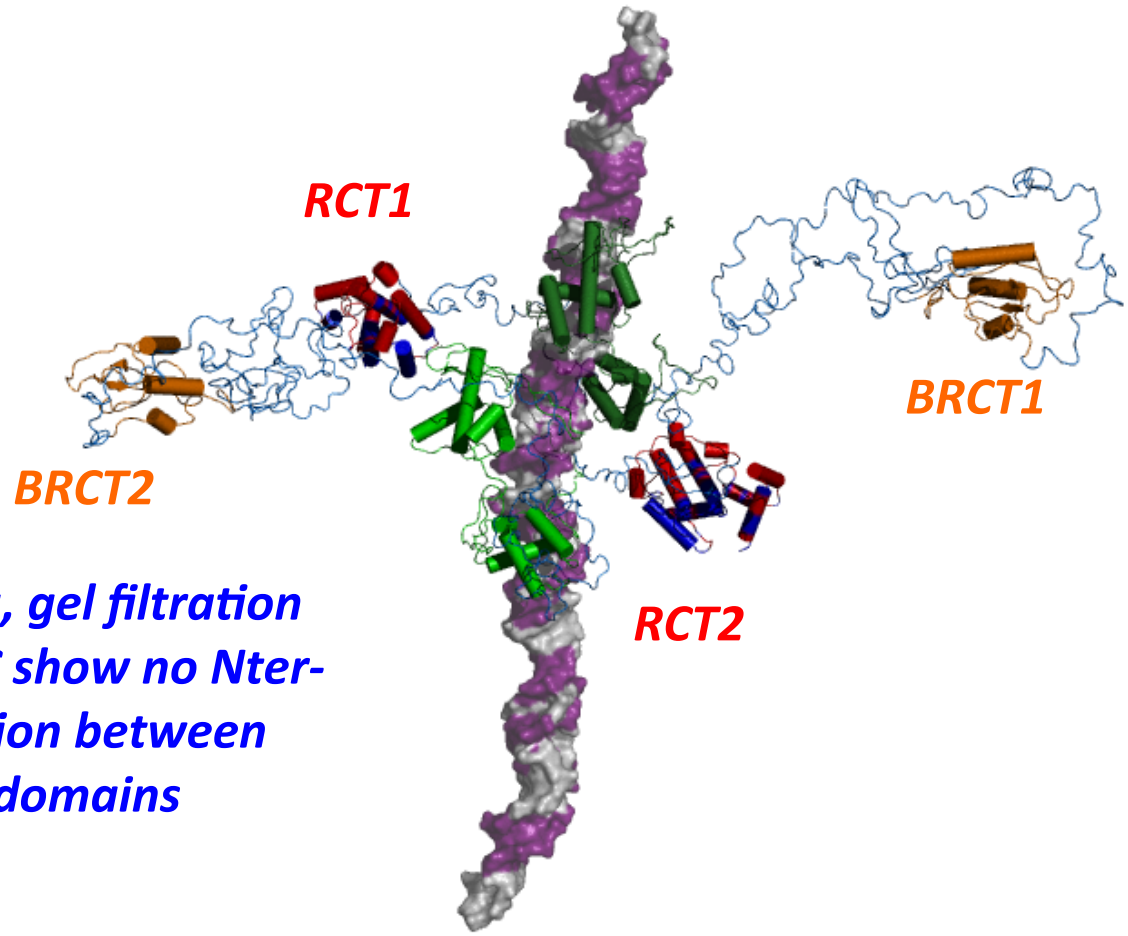
Toward telomeric assembly



Toward telomeric assembly

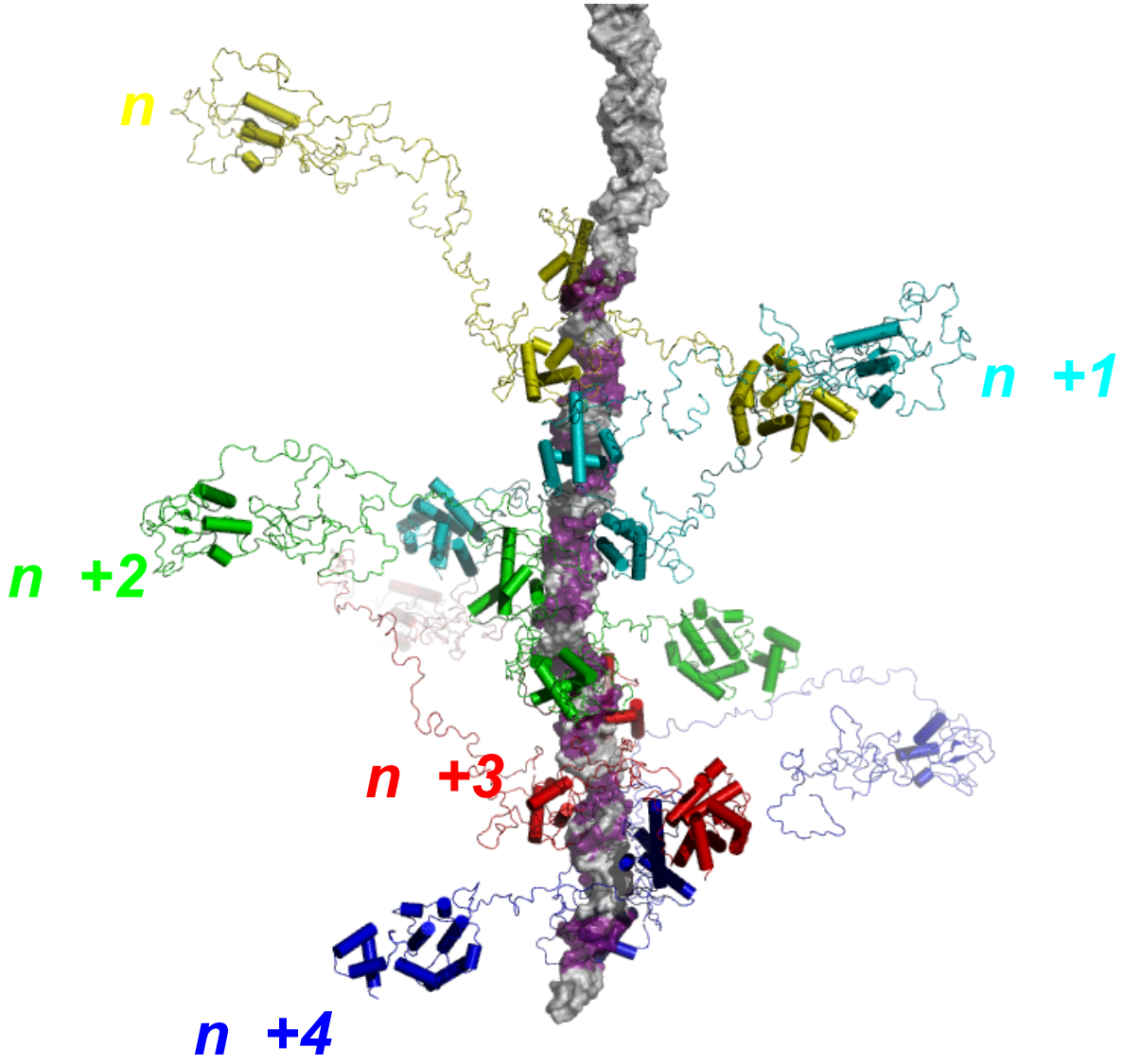


Toward telomeric assembly

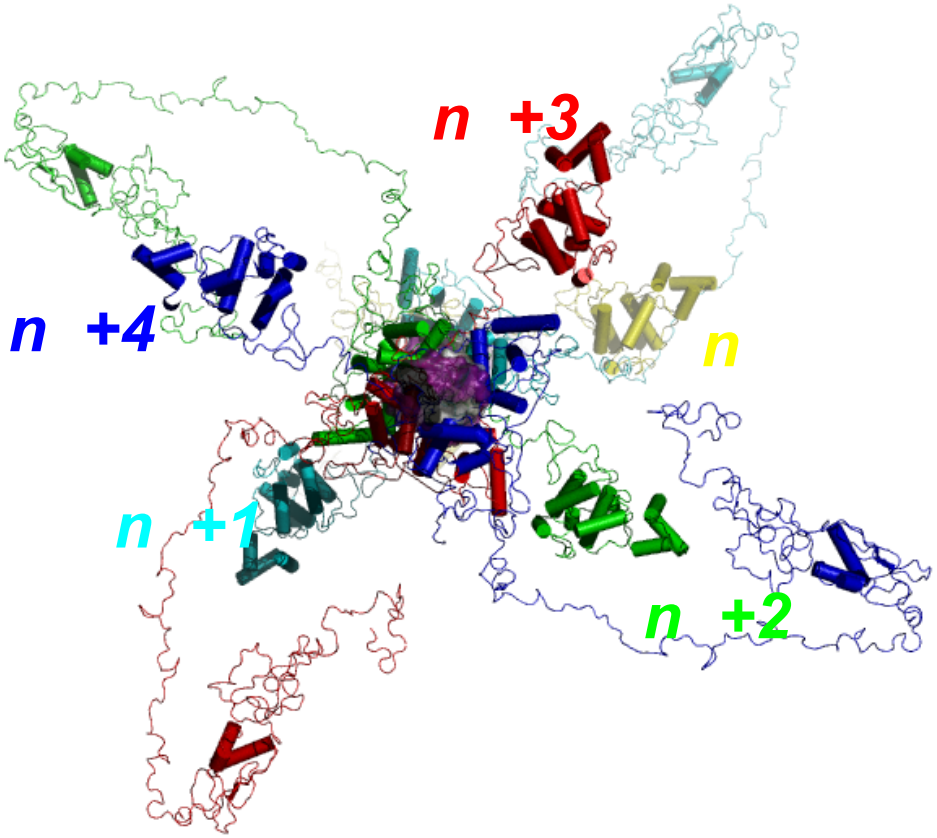


NMR mapping, gel filtration profiles and ITC show no Nter-Cter interaction between isolated domains

Toward telomeric assembly



Toward telomeric assembly



RCTs remain accessible to protein partners

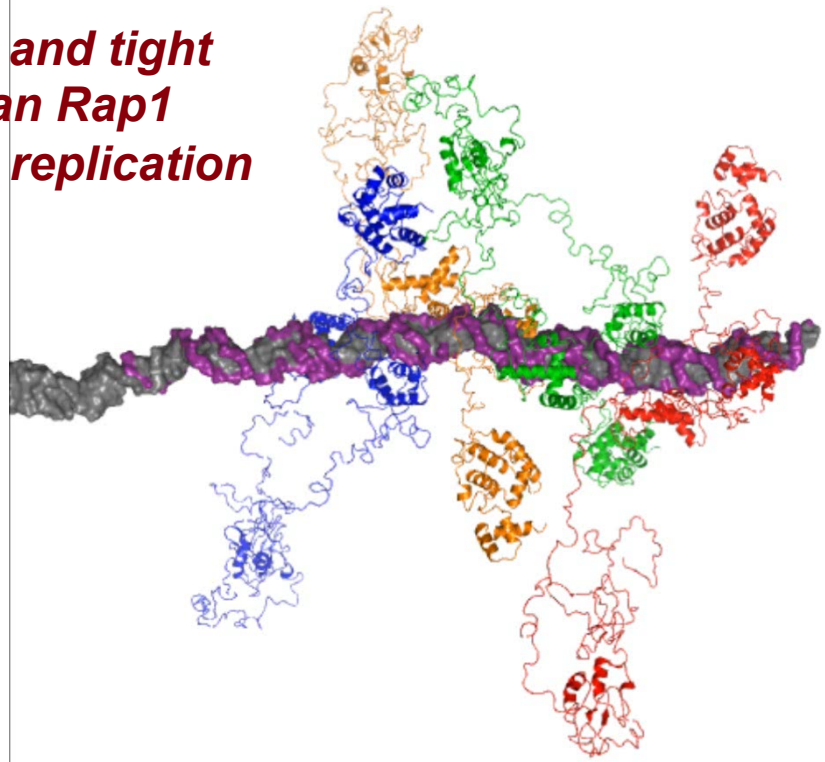
BRCT-DNA distance ≈ 50 Å

Matot, Le Bihan, et al., Nucl Acid Res, 2012.

New arising questions:

With such promiscuity of tightly bound molecules, does 'affinity' still mean anything ?

With such wrapping and tight interaction, how can Rap1 efficiently unbound at replication stage ?



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Thank you !!