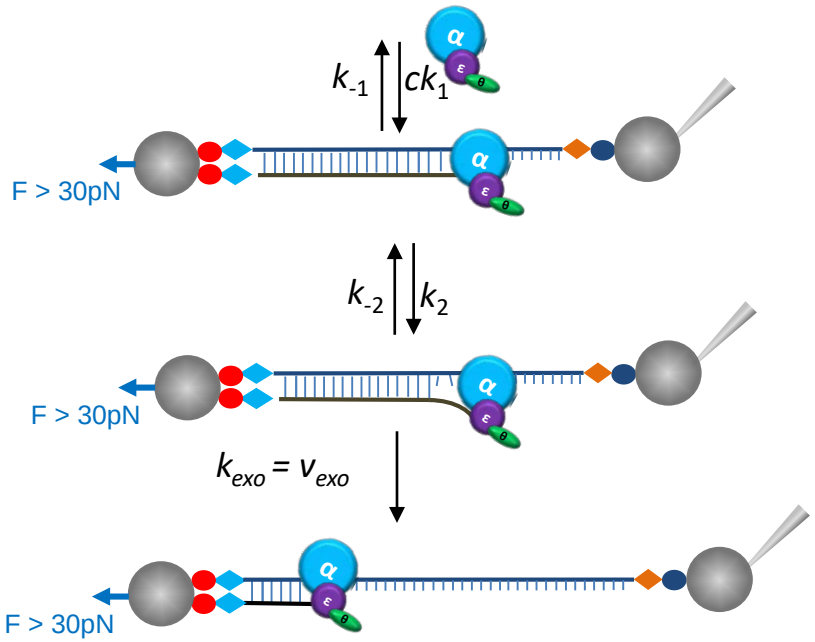
Controlling enzyme and polymerase activity on single DNA molecules

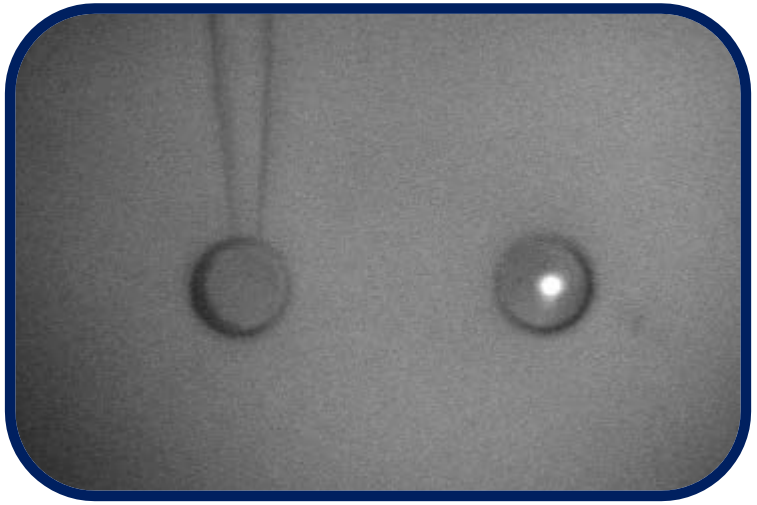


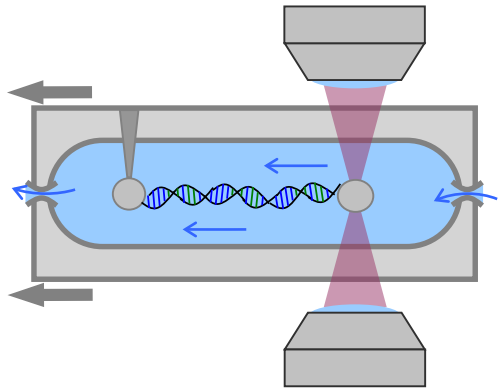
Mark C. Williams

Northeastern University,
Boston, MA, USA

Collective Dynamics of-, on- and around Filaments in Living Cells: Motors, MAPs, TIPs and Tracks
International Center for Theoretical Sciences
Tata Institute of Fundamental Research
Bangalore, India
28 October 2017 to 02 November 2017

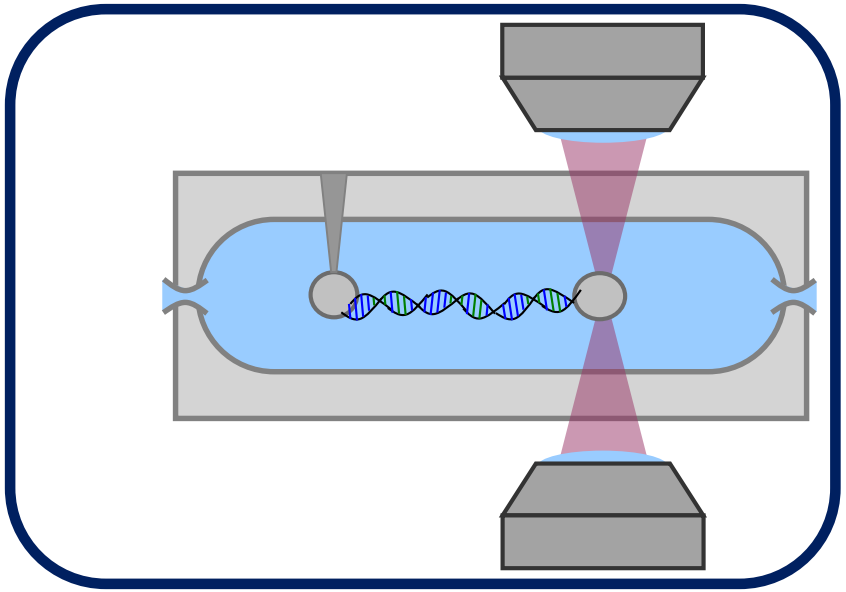
Optical tweezers enable single molecule protein-DNA interaction experiments

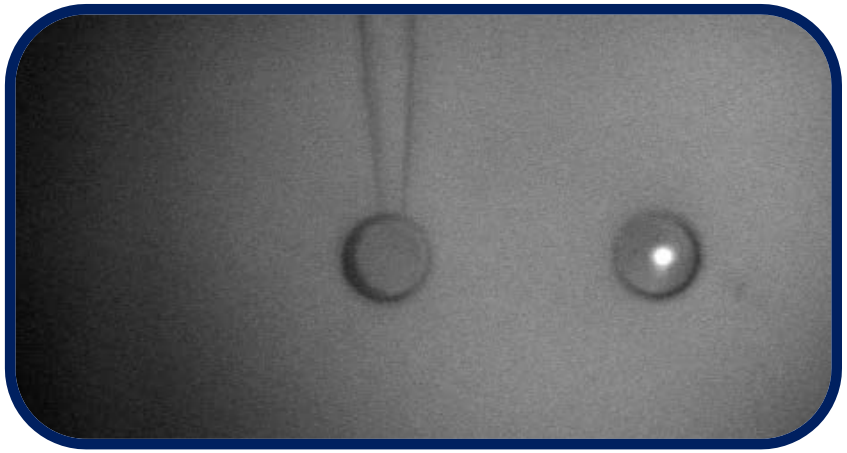


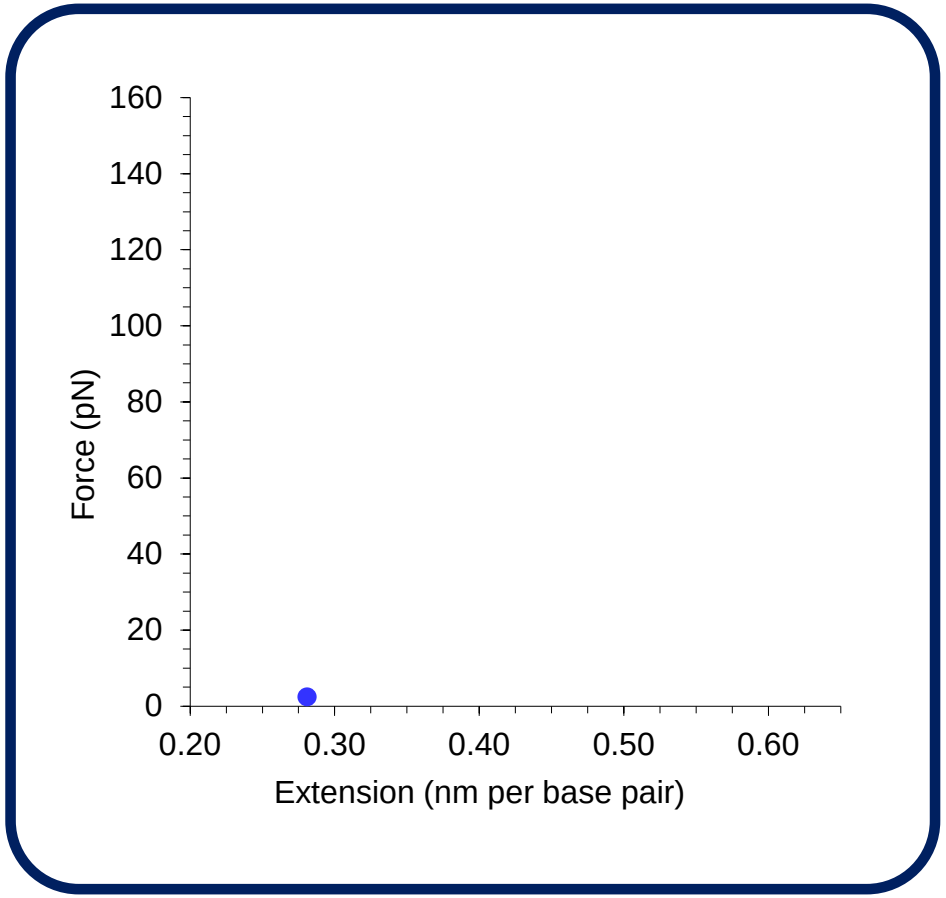


- Single DNA molecule is tethered between two polystyrene beads
- Pipette tip is moved with sub-nanometer resolution
- Force exerted by trap is measured with picoNewton resolution
- The binding of proteins is detected by change in DNA length/tension
- Replication of DNA can be detected in real time

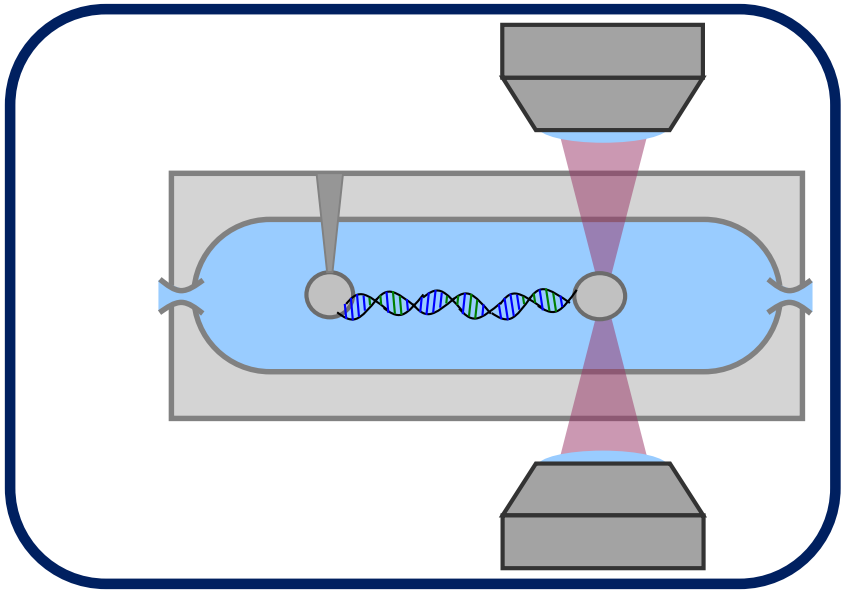
Force-extension reveals DNA structural changes

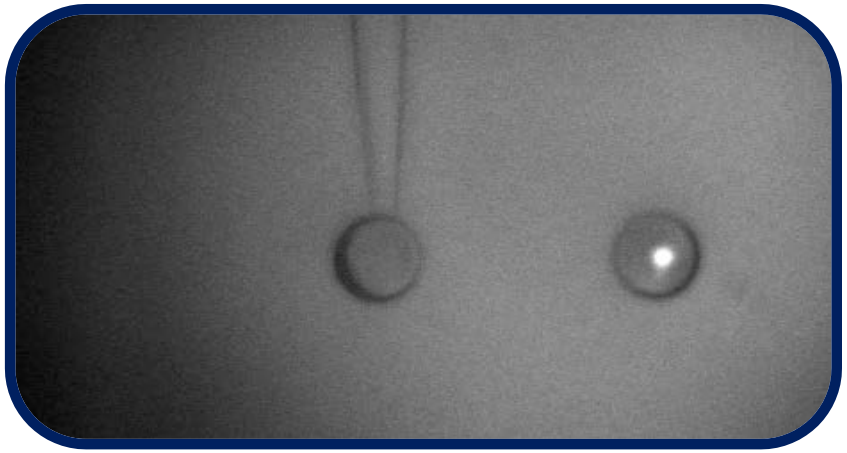


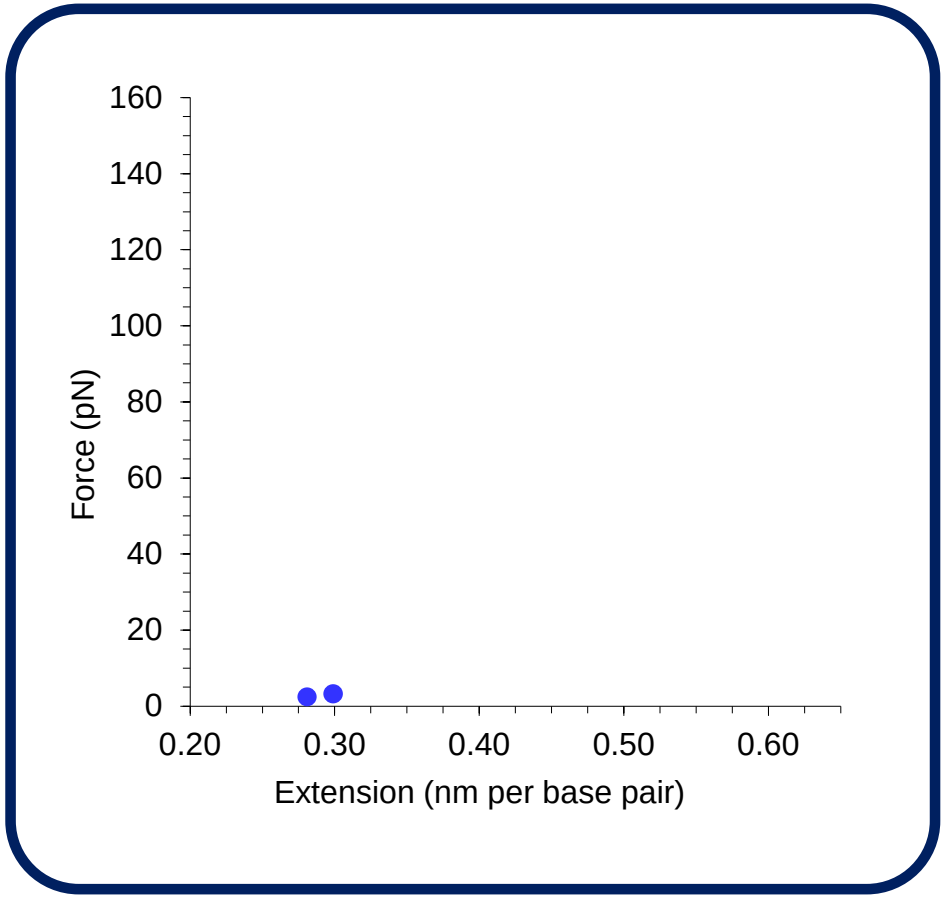




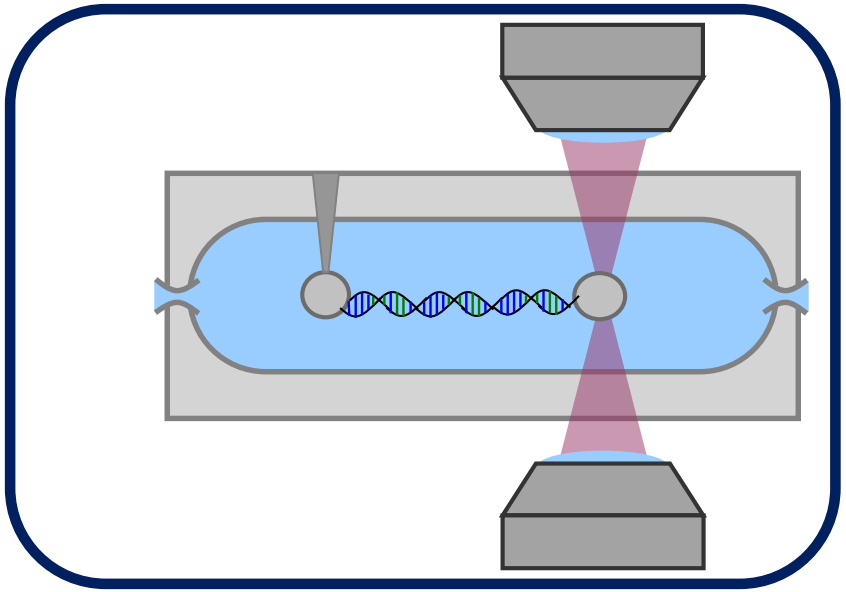
Force-extension reveals DNA structural changes

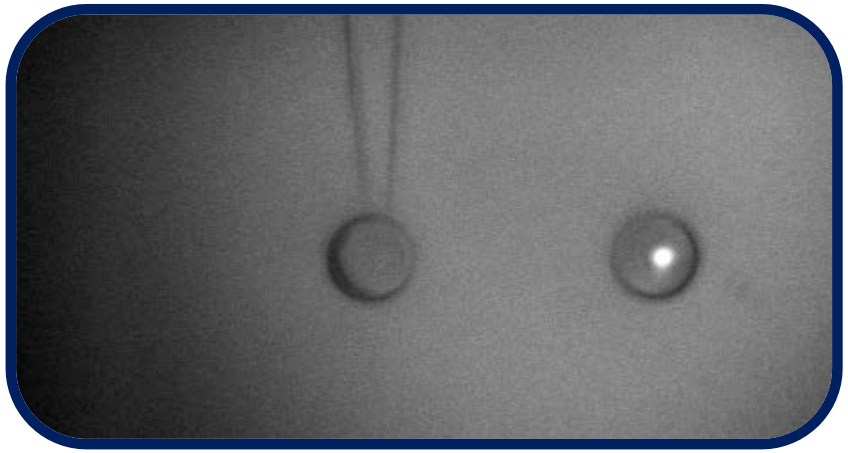


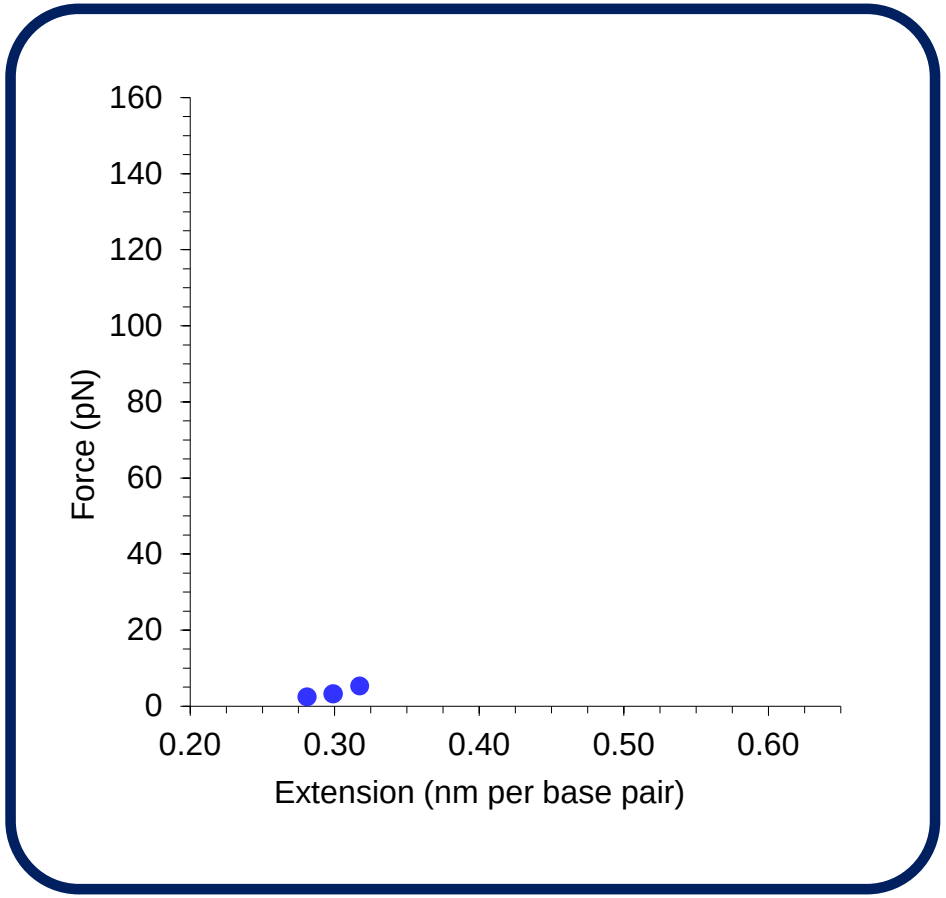




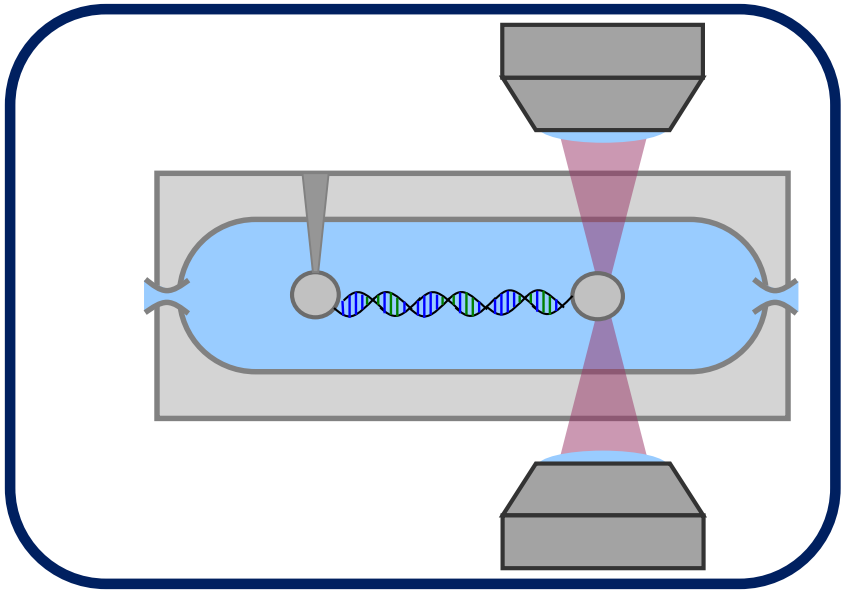
Force-extension reveals DNA structural changes

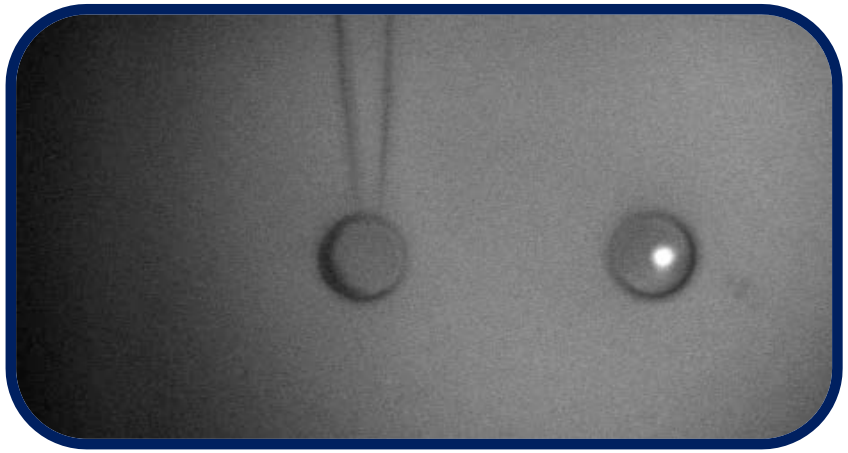


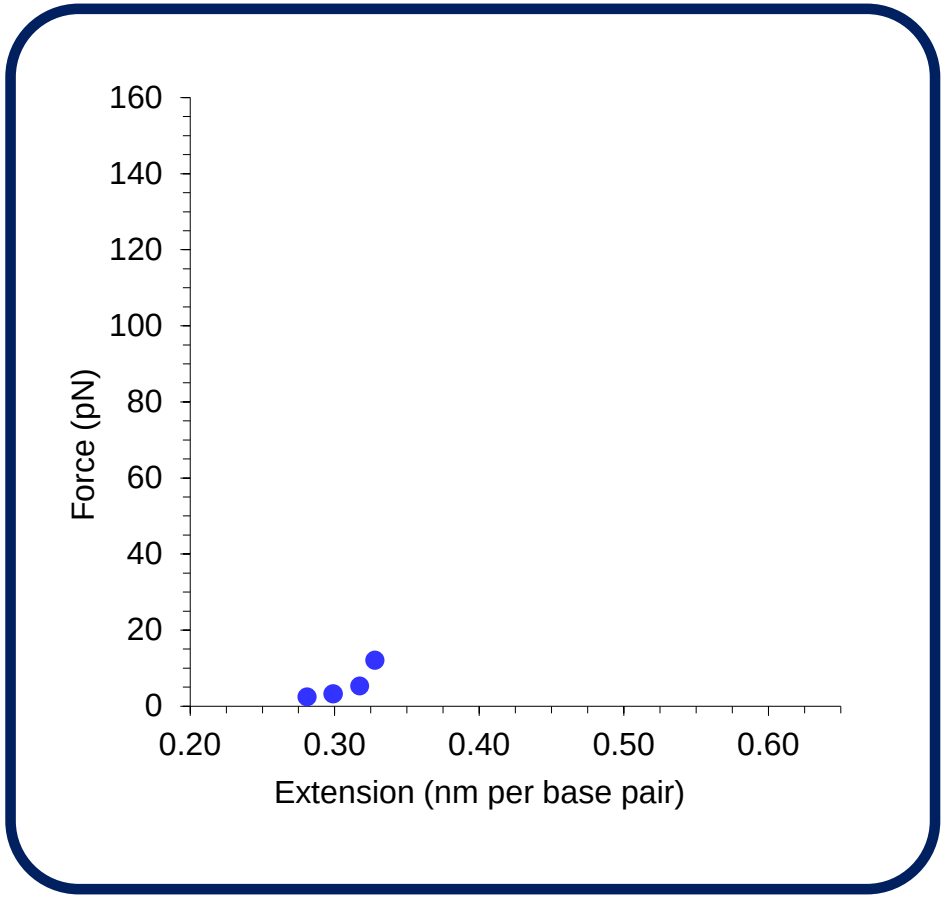




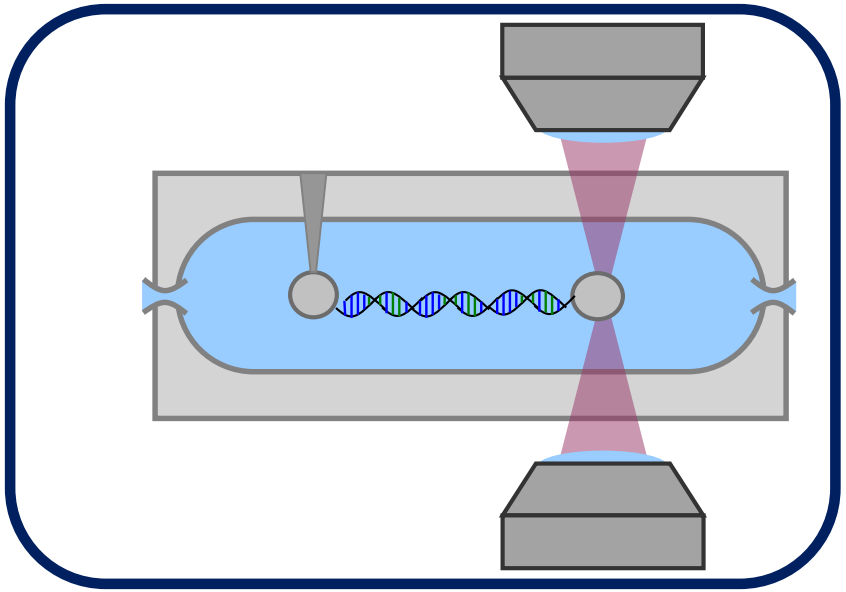
Force-extension reveals DNA structural changes

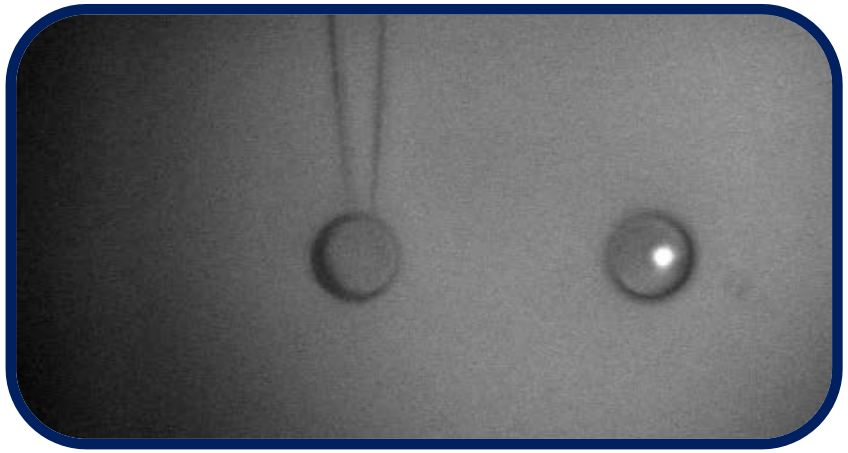


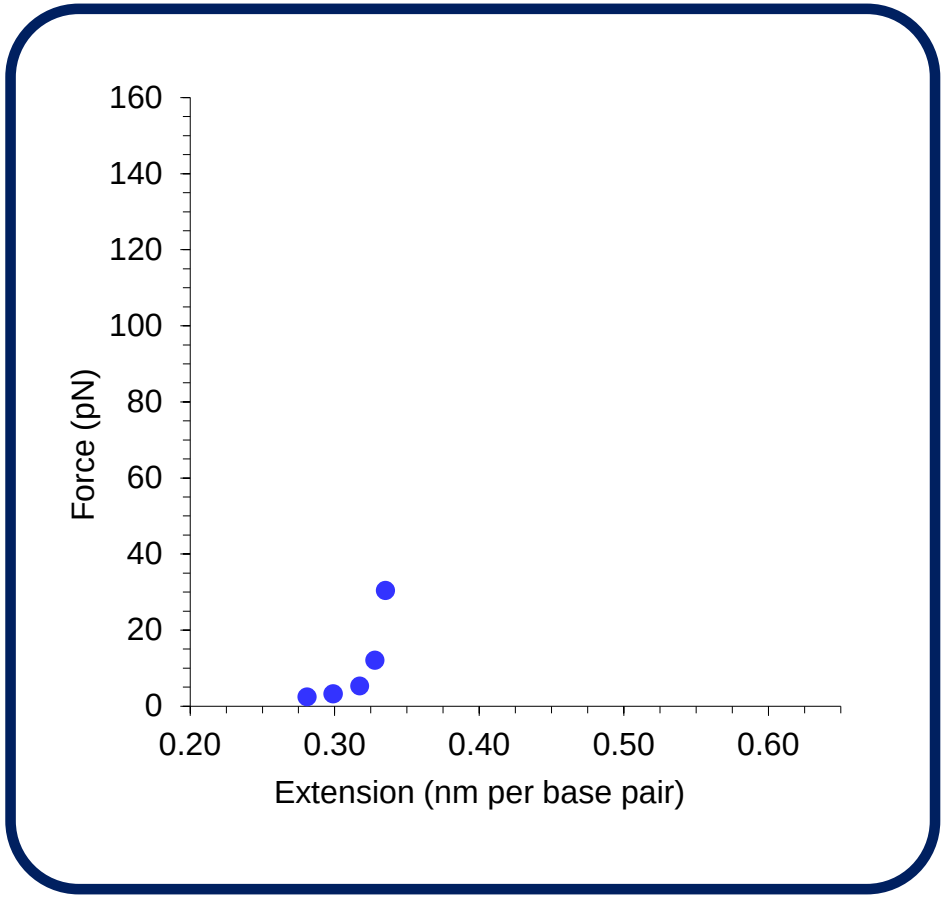




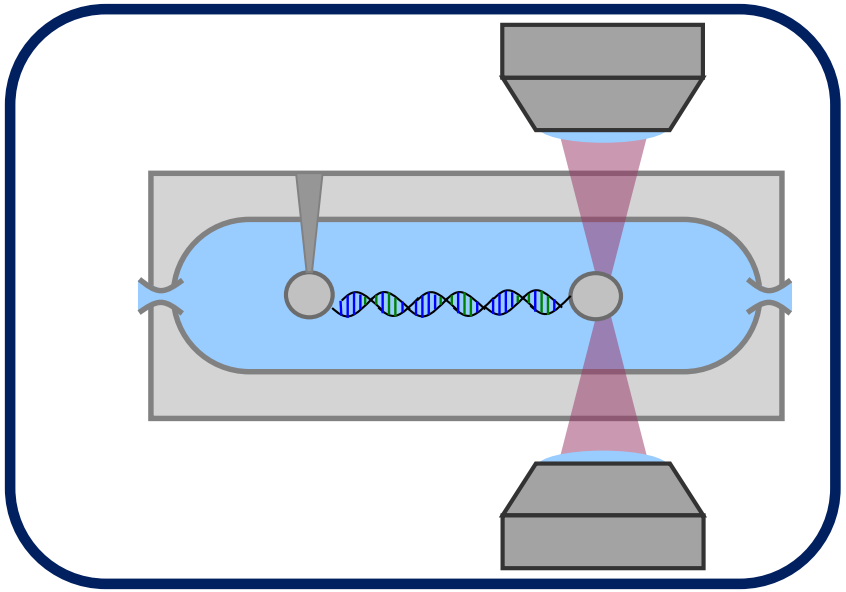
Force-extension reveals DNA structural changes

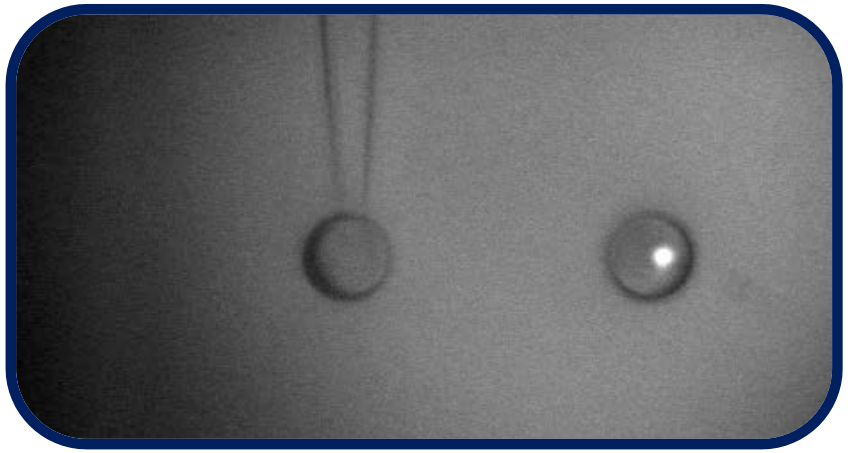


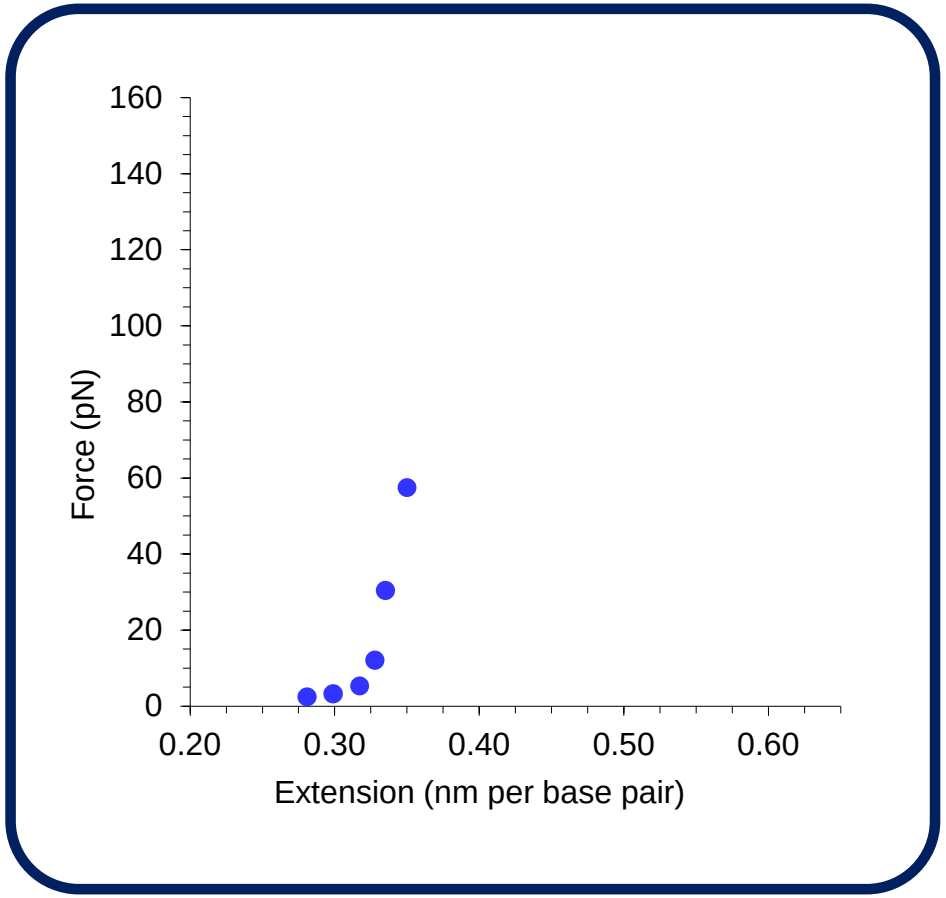




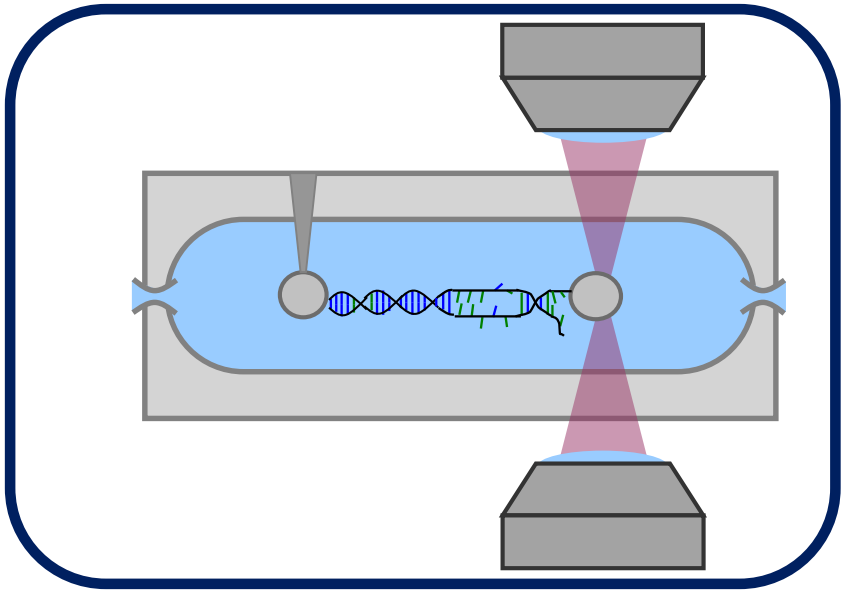
Force-extension reveals DNA structural changes

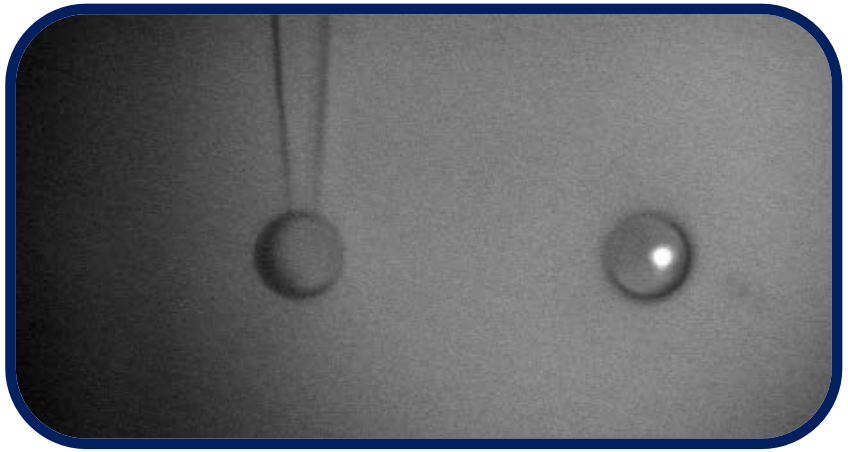


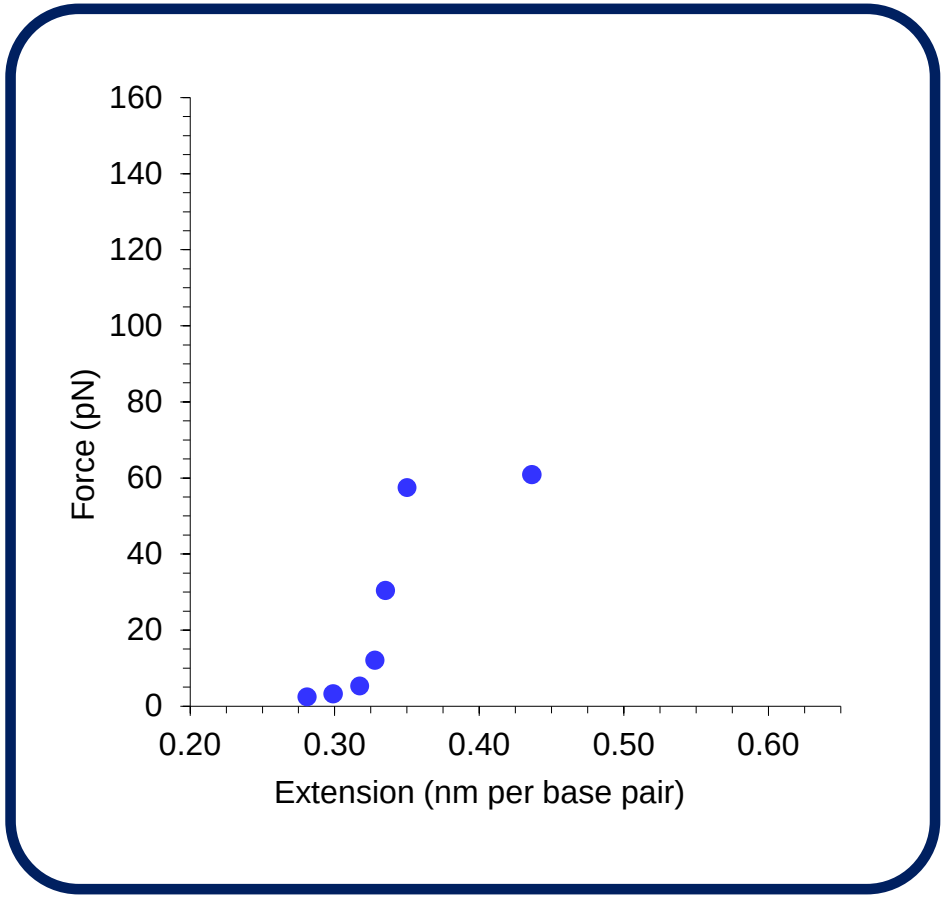




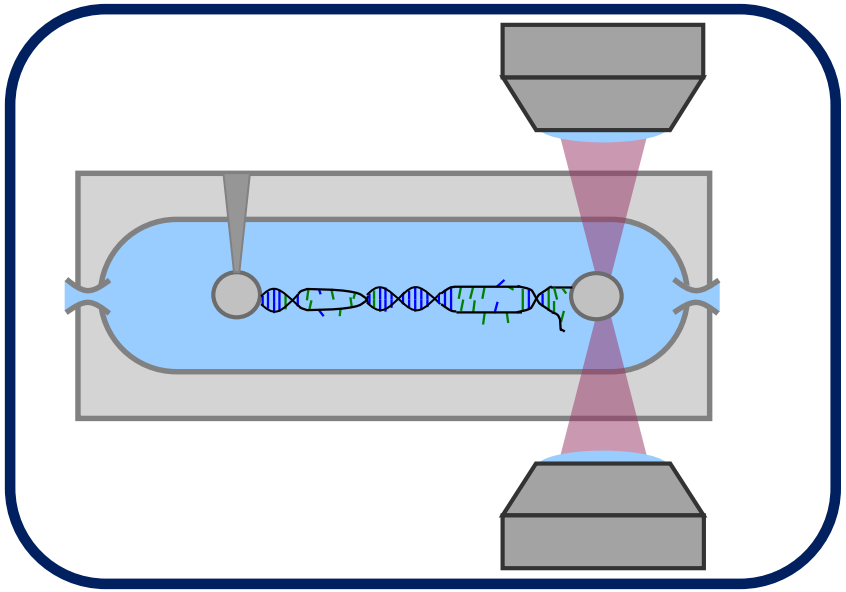
Force-extension reveals DNA structural changes

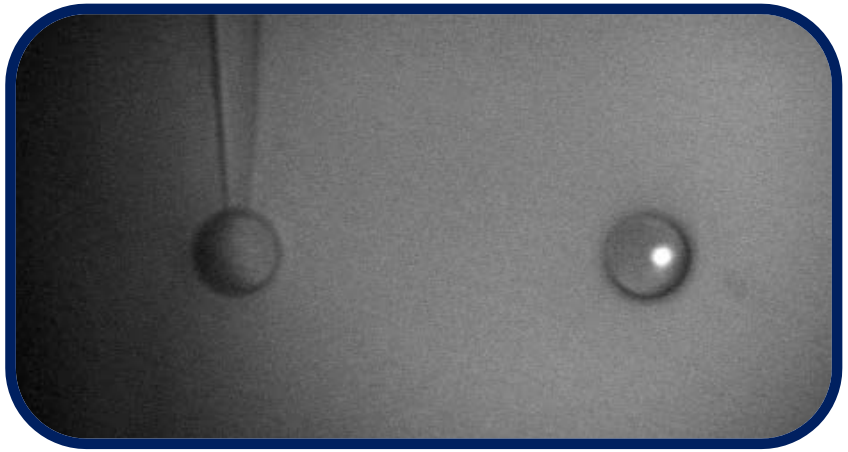


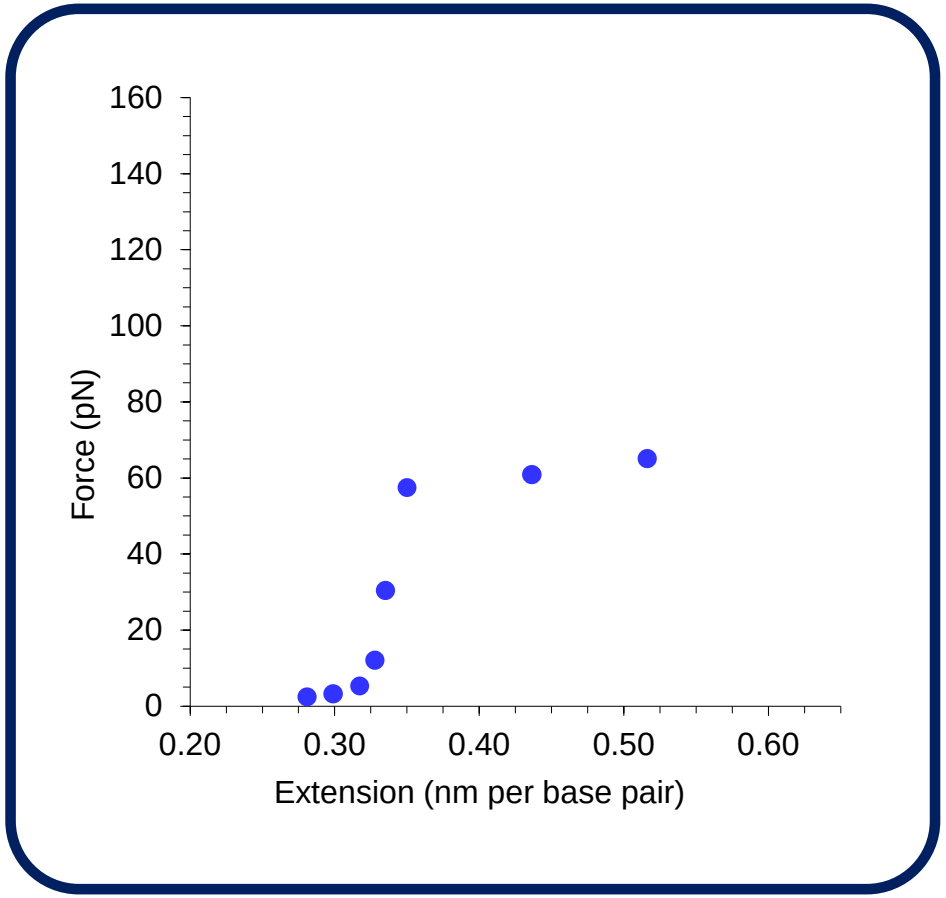




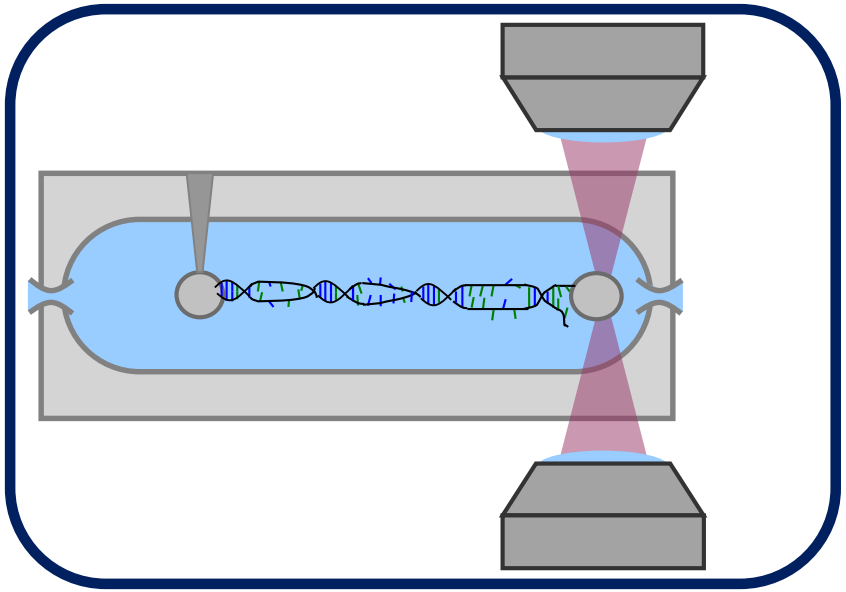
Force-extension reveals DNA structural changes

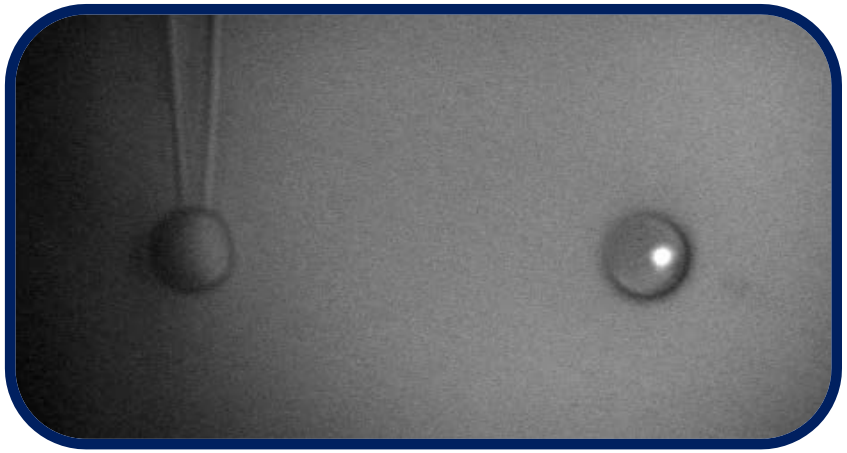


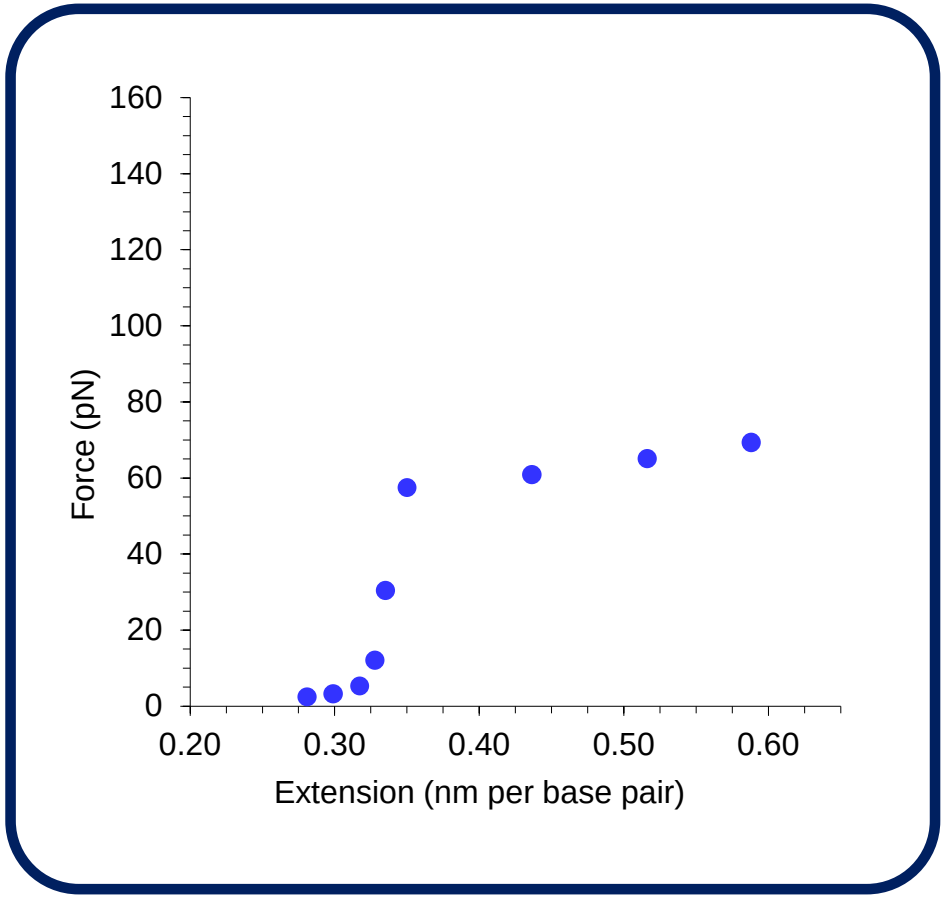




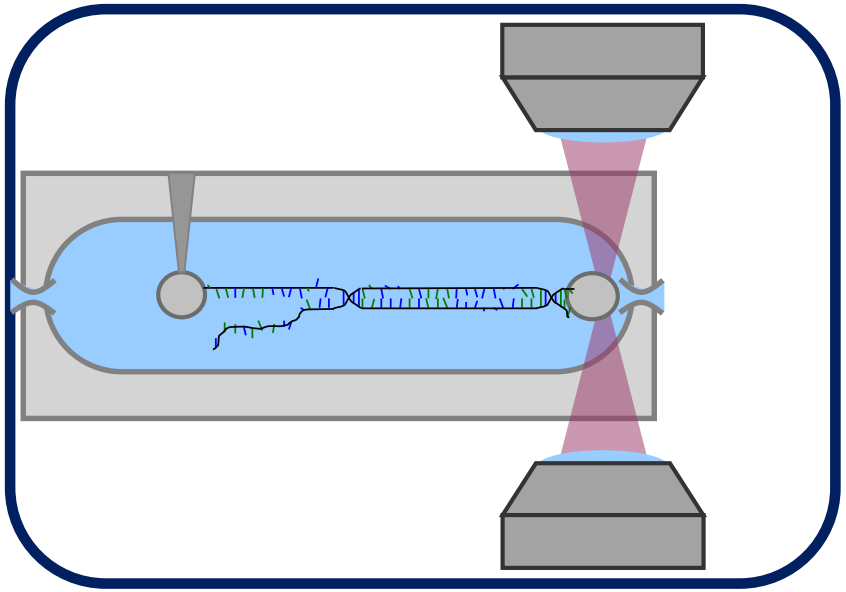
Force-extension reveals DNA structural changes

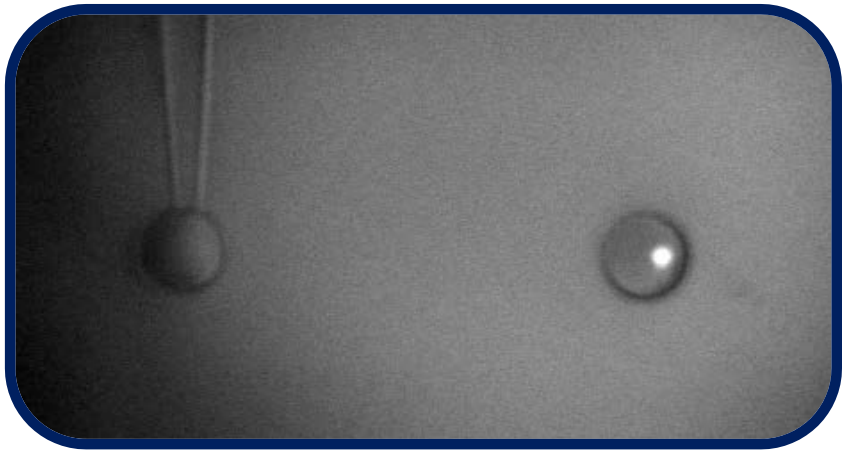


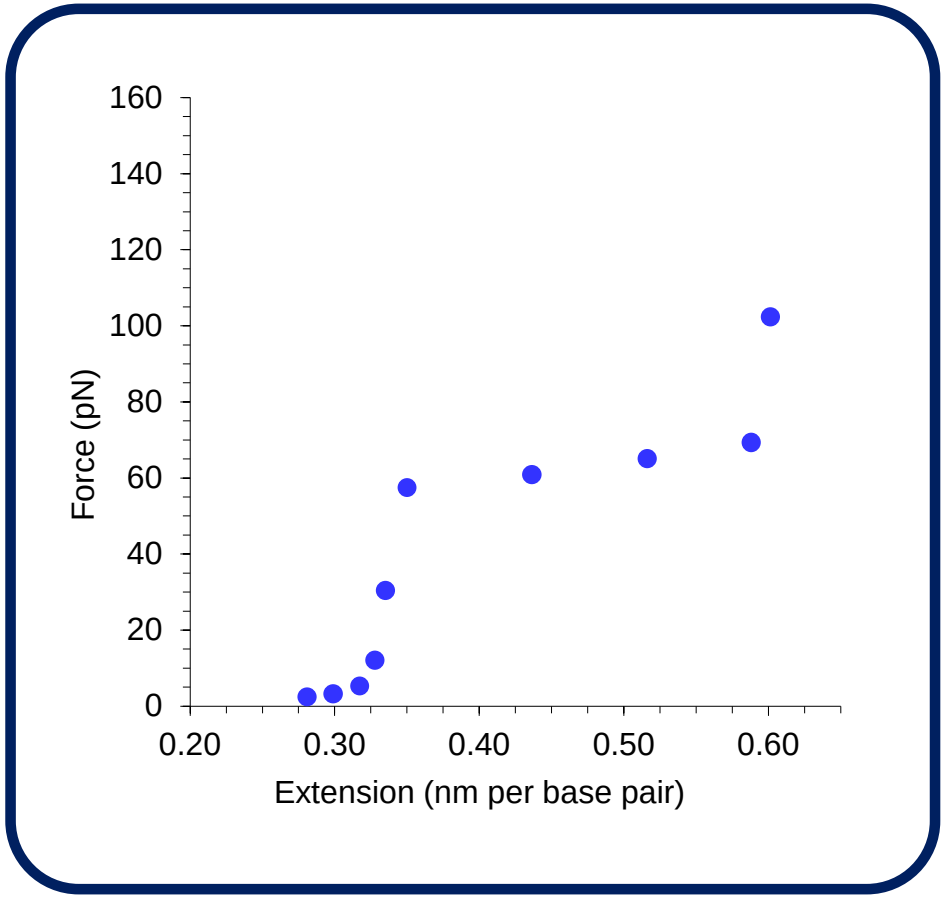




Force-extension reveals DNA structural changes

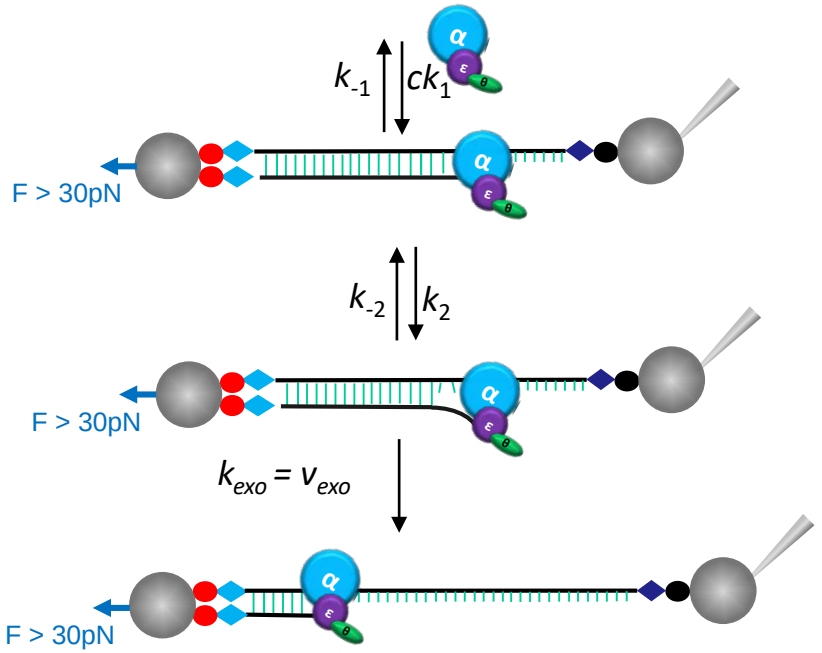




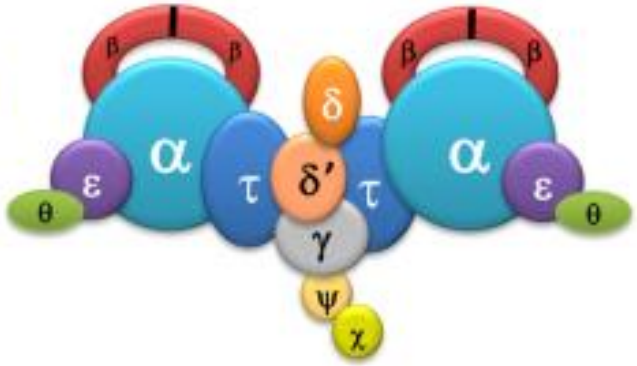


Summary

- Optical tweezers and DNA stretching
- E. coli pol III core switching between pol and exo activity
- DNA binding by human innate immune system protein APOBEC3G, which inhibits HIV-1 replication



There are at least five types of DNA polymerases in *E. coli*

	Pol I	Pol II	Pol III	Pol IV	Pol V
DNA polymerase family	A	B	C	Y	Y
Activity	5'-3' polymerase 3'-5' exonuclease 5'-3' exonuclease	5'-3' polymerase 3'-5' exonuclease	5'-3' polymerase 3'-5' exonuclease	5'-3' polymerase	5'-3' polymerase
	PolA	PolB		DinB	UmuC UmuD' UmuD'
Number of molecules/ cell					
- SOS	400	50 - 75	10 - 20	150 - 250	< 15
+ SOS	400	350 - 1000	10 - 20	1200 - 2500	200
Biological functions in the cell	DNA replication, Okazaki fragment maturation, DNA repair	DNA replication (backup DNA polymerase), DNA repair, TLS	DNA replication DNA repair	TLS	TLS

Fijalkowska, I. J., et al. (2012). FEMS Microbiol Rev.

DNA polymerase III (pol III) holoenzyme is the replicative polymerase in *E. coli*

Subunit	Function	Groupings
α	5′–3′ polymerization	Core enzyme: Elongates polynucleotide chain and proofreads
ϵ	3′–5′ exonuclease	
θ	core assembly	
γ	Loads enzyme on template (Serves as clamp loader)	γ complex
δ		
δ'		
χ		
ψ		
β	Sliding clamp structure (processivity factor)	
τ	Dimerizes core complex	

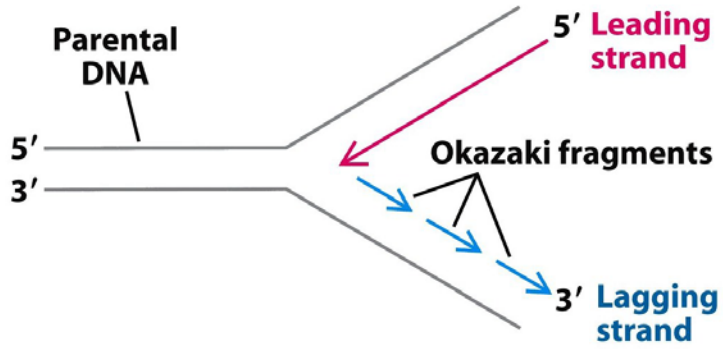


Figure 28.9
Biochemistry, Seventh Edition
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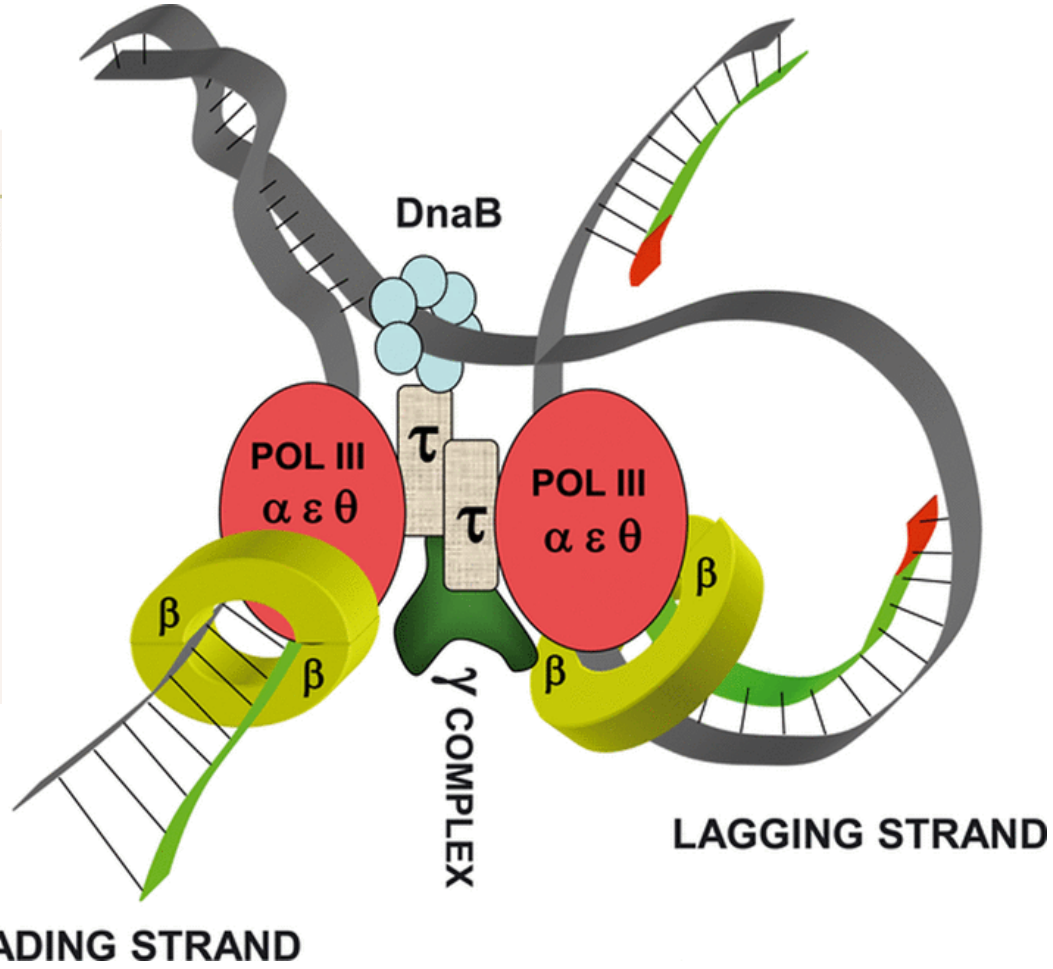

Fijalkowska, I. J., et al. (2012). FEMS Microbiol Rev.

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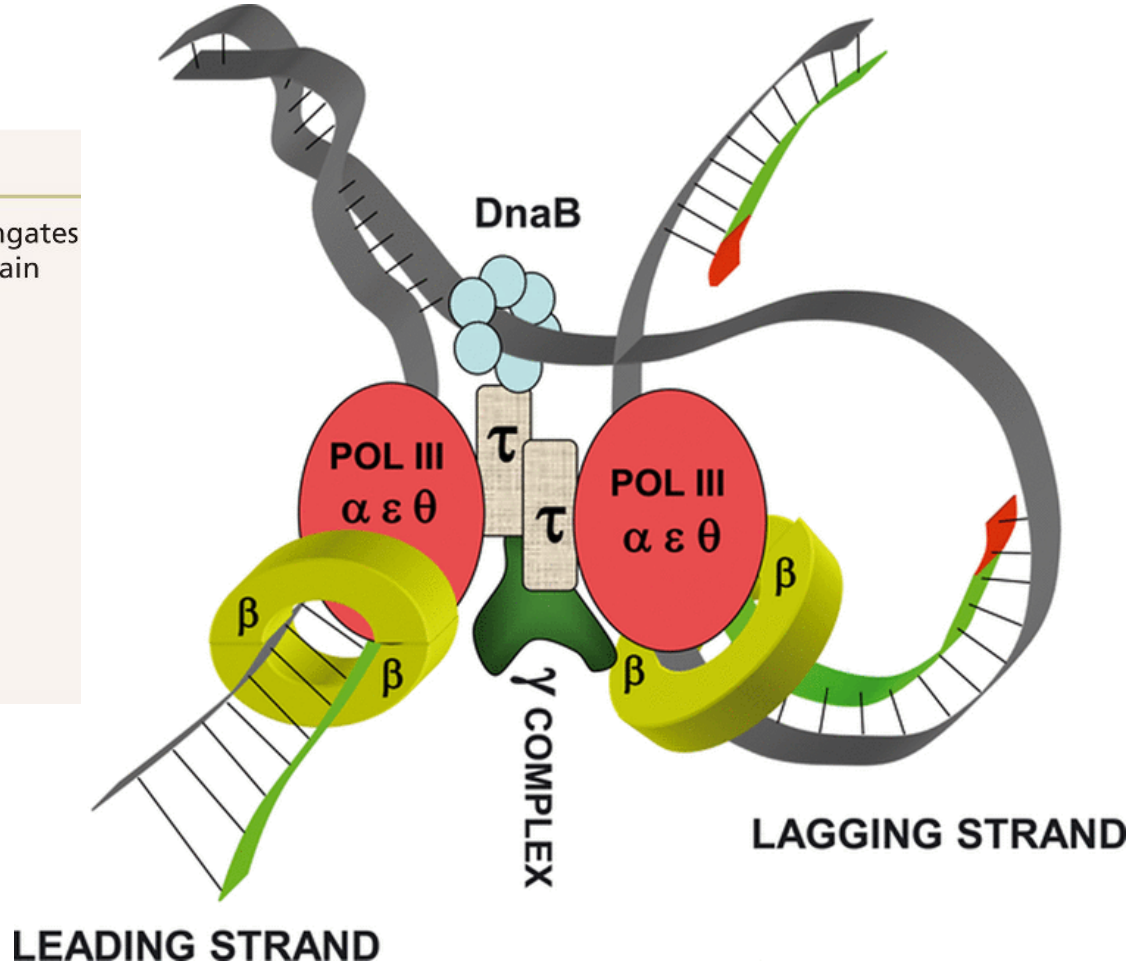
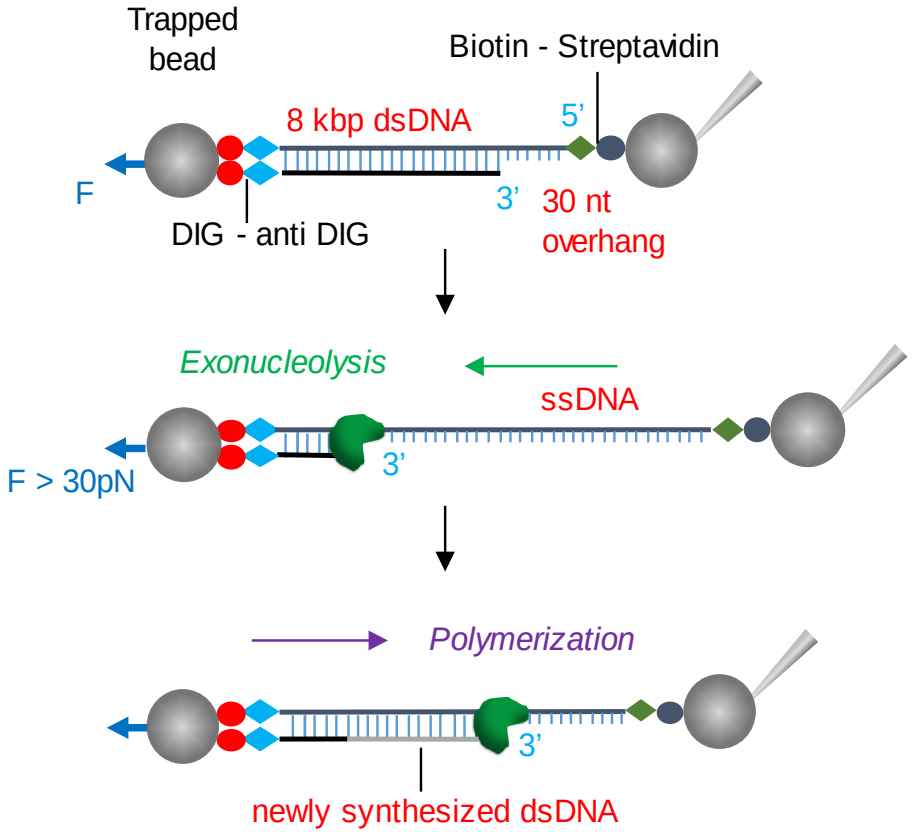
θ	core assembly	
γ	Loads enzyme on template (Serves as clamp loader)	γ complex
δ		
δ'		
χ		
ψ		
β	Sliding clamp structure (processivity factor)	
τ	Dimerizes core complex	

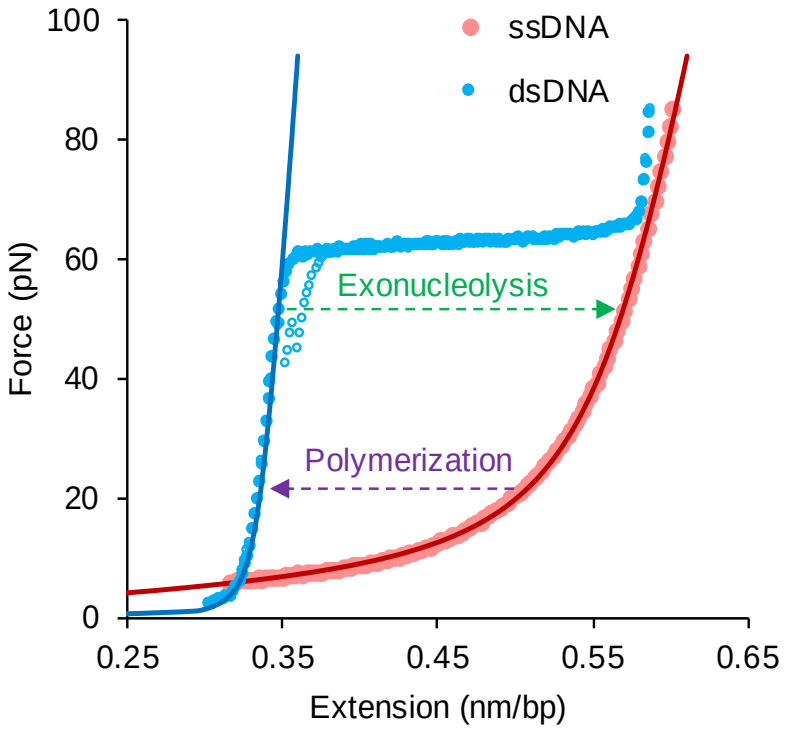
Error frequency	
Without proofreading	With proofreading
10^{-5}/bp	10^{-8}/bp



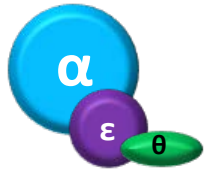
Fijalkowska, I. J., et al. (2012). FEMS Microbiol Rev.

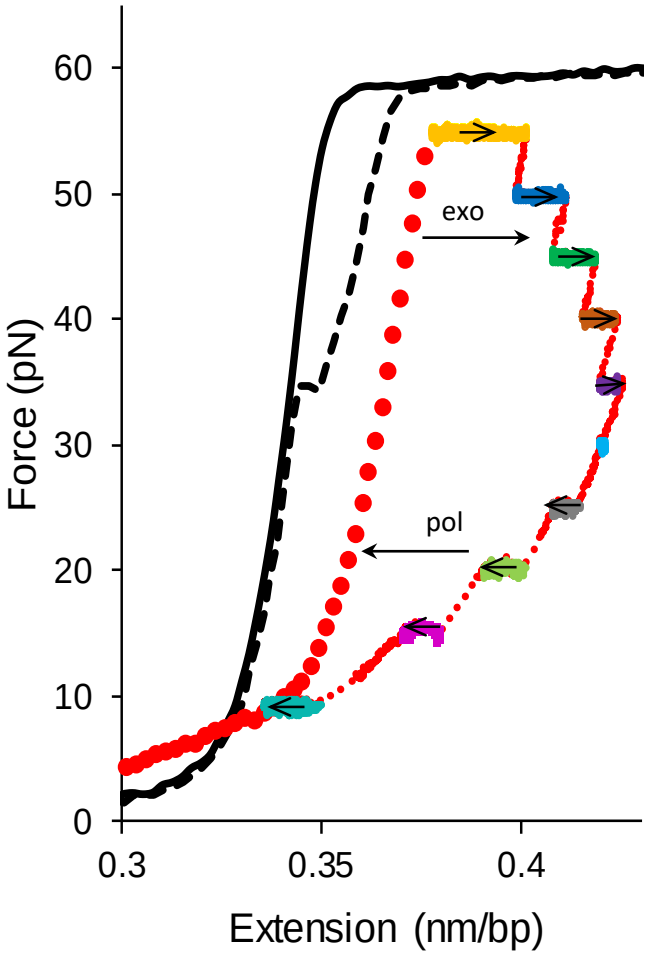
Polymerization and exonucleolysis are controlled by force

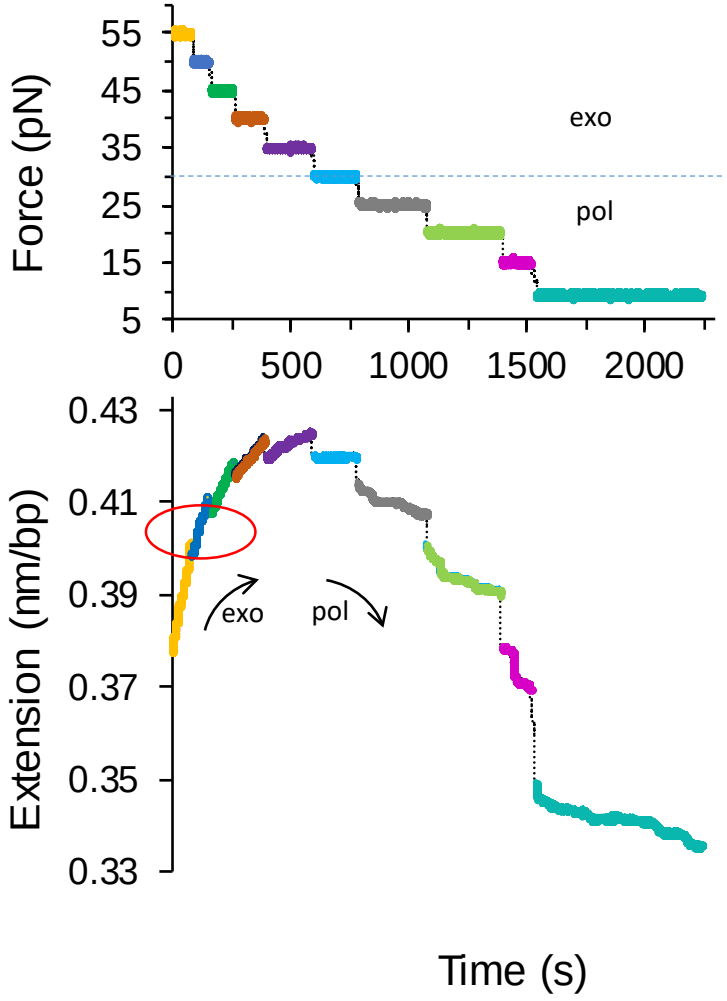




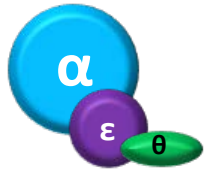
Pol III core activity at constant forces

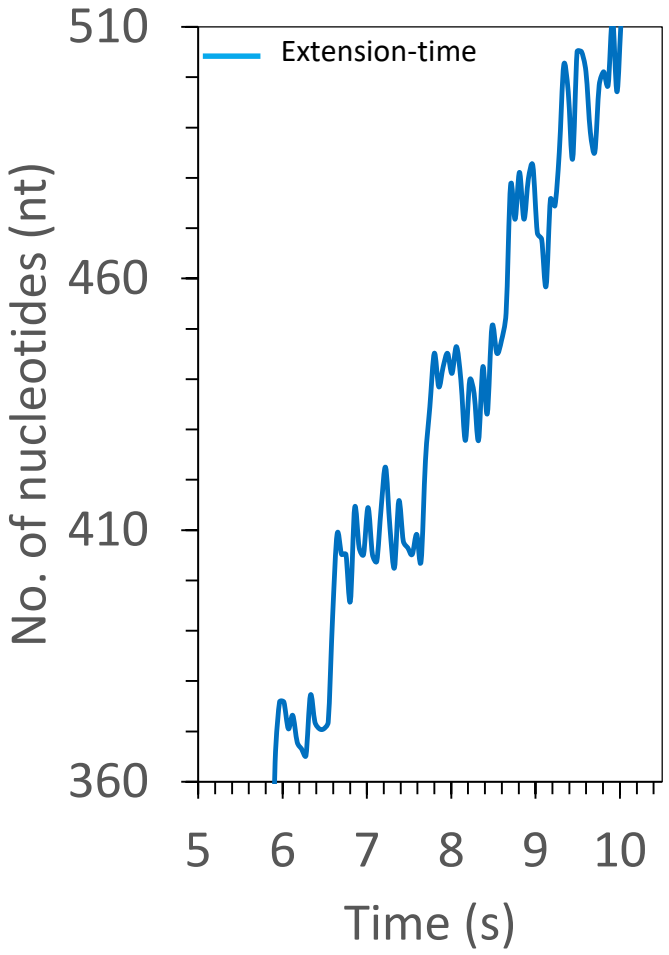




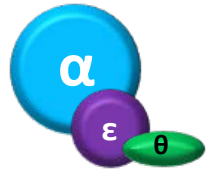


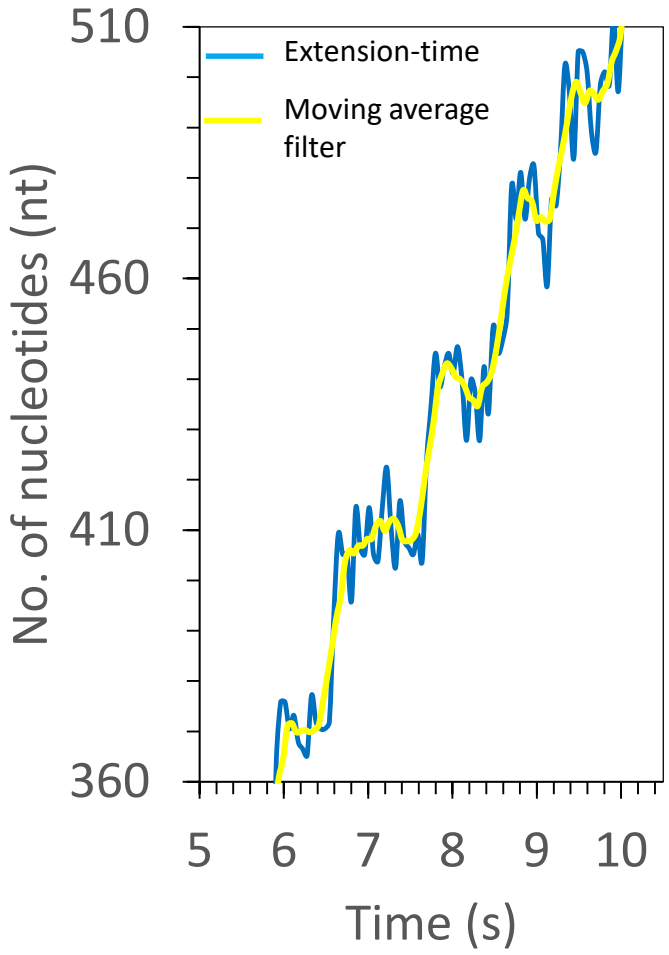
Pol III core activity at constant force

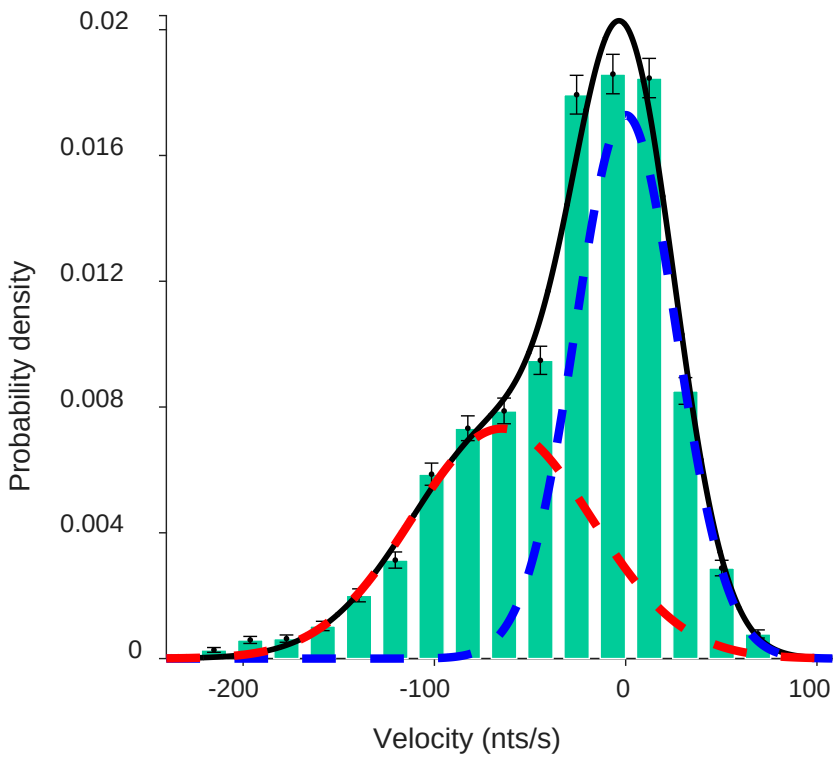




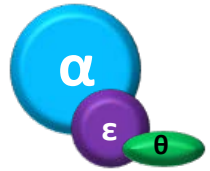
Pol III core activity at constant force

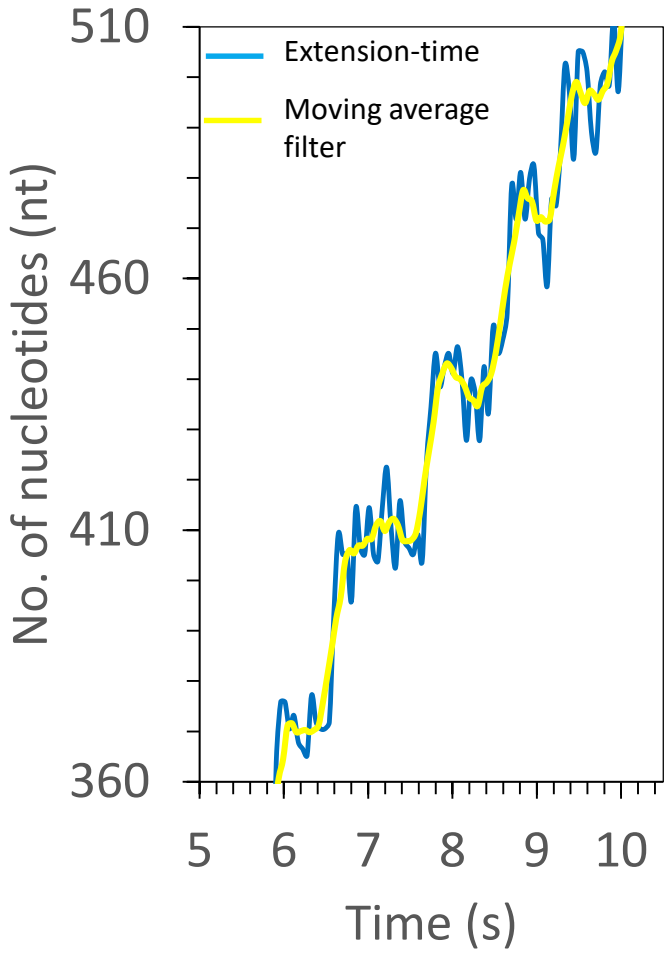




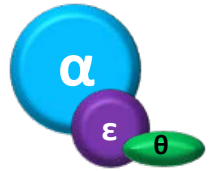


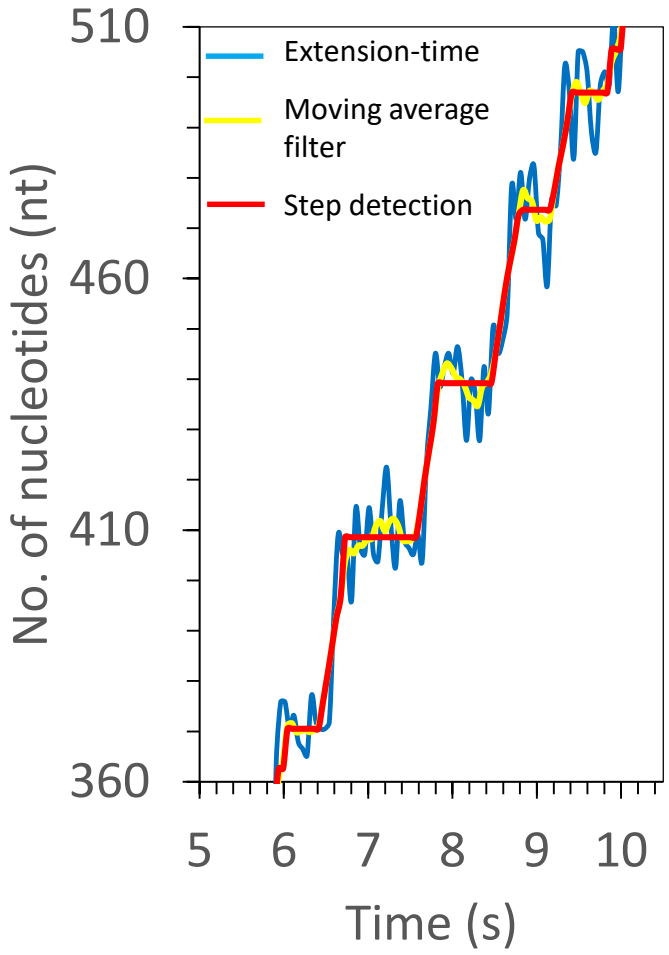
Pauses during exonucleolysis



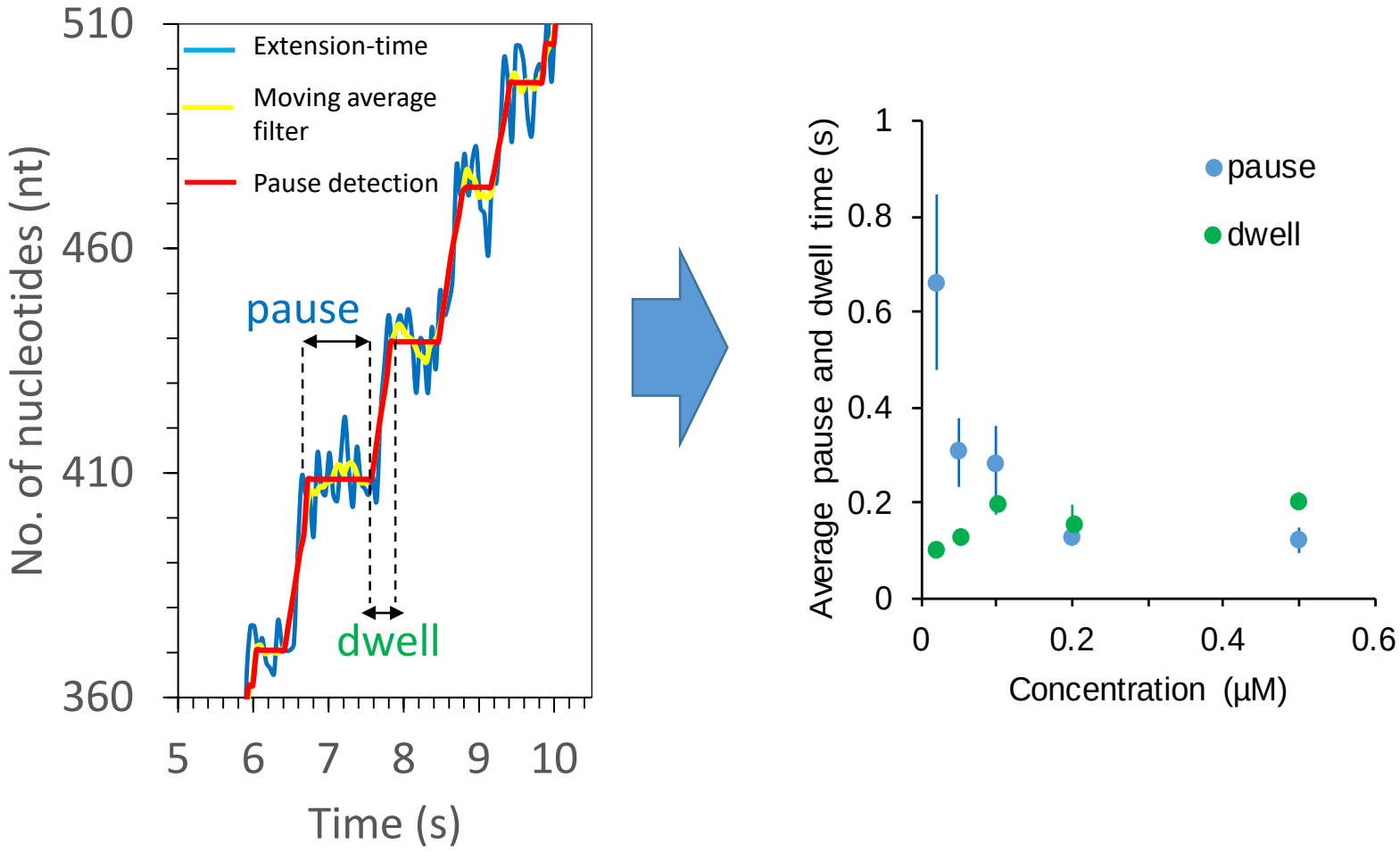


Pauses during exonucleolysis

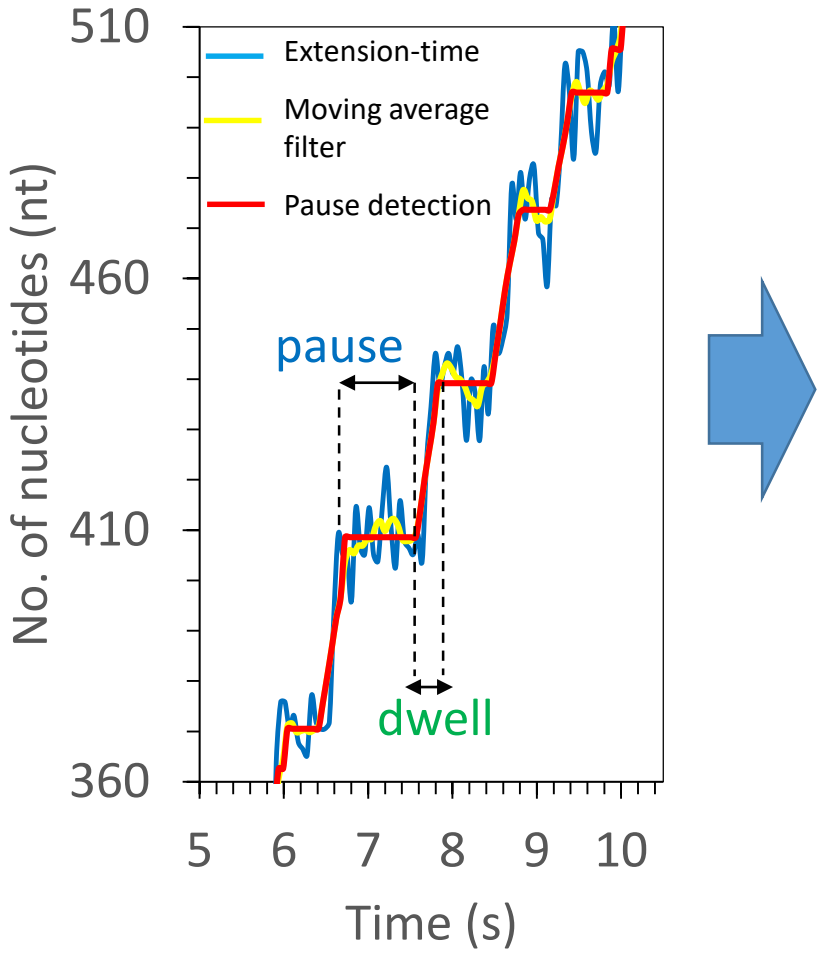




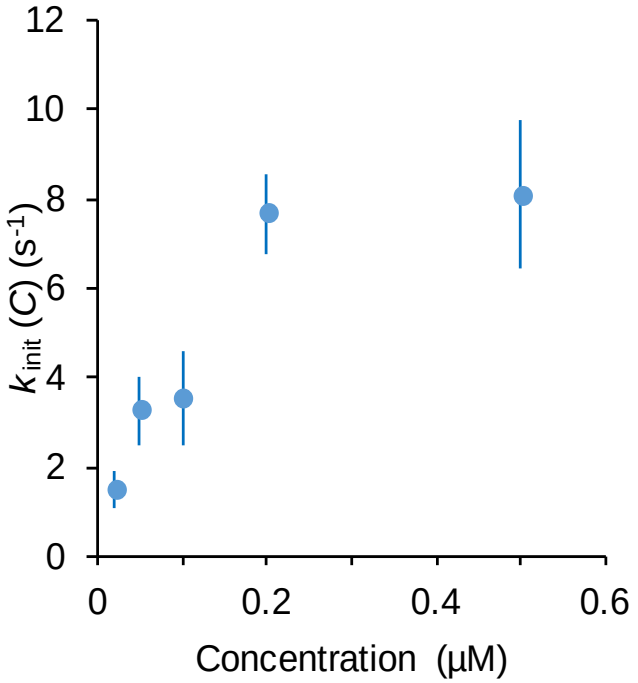
Pauses during exo depends on pol III concentration



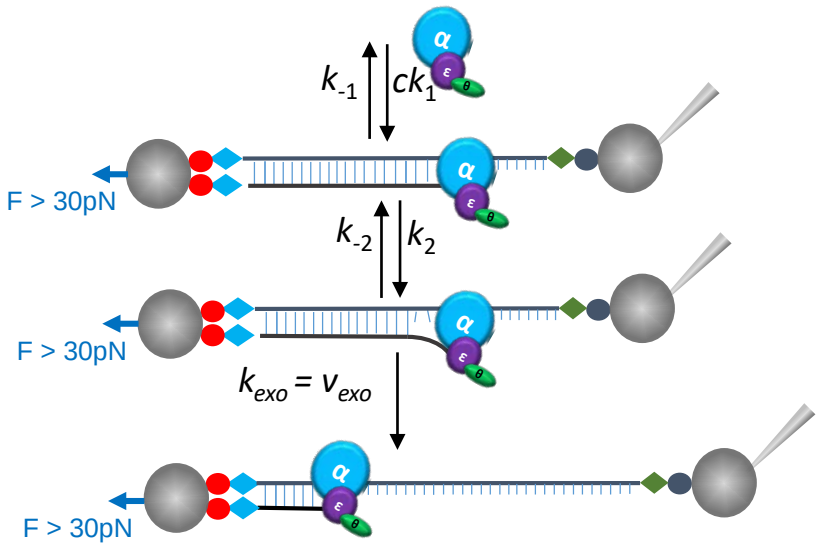
Exo-initiation rate depends on pol III core concentration



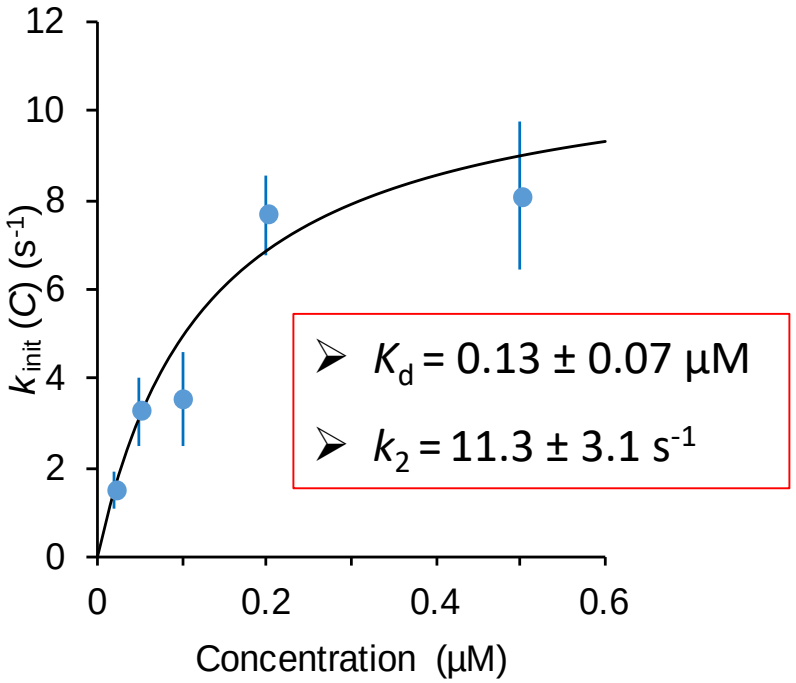
$$k_{init} = \frac{1}{<pause>}$$ → Rate of initiating an exo event



Two-step mechanism of exo-initiation



$$k_{init} = \frac{1}{<pause>} \rightarrow \text{Rate of initiating an exo event}$$

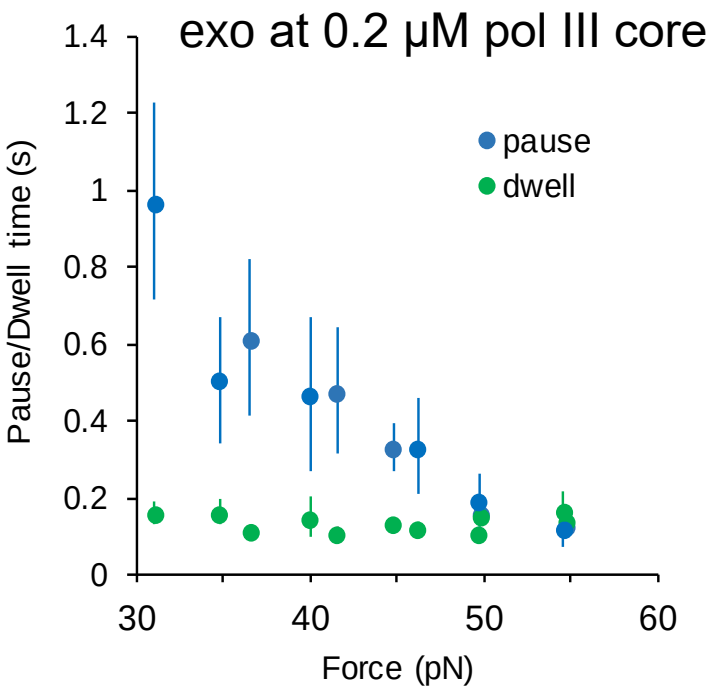


$$E + DNA_n \underset{k_{-1}}{\overset{k_1 C}{\rightleftarrows}} E \bullet DNA_n \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E \bullet DNA_n^* \xrightarrow{k_{exo}} E \bullet DNA_{n-1}^*$$

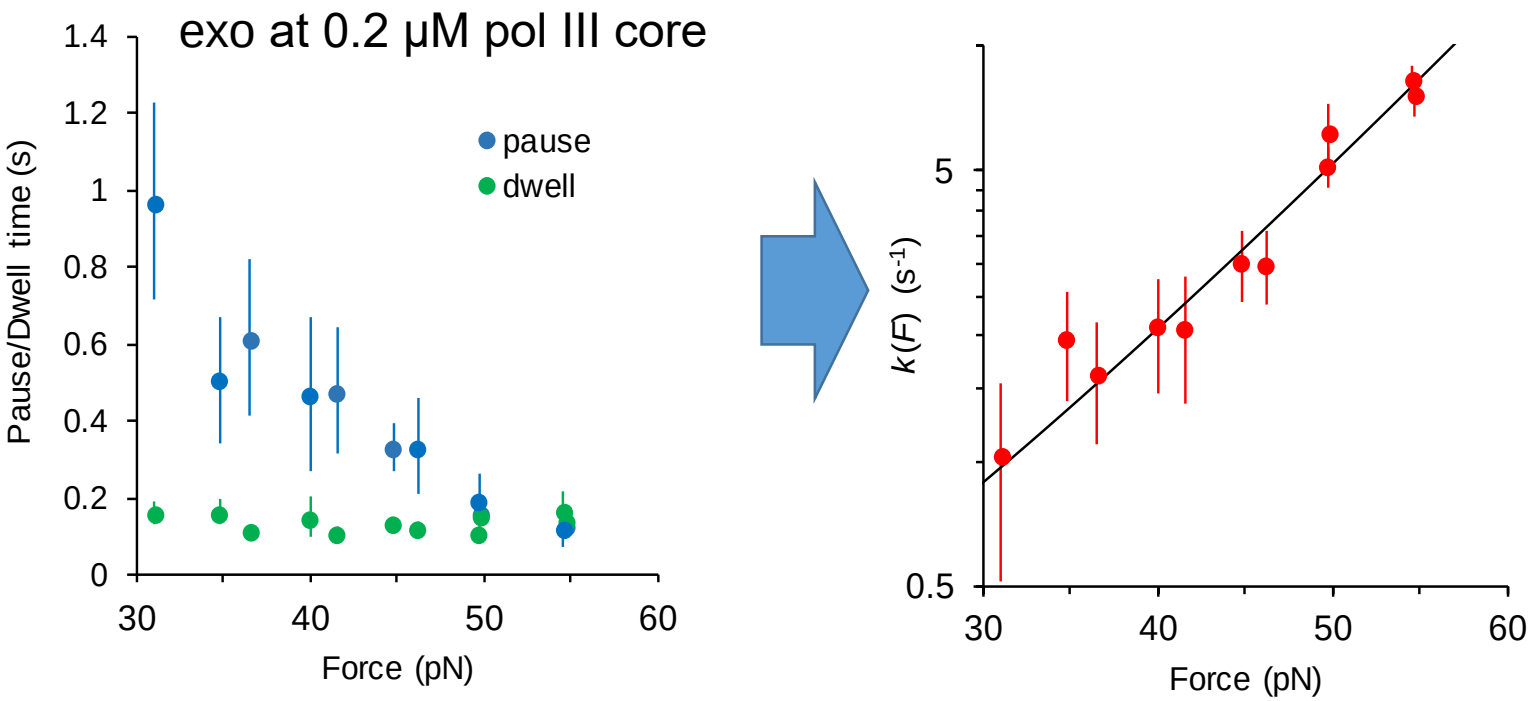
$$k_{init}(C) = \frac{k_1 C}{k_1 C + k_{-1}} k_2 = \frac{1}{1 + K_d / C} k_2$$

where, $K_d = k_{-1} / k_1$

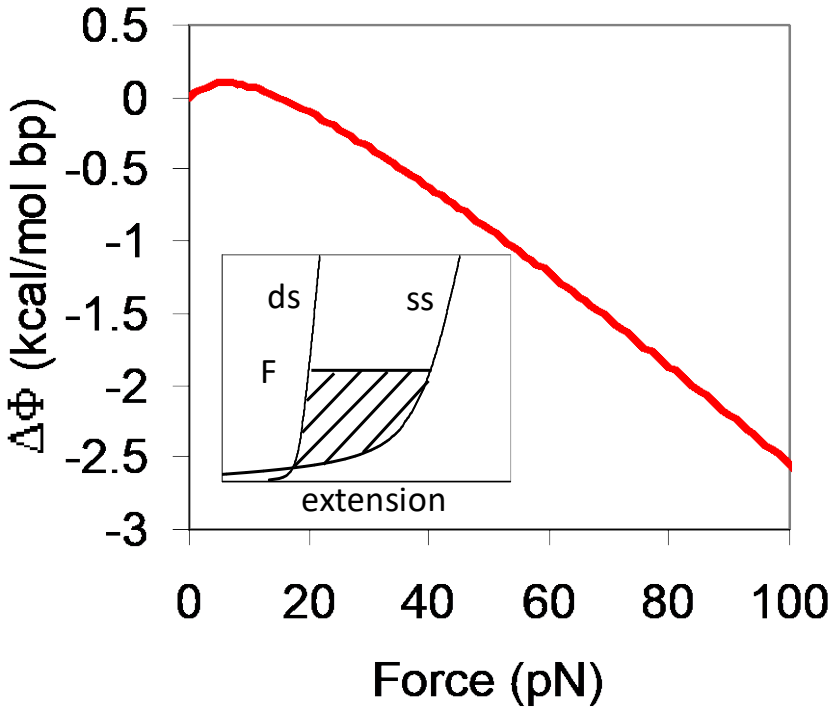
Pauses during exo depend on the template tension

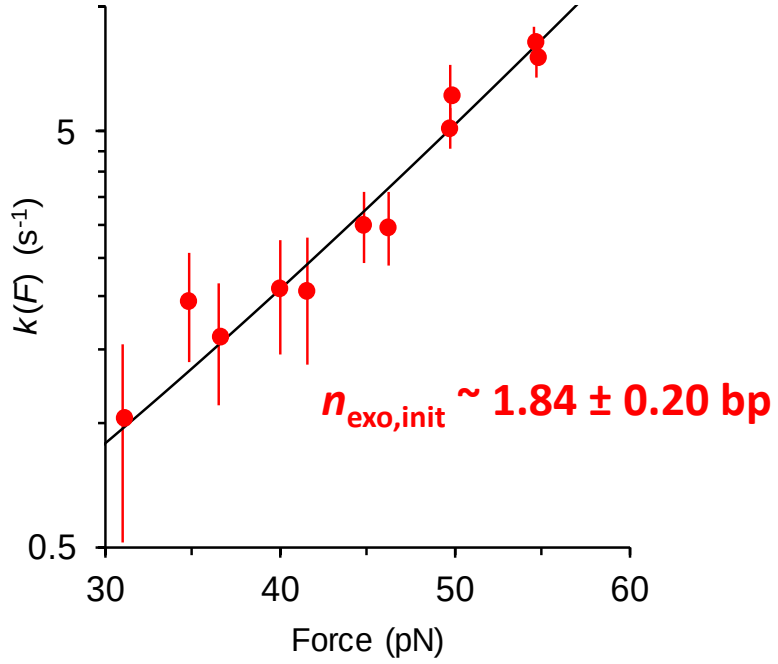


Pauses during exo depend on the template tension



Pauses during exo depends on the template tension





$$\Delta G(F) = \int_0^F x_{ss}\,dF - \int_0^F x_{ds}\,dF$$

$$k_{\text{init}}(F) = k_F e^{n_{\text{exo,init}}(\Delta G(F)-\Delta G_0)/k_B T}$$

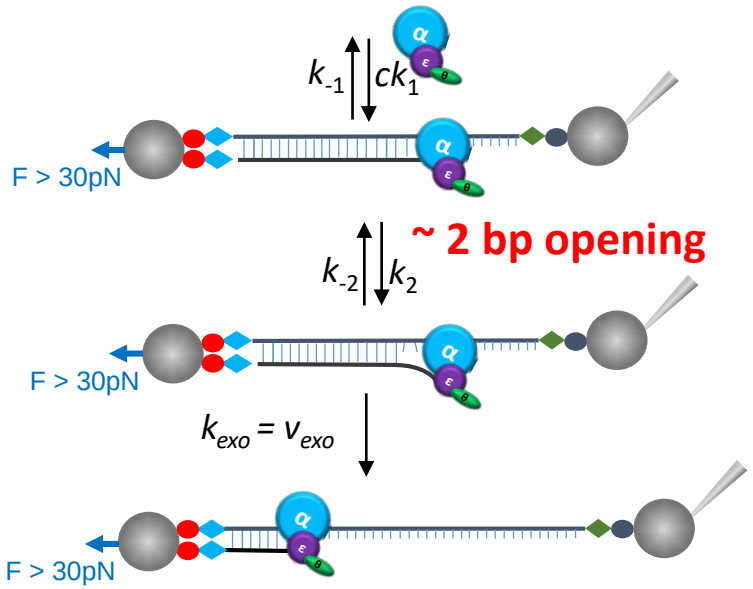
$$\Delta G_0 = \Delta G(F = 62\,\text{pN})$$

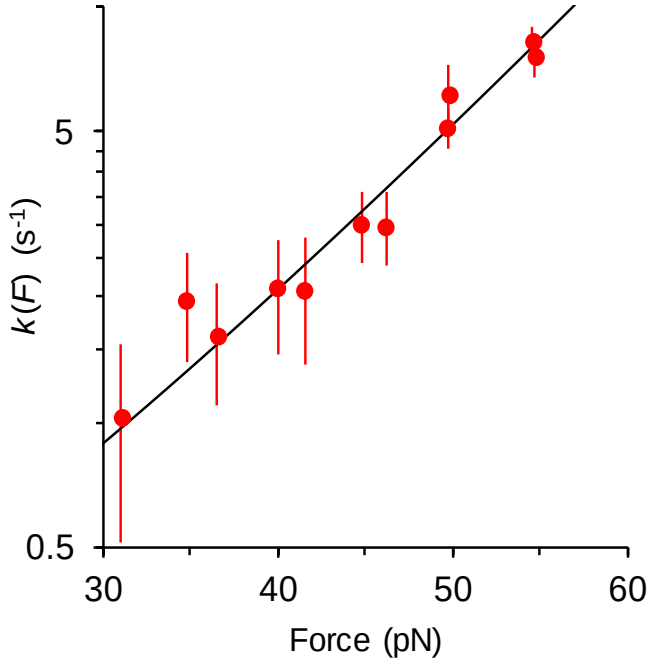
$\Delta G(F) - \Delta G_0$ is the free energy required to open one terminal bp

At least 2 bp are required to fluctuate open at the primer-template junction for exo-initiation

$$k_{\text{init}} = k_F e^{n_{\text{exo,init}}(\Delta G(F)-\Delta G_0)/k_B T}$$

$$k_{init}(\text{F}) = \frac{k_1 C}{k_1 C + k_{-1}} k_2 = \frac{1}{1 + K_d/C} k_2 \approx k_2 \quad for \quad C > K_d$$





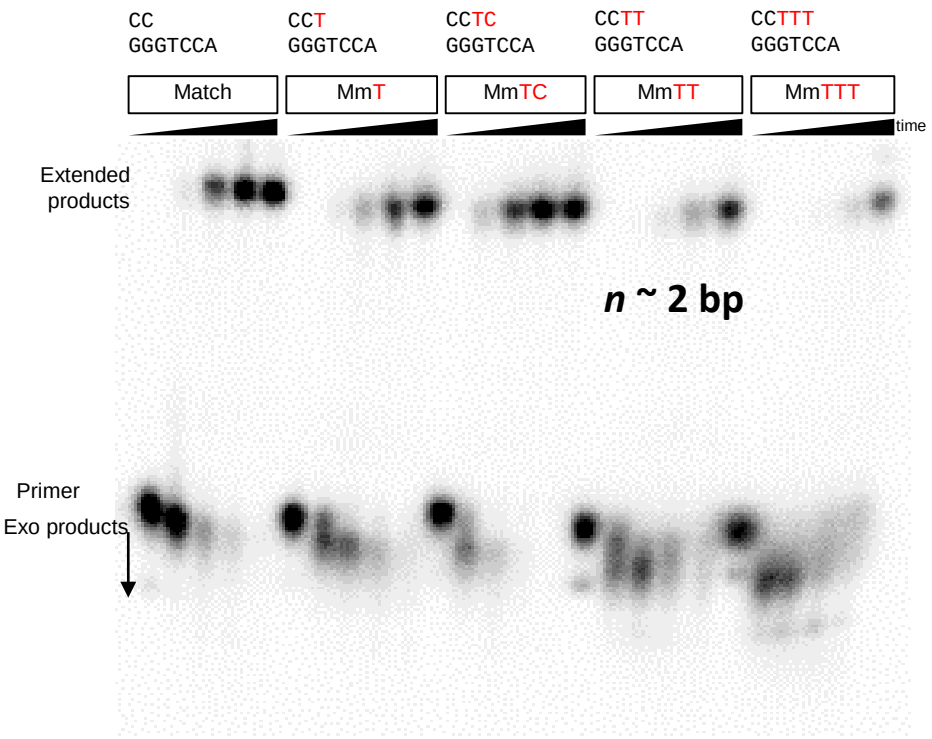
$$k_{\text{init}}(F) = k_F e^{n_{\text{exo,init}}(\Delta G(F)-\Delta G_0)/k_B T}$$

$\Delta G(F) - \Delta G_0$ is the free energy required to open one terminal bp

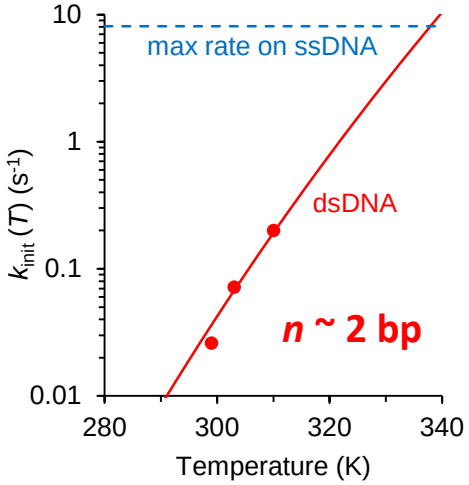
$$\Delta G(F) = \int_0^F x_{ss} dF - \int_0^F x_{ds} dF$$

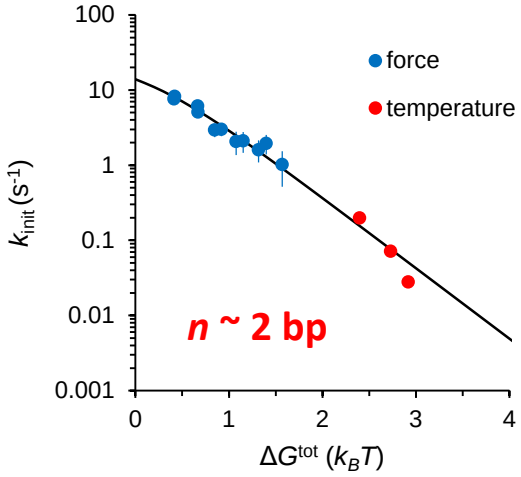
$$\Delta G_0 = \Delta G(F = 62\,\text{pN})$$

Mismatches and temp-dependence mimic destabilization by force



Brenowitz S et al.
(1991) J Biol Chem.





$$k_{init}(T) = k_T e^{-\frac{n_{exo,init}\Delta G^{\dagger}(T)}{k_B T}}$$

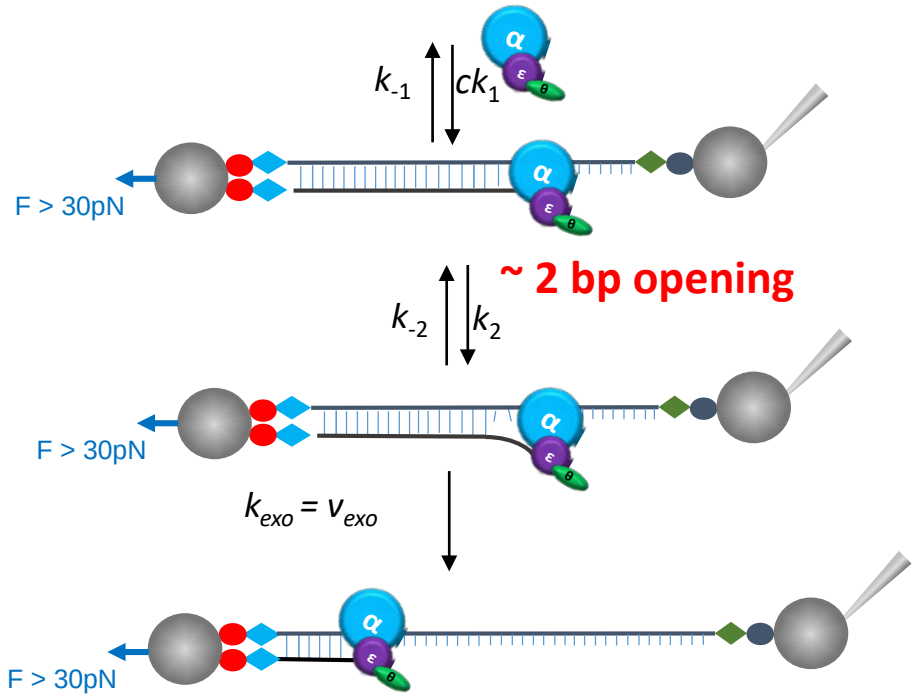
$$= k_T e^{-\frac{n_{exo,init}(\Delta H_0 - T\Delta S_0)}{k_B T}}$$

$$k_{init}(T) = k_0 e^{-\frac{n_{exo,init}\Delta G^{\dagger}(F,T)}{k_B T}}$$

Binding to an unstable primer is the primary mechanism for mismatch recognition during proofreading

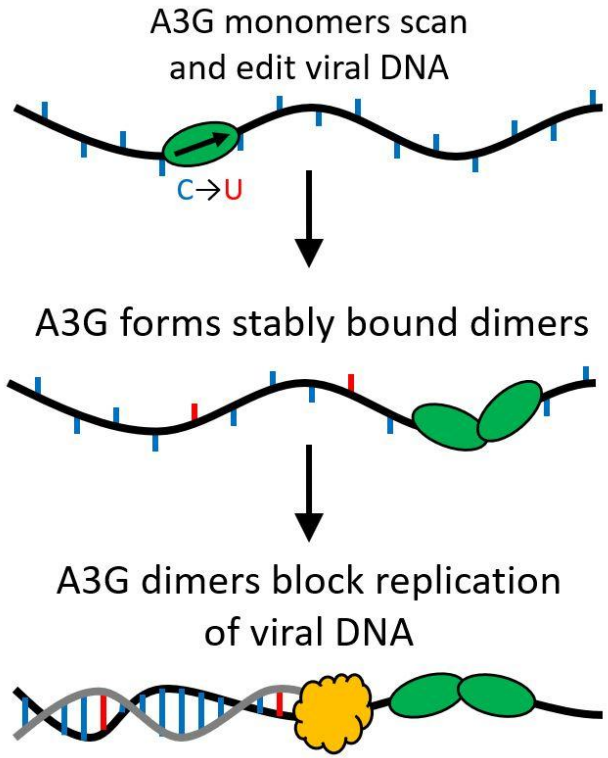
Summary: E. coli pol III exo and pol switching

- **Single molecule system** allows us to study exo and pol acitivity with high spatial and temporal resolution
- Observe force-induced switching between two separate exo and pol proteins as part of 3-protein complex
- Show that pol and exo activities are effectively independent
- Type of enzymatic activity determined by DNA substrate stability alone
- Only 2 bp must be destabilized to induce exo activity

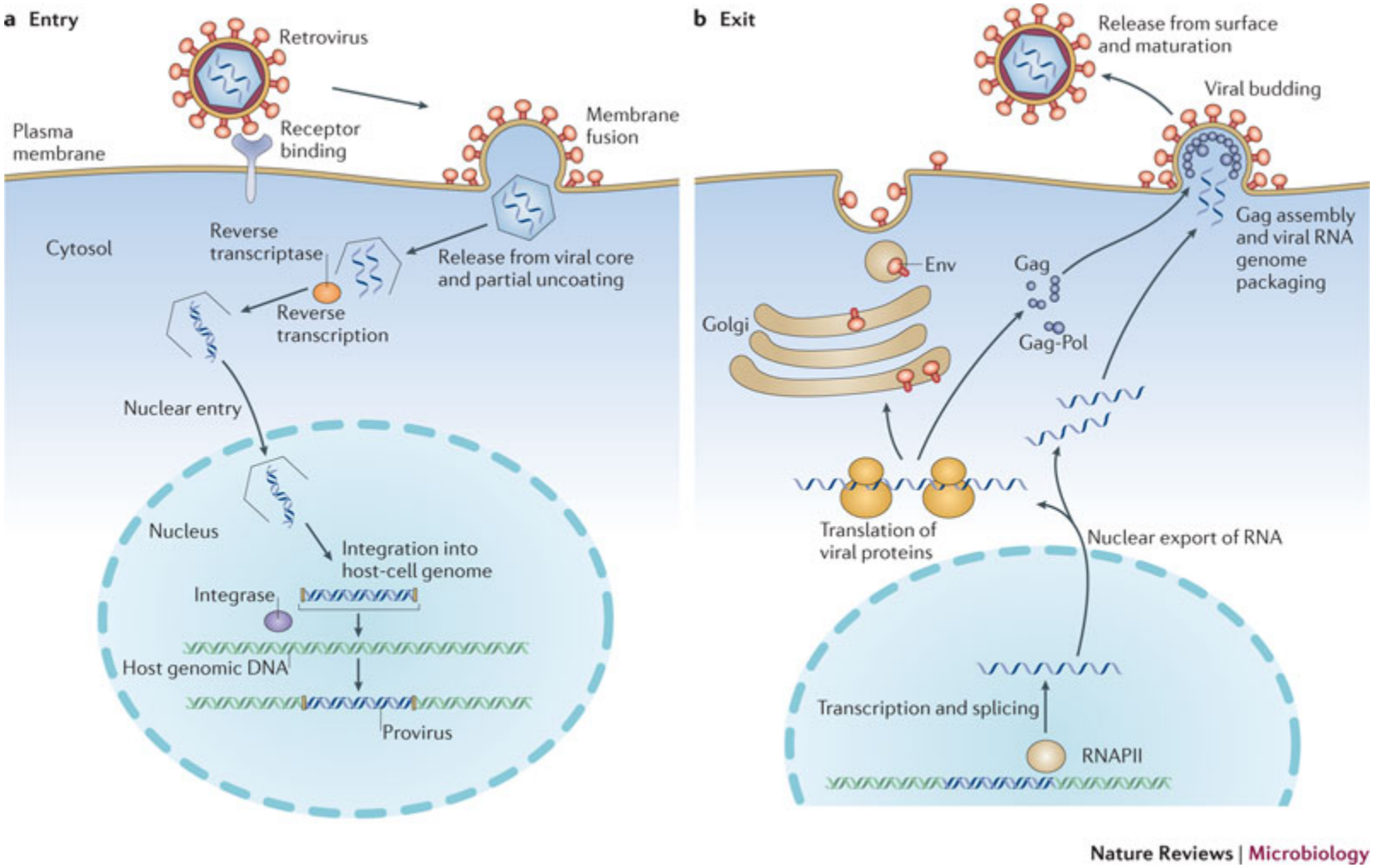


Summary

- Optical tweezers and DNA stretching
- E. coli pol III core switching between pol and exo activity
- DNA binding by human innate immune system protein APOBEC3G, which inhibits HIV-1 replication

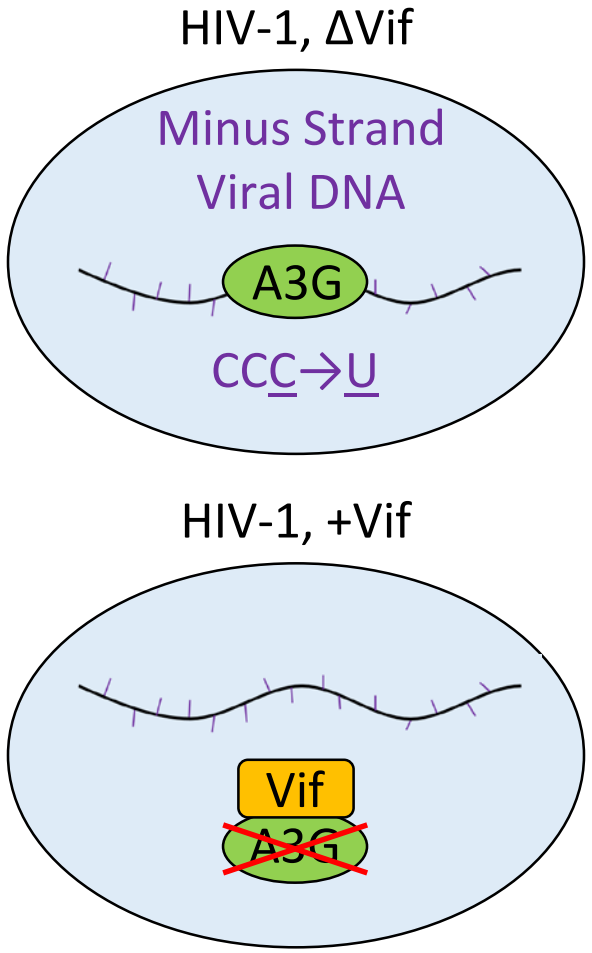


Life cycle of retroviruses



Jonathan P. Stoye, *Nature Reviews Microbiology* **10**, 395-406 (2012)

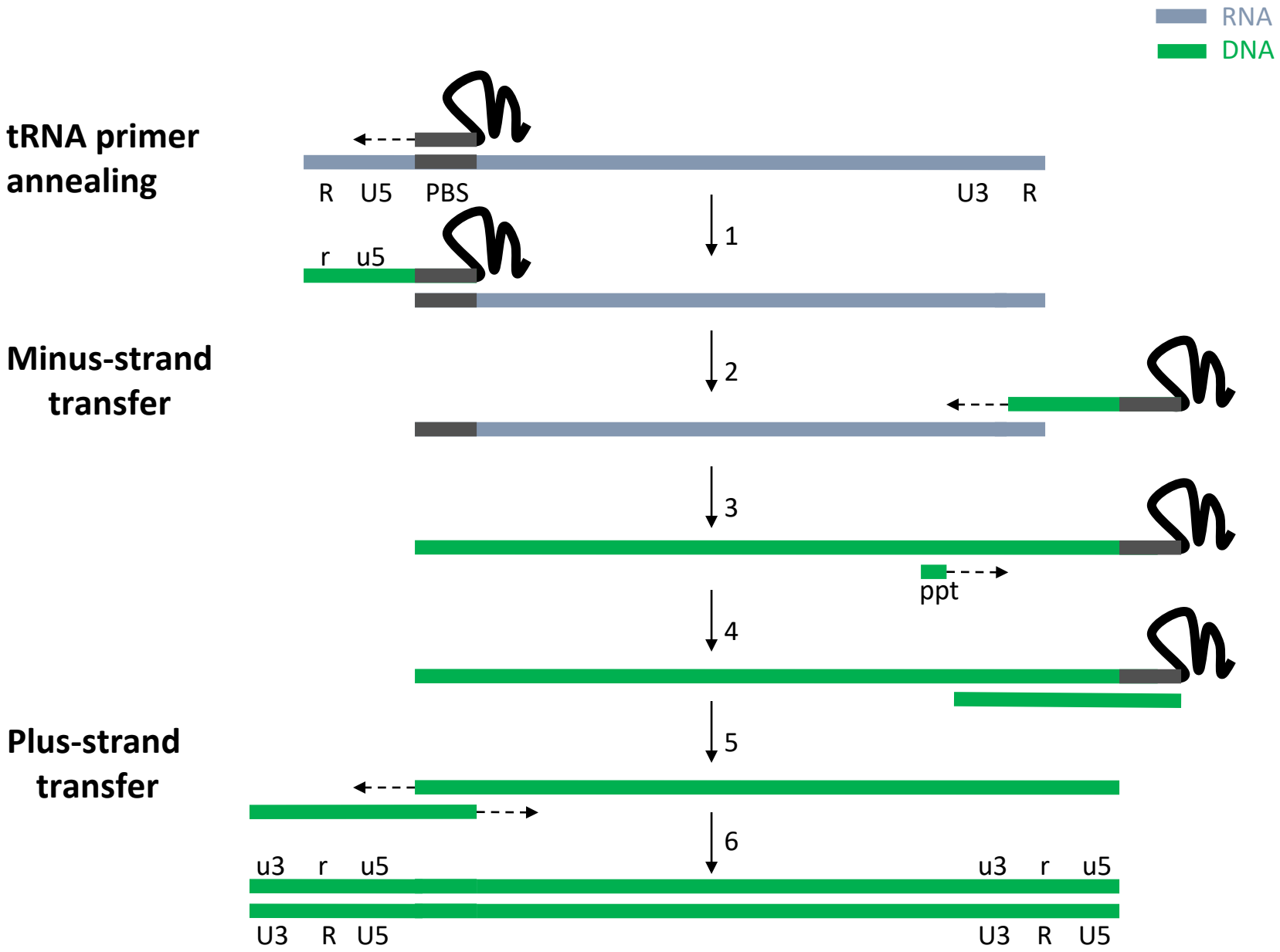
APOBEC3G (A3G), anti-viral enzyme



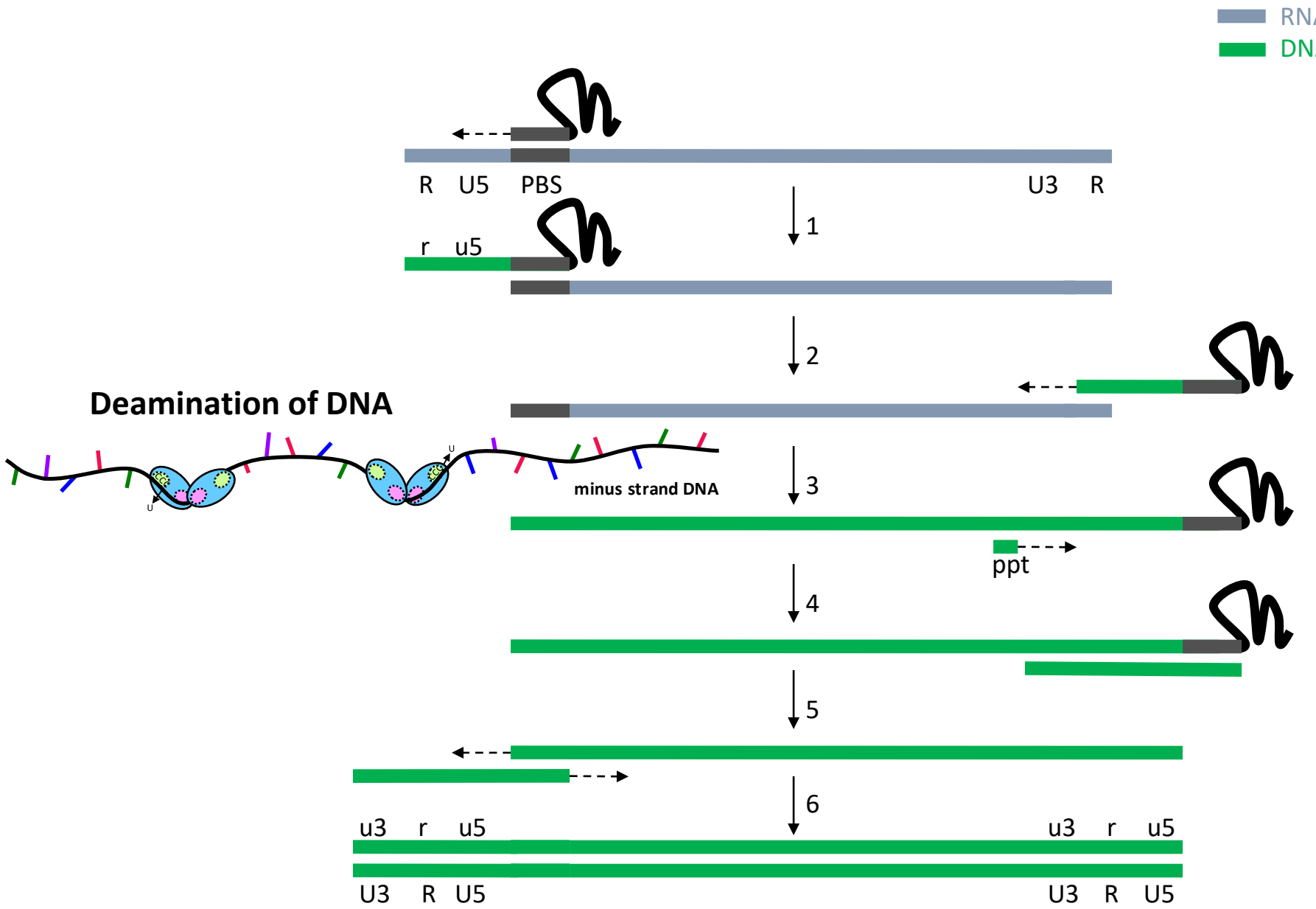
- Inhibits HIV-1 replication in the absence of HIV-1 Vif
- Targeted for degradation by HIV-1 Vif
- ssDNA deoxycytidine deaminase, creates C to U mutations on minus strand viral ssDNA
- Deaminase-deficient mutants still partially inhibit HIV-1 replication
- Disrupts reverse transcription in human T cells at endogenous levels of A3G*
- Forms dimers, tetramers, and higher order oligomers, especially when bound to RNA/ssDNA

*Gillick et al. Suppression of HIV-1 Infection by APOBEC3 Proteins in Primary Human CD4+T Cells Is Associated with Inhibition of Processive Reverse Transcription as Well as Excessive Cytidine Deamination. J. Virol. 87: 1508-1517 (2013).

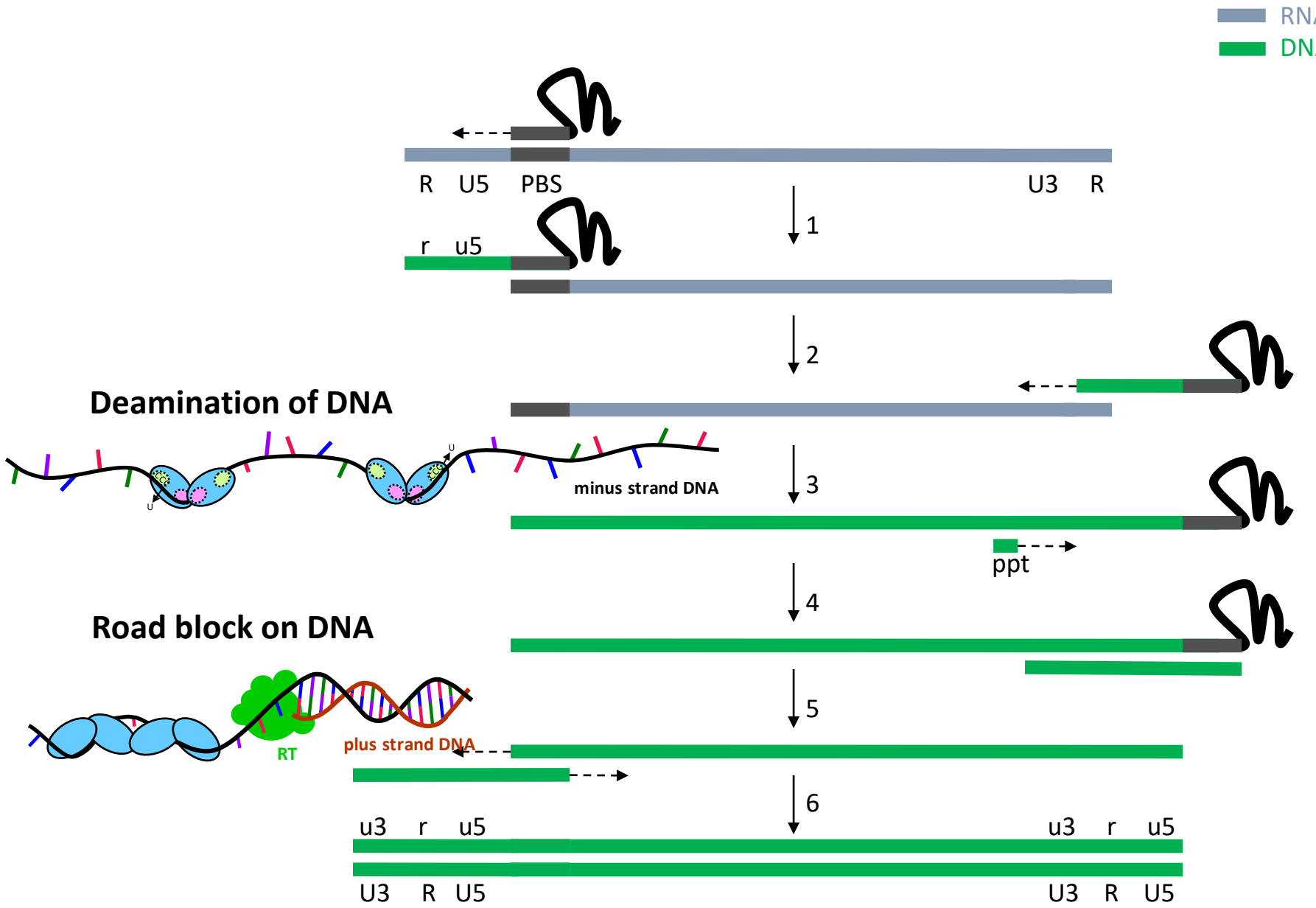
Steps in Retroviral Reverse Transcription



A3G inhibits reverse transcription in two ways

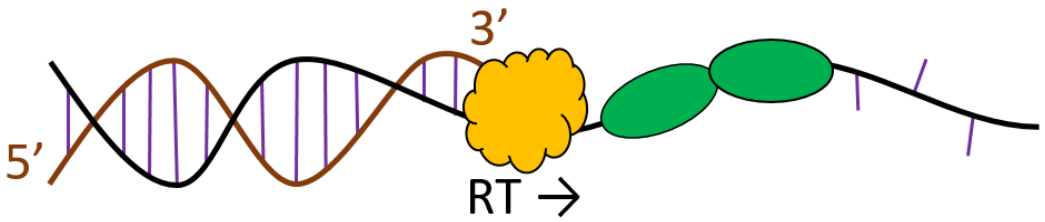


A3G inhibits reverse transcription in two ways



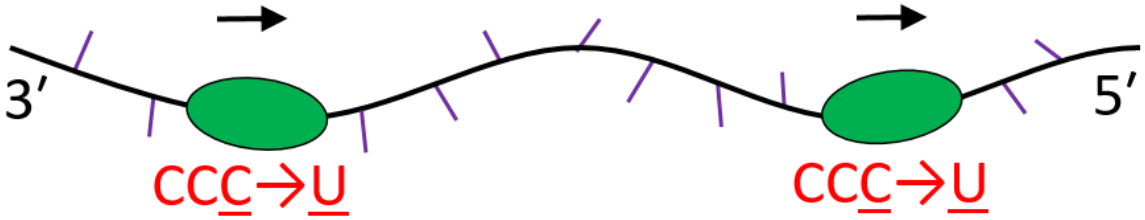
Roadblock model for deaminase-independent HIV-1 inhibition mechanism

- Model*: bound A3G physically blocks and interrupts the activity of reverse transcriptase



- Requires slow binding kinetics

- However: a small number of A3G subunits must rapidly deaminate approximately 1,000 sites within minutes



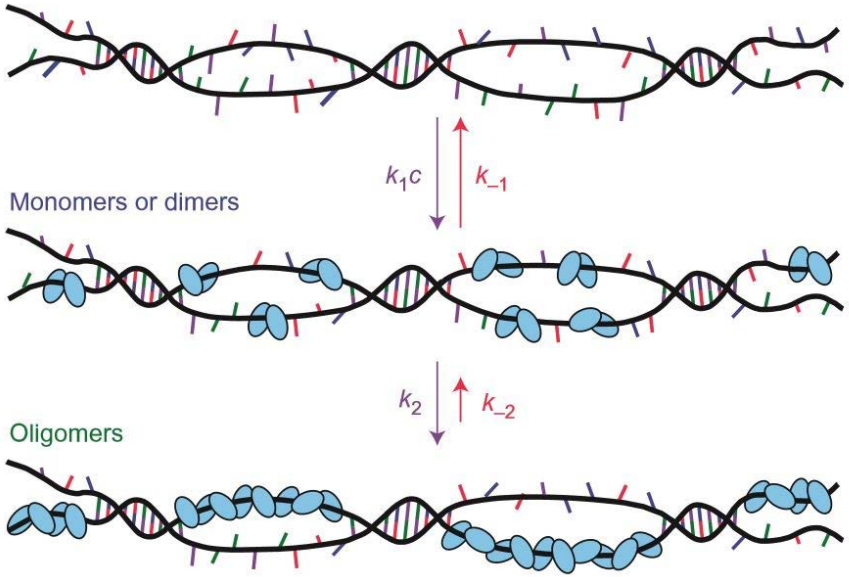
- Requires fast binding kinetics

To what extent does A3G have fast and slow binding kinetics?

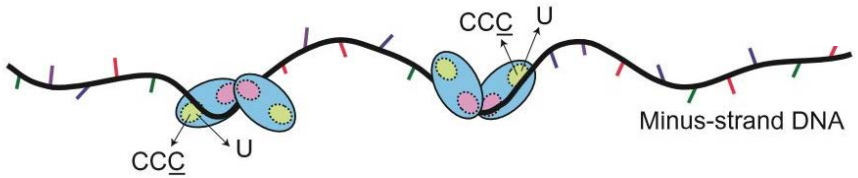
*Iwatani et al. Deaminase-independent inhibition of HIV-1 reverse transcription by APOBEC3G. Nucleic Acids Research 35: 7096-7108 (2007)

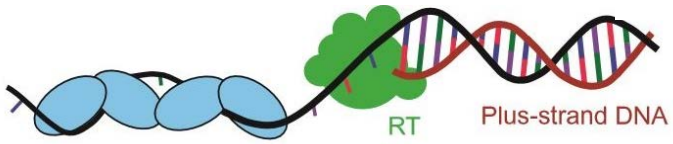
Two step binding model, two modes of HIV-1 inhibition

In vitro



In vivo





- Monomers/dimers rapidly slide along and deaminate minus strand DNA
- Oligomers remain stably bound and act as a roadblock for RT activity

$$A3G + ssDNA \underset{k_{-1}}{\overset{ck_1}{\rightleftarrows}} (A3G + ssDNA)_{fast} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} (A3G + ssDNA)_{slow}$$

Chaurasiya et al. Oligomerization transforms human APOBEC3G from an efficient enzyme to a slowly dissociating nucleic acid-binding protein. Nature Chemistry (2014)

Mechanisms of A3G oligomerization and its effects on A3G-nucleic acid interactions

- To what extent are previously observed slow binding kinetics due to oligomerization?
- What degree of oligomerization (dimer/tetramer etc.) is required to slow A3G sliding and dissociation?
- How does oligomerization alter enzyme activity?

- To understand oligomerization, examine mutants that lack some oligomerization capability

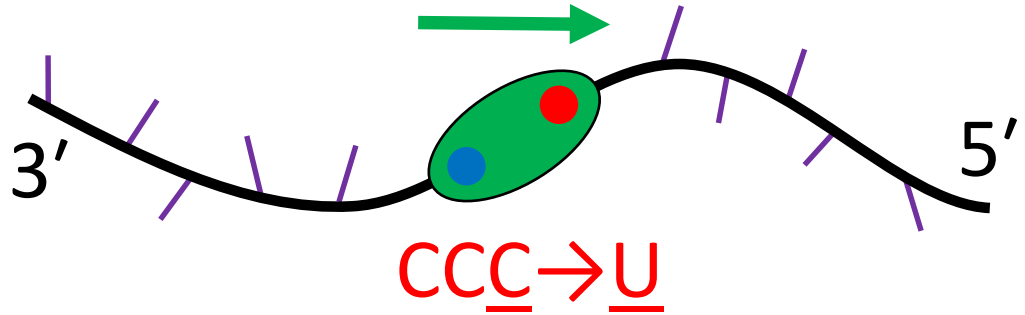
A3G has two potential cytidine deaminase domains

N-Terminal Domain (NTD)

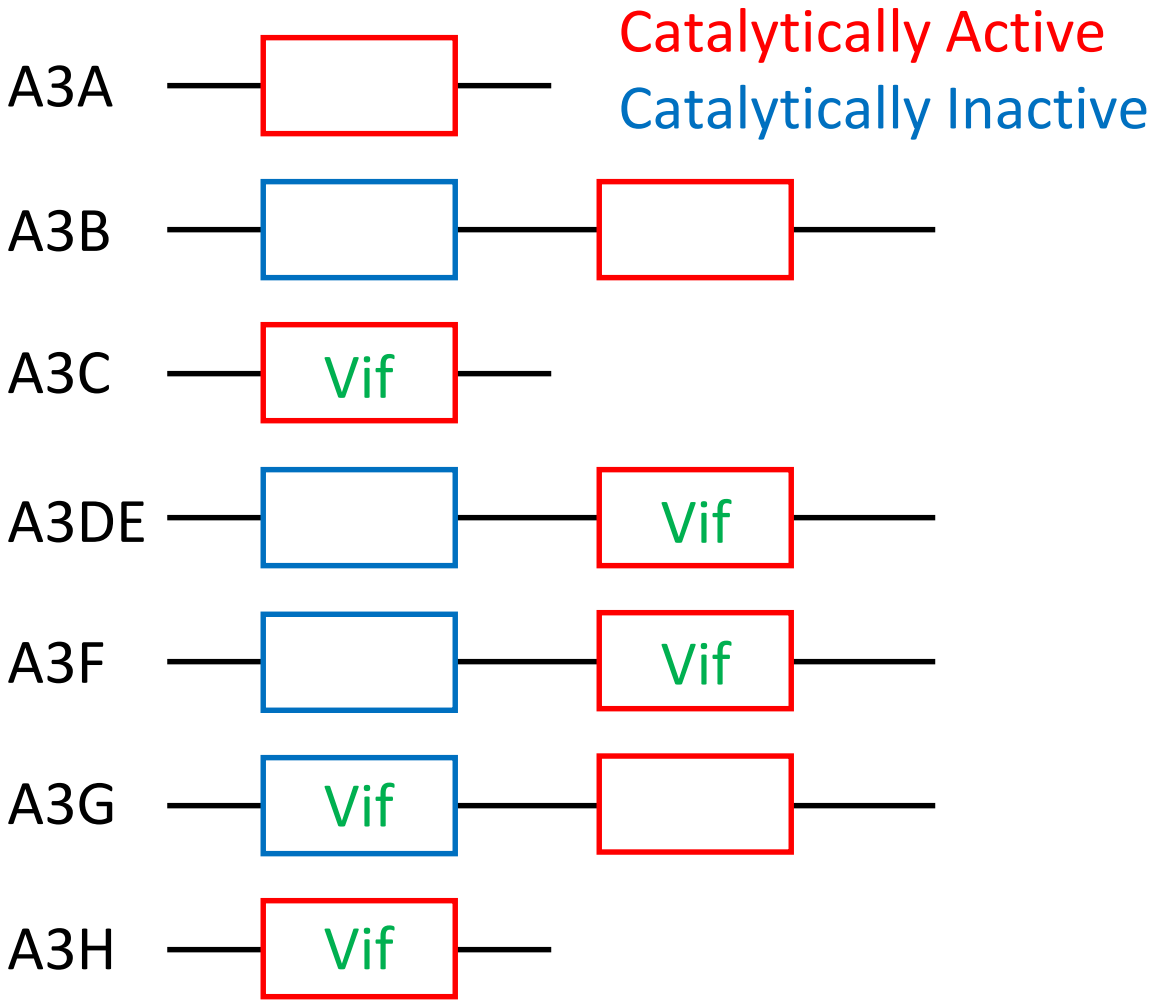
- Catalytically inactive
- Binds RNA/ssDNA
- Required for packaging in virion
- Primary dimerization interface
- Interacts with Vif

C-Terminal Domain (CTD)

- Catalytically active
- Weakly binds ssDNA
- Additional weak dimerization interface



Common structure and function among APOBEC3 family proteins



- Many A3 proteins have anti-viral functionality
- Each A3 has one or two CD domains, but only one is catalytically active
- HIV-1 Vif binds certain CD domains to restore infectivity
- Results with A3G could impact understanding of other multi-CD domain A3 proteins

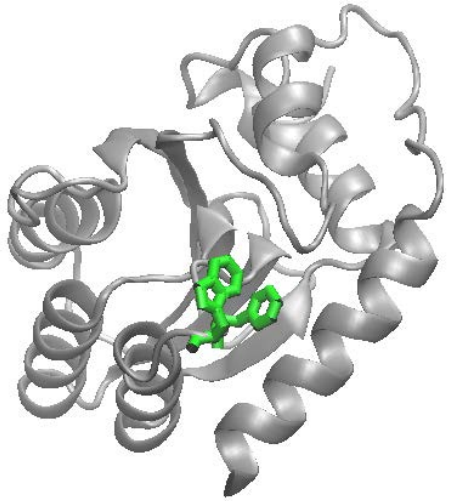
Dimerization can occur between both the N- and C-terminal domains of A3G

- Dimerization interfaces can be disrupted by targeted mutations
- Dimerization interfaces originally determined based on the structure of the homologous A2 tetramer

Chelico et al. Structural Model for Deoxycytidine Deamination Mechanisms of the HIV-1 Inactivation Enzyme APOBEC3G. JBC (2010)

NTD mutant

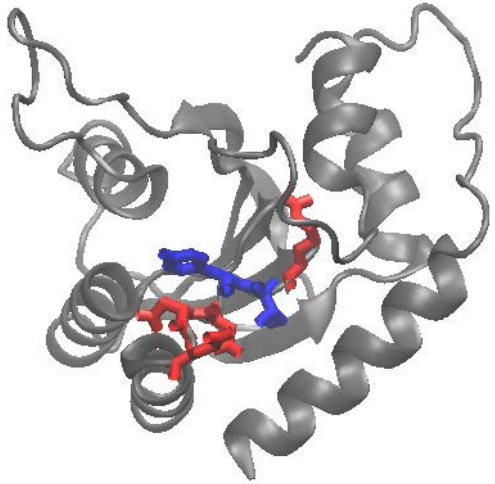
F126A/W127A (**FW**, green)
FW has deaminase activity



Structure from Xiao et al. Crystal structures of APOBEC3G N-domain alone and its complex with DNA. Nature Communications (2016)

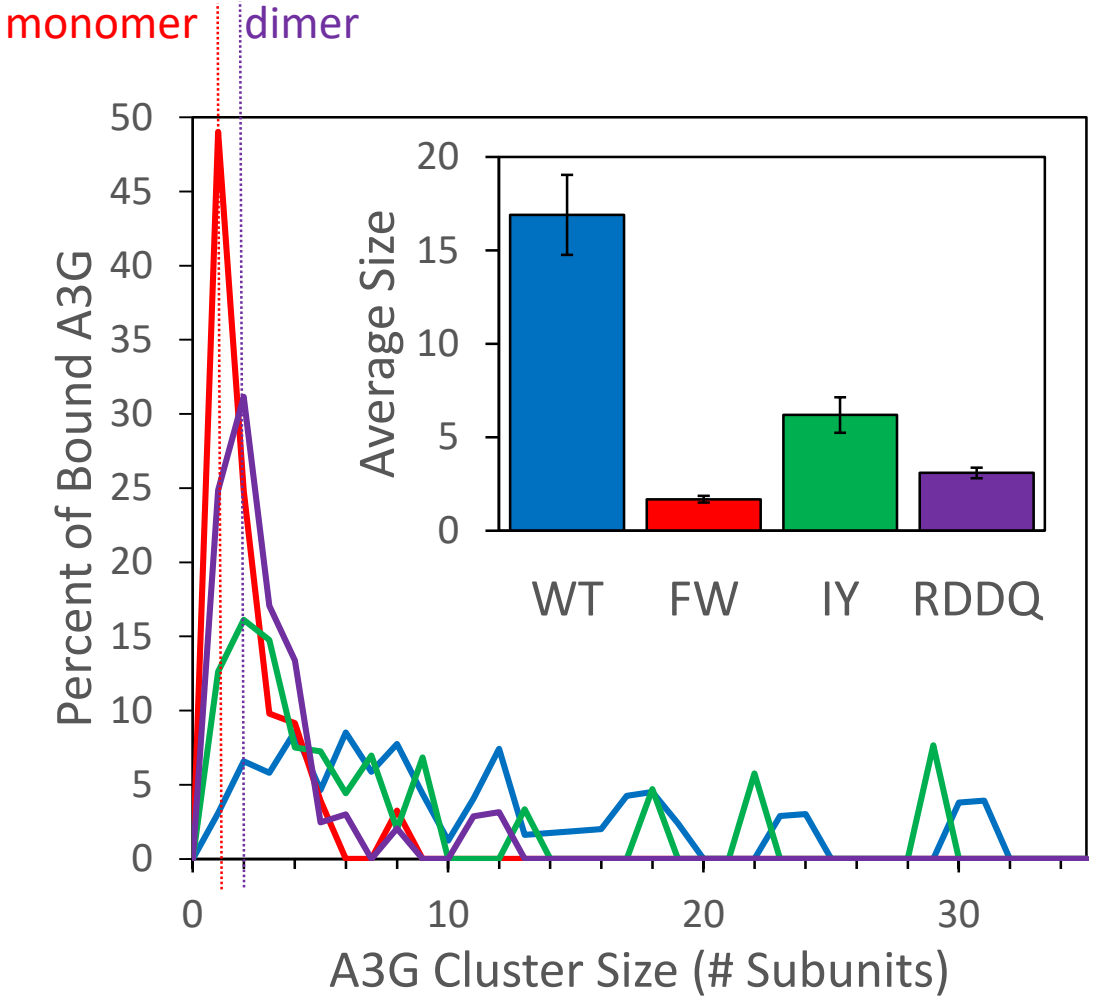
CTD mutants

I314A/Y315A (**IY**, blue) and R313A/D316A/D317A/Q318A (**RDDQ**, red)

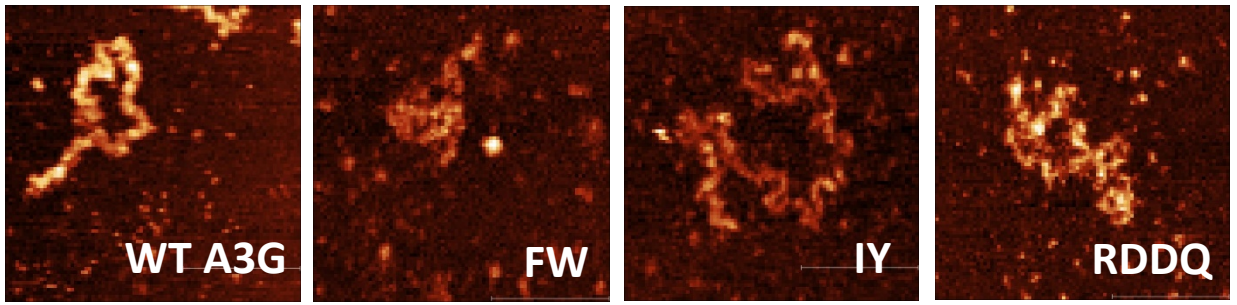


Structure from Holden et al. Crystal structure of the anti-viral APOBEC3G catalytic domain and functional implications. Nature (2008)

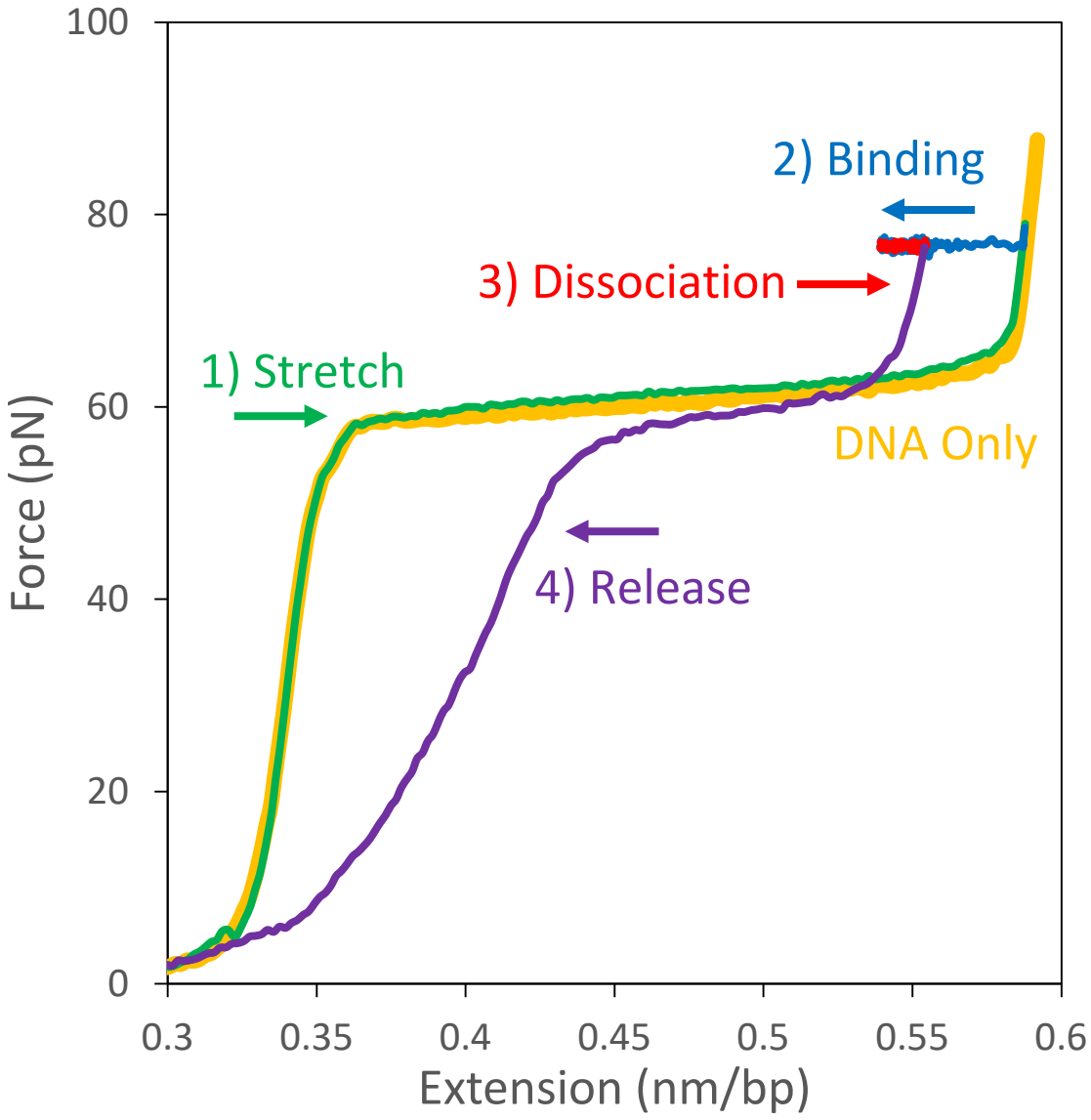
AFM imaging shows NTD and CTD mutations inhibit oligomerization



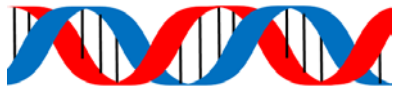
- 70 nM A3G bound to linearized m13 ssDNA
- Most WT A3G forms very large oligomers of varying sizes
- NTD mutant (FW) is mostly monomeric, with some dimers/tetramers
- CTD mutants (IY and RDDQ) are mostly dimeric with some larger oligomers



Experimental procedure

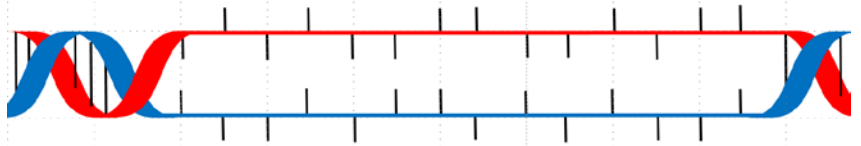


1) dsDNA is captured by optical trap



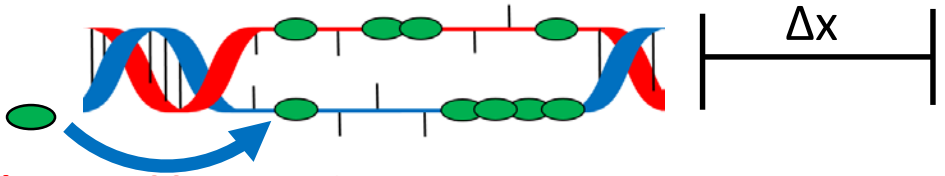
F = 0 pN

2) DNA is stretched to a force of 80 pN



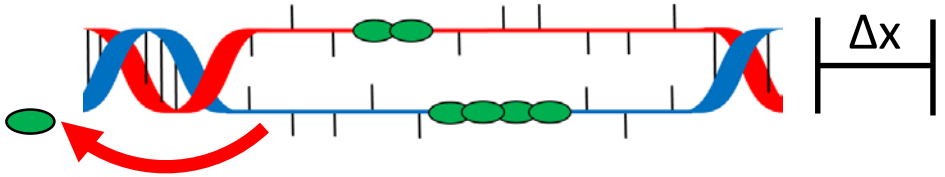
F = 80 pN

3) Protein solution: DNA length decreases as A3G binds ssDNA and forms oligomers



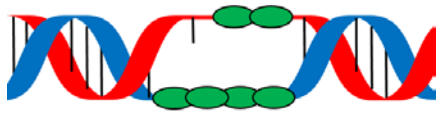
F = 80 pN

4) Buffer solution: Some A3G dissociates



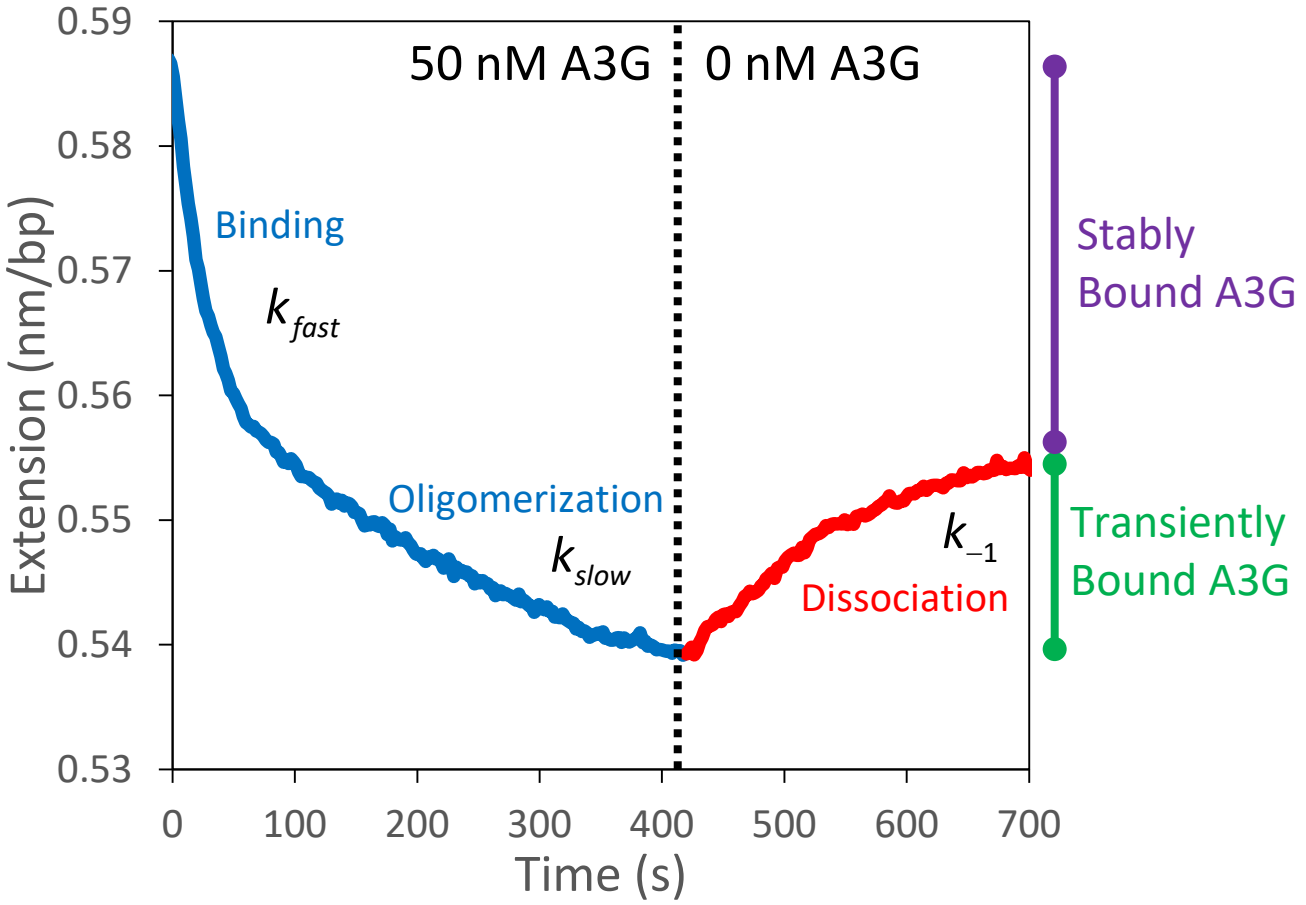
F = 80 pN

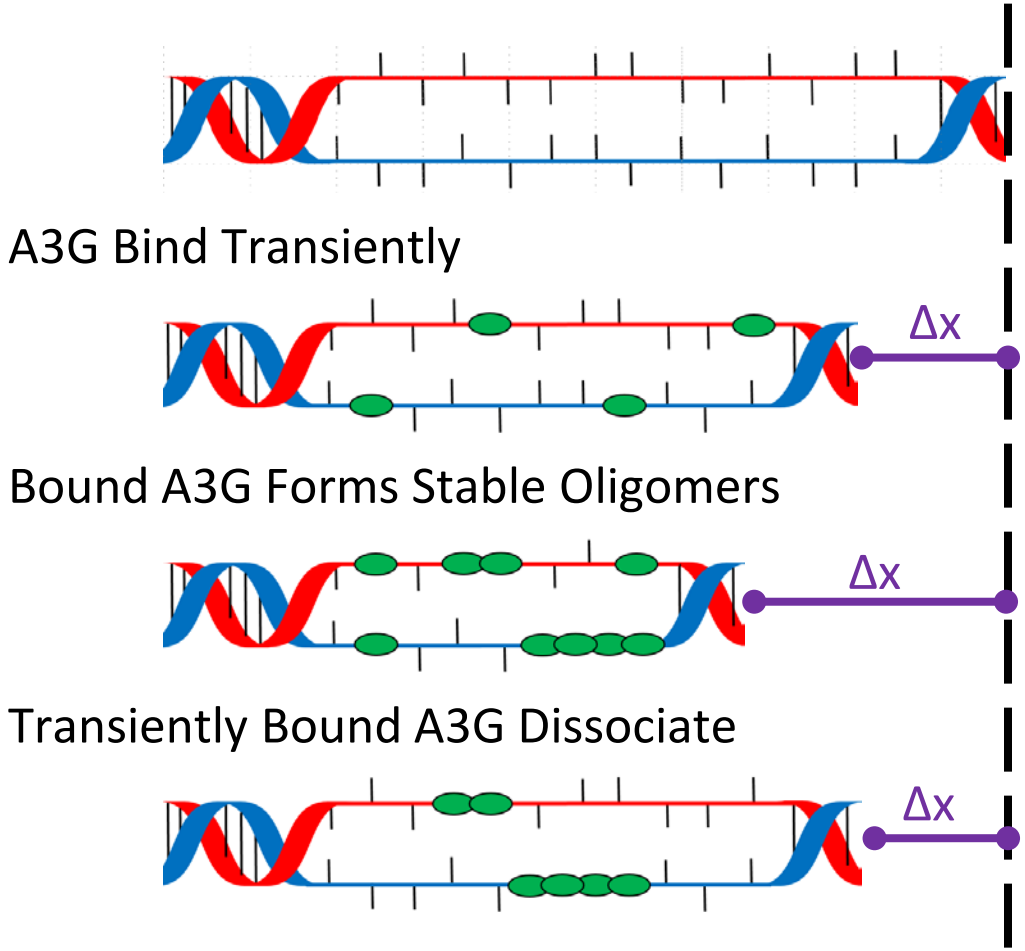
5) DNA is released back to zero force



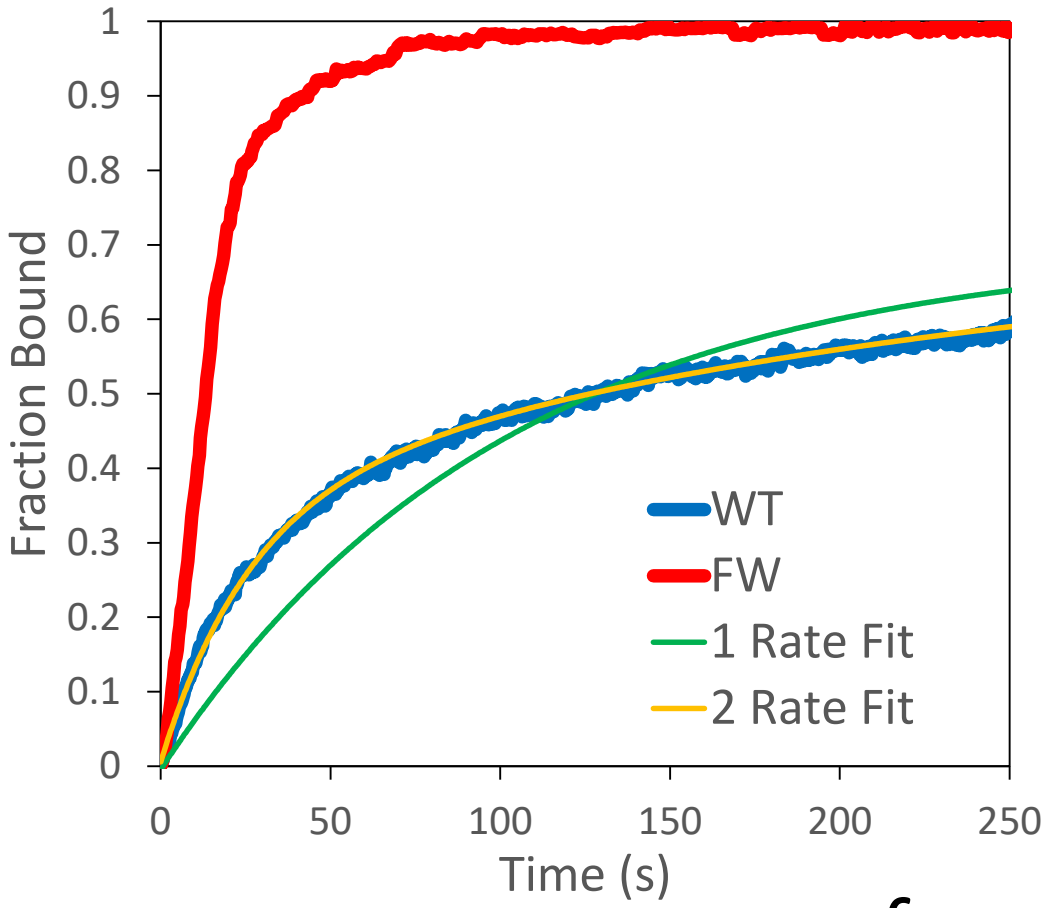
F = 0 pN

Real time measurements as A3G binds ssDNA, forms oligomers, and partially dissociates





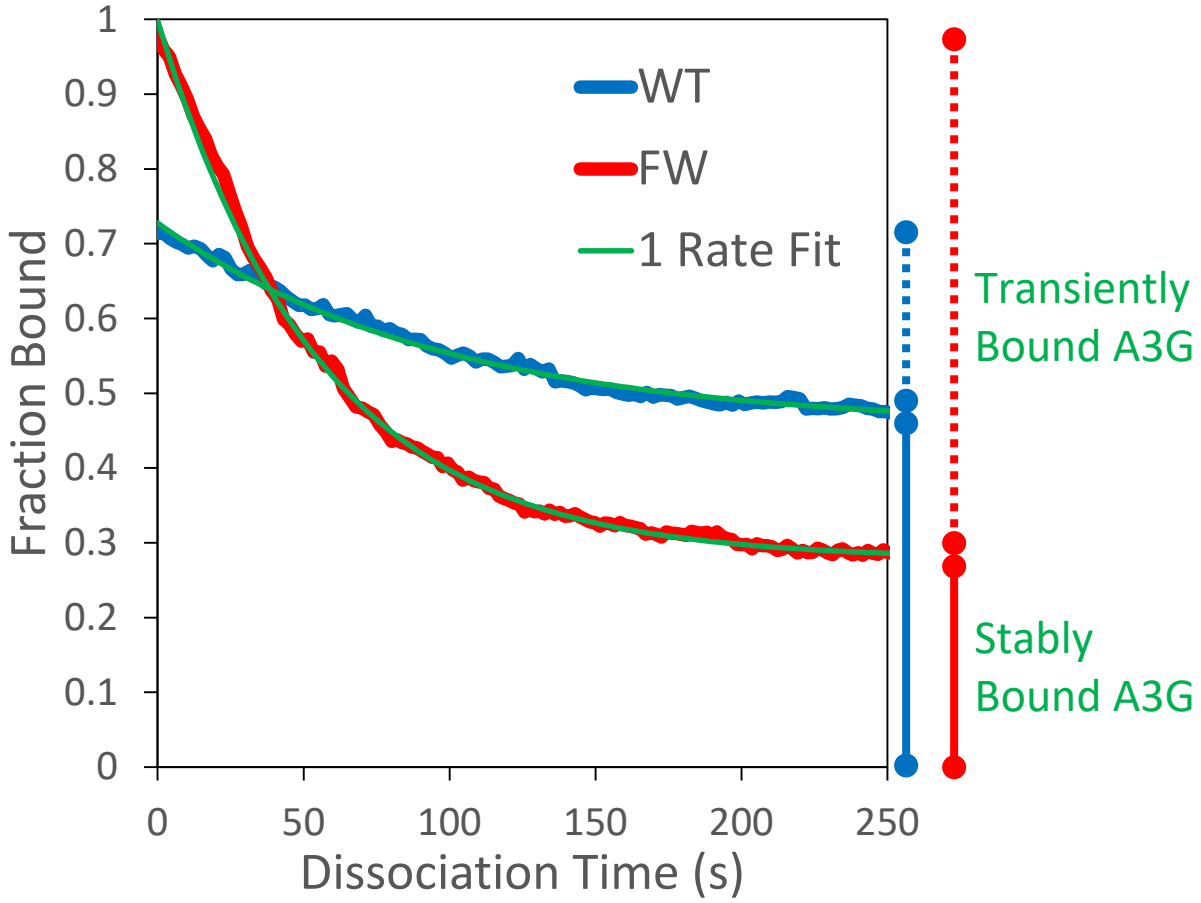
A3G binds then forms oligomers on two different timescales



- A3G binding curve must be fit with two rates
- Fast rate is free A3G binding from buffer
- Slow rate is bound A3G forming oligomers
- WT binding does not reach full saturated binding as compared to the FW mutant

$$f_{\text{incubation}}(t) = A_{fast}(1 - e^{-k_{\text{fast}}t}) + A_{slow}(1 - e^{-k_{\text{slow}}t})$$

Transiently bound A3G dissociates, oligomers remain bound



- Dissociation measured after rinsing all A3G out of the flow cell
- Some A3G remains bound even after dissociation
- Dissociation occurs at a single rate
- The initial and final extension quantify how much A3G is transiently or stably bound
- Less A3G forms stable oligomers for NTD mutant (FW)

Oligomerization as a two-step chemical reaction

$$A3G + ssDNA \underset{k_{-1}}{\overset{k_1 c}{\rightleftharpoons}} (A3G \bullet ssDNA)_{\text{fast}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (A3G \bullet ssDNA)_{\text{slow}}$$

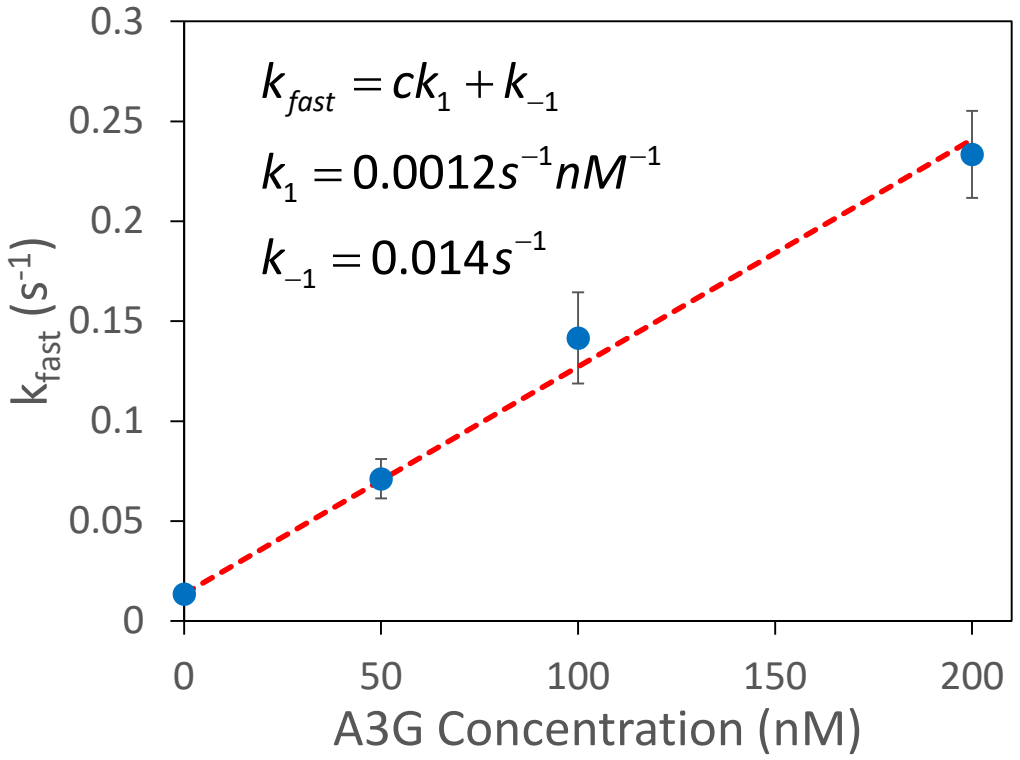
Fraction bound as a function of time

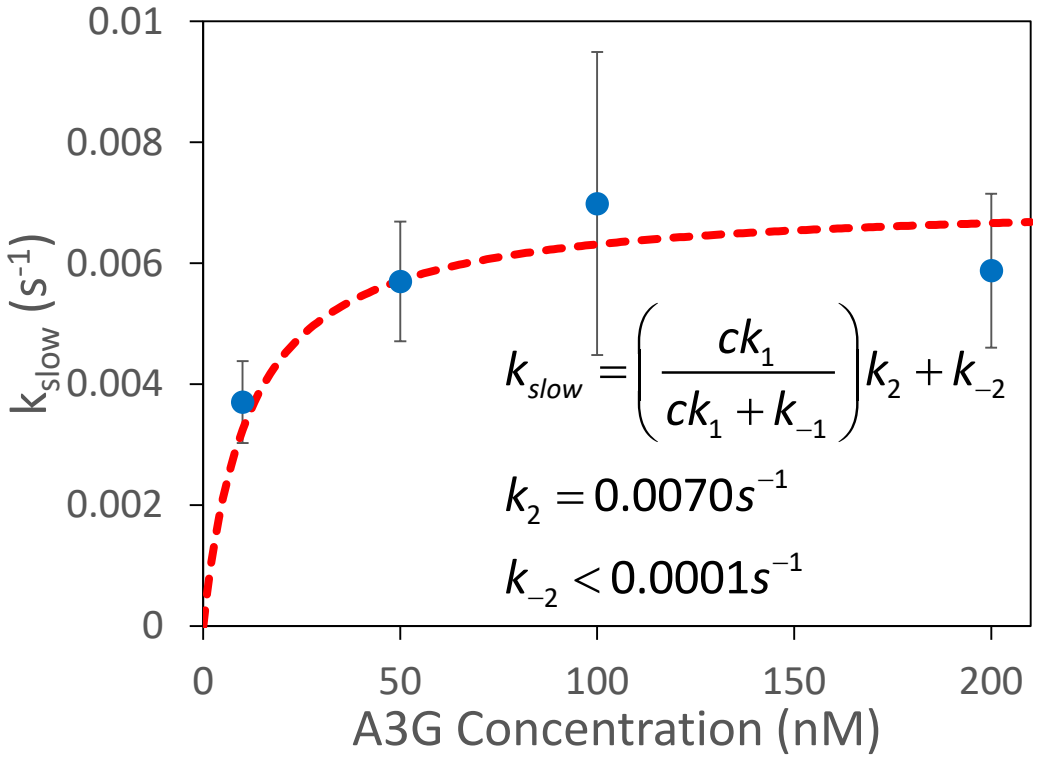
$$f_{\text{incubation}}(t) = A_{fast}(1 - e^{-\boldsymbol{k}_{\text{fast}} t}) + A_{slow}(1 - e^{-\boldsymbol{k}_{\text{slow}} t})$$

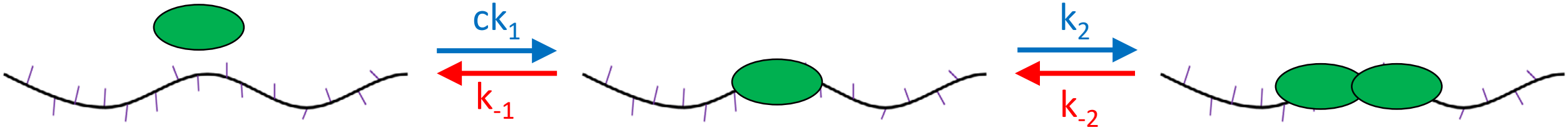
$$f_{dissociation}(t) = A_{total} - A_{dis}(1 - e^{-\boldsymbol{k}_{-1} t})$$

$$\boldsymbol{k}_{\text{fast}} = k_1 c + k_{-1} \qquad \boldsymbol{k}_{\text{slow}} = k_2 \left(\frac{k_1 c}{k_1 c + k_{-1}} \right) + k_{-2}$$

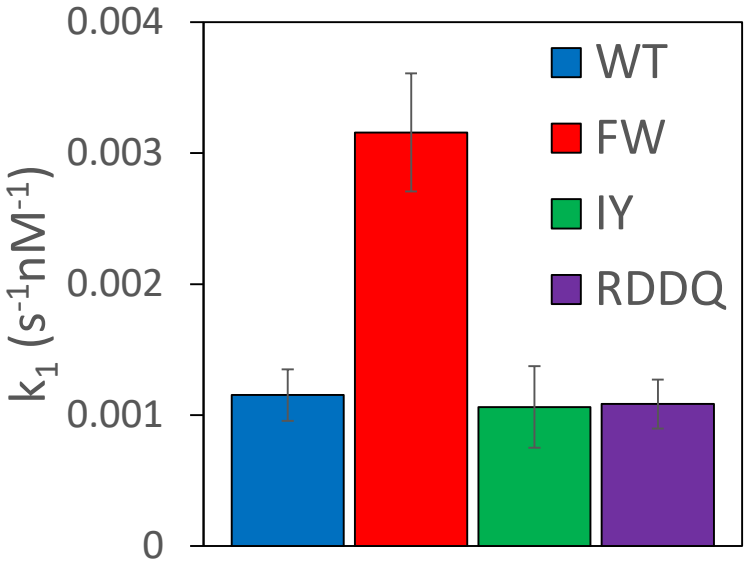
Binding is concentration-dependent
Oligomerization saturates at high concentration



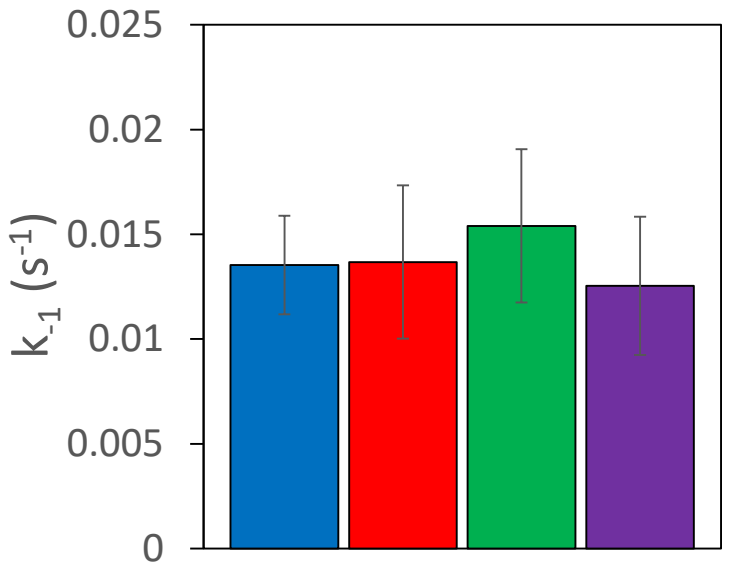




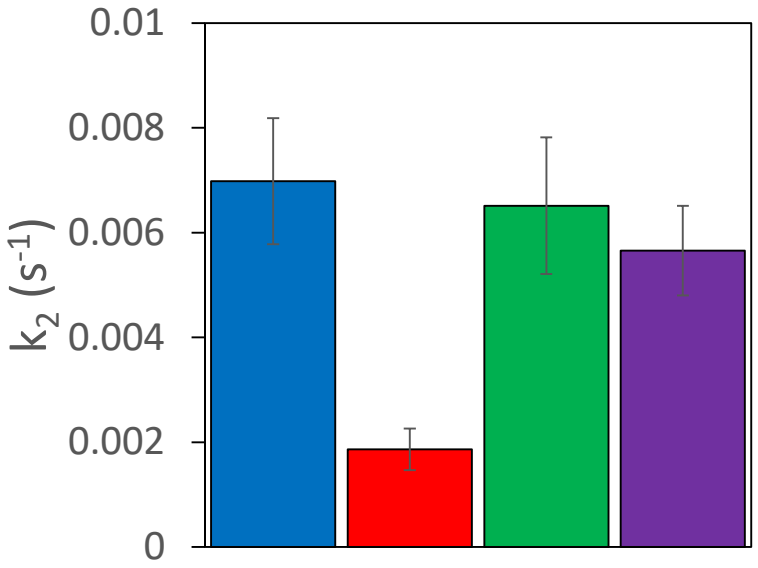
NTD mutant (FW) binds faster but forms oligomers significantly more slowly than WT and CTD mutants



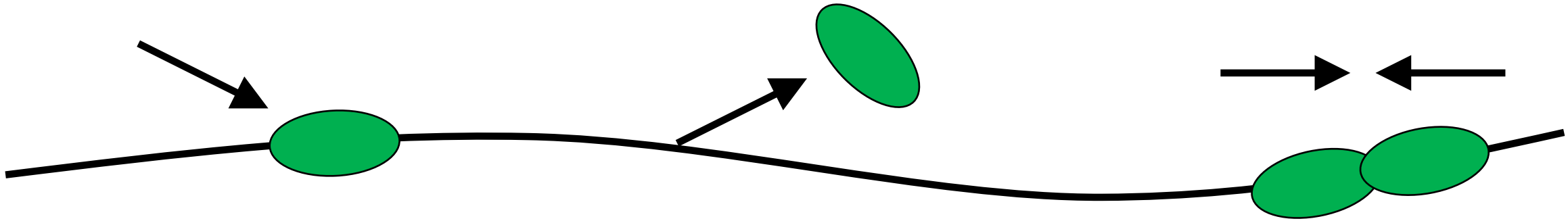
Binding



Dissociation

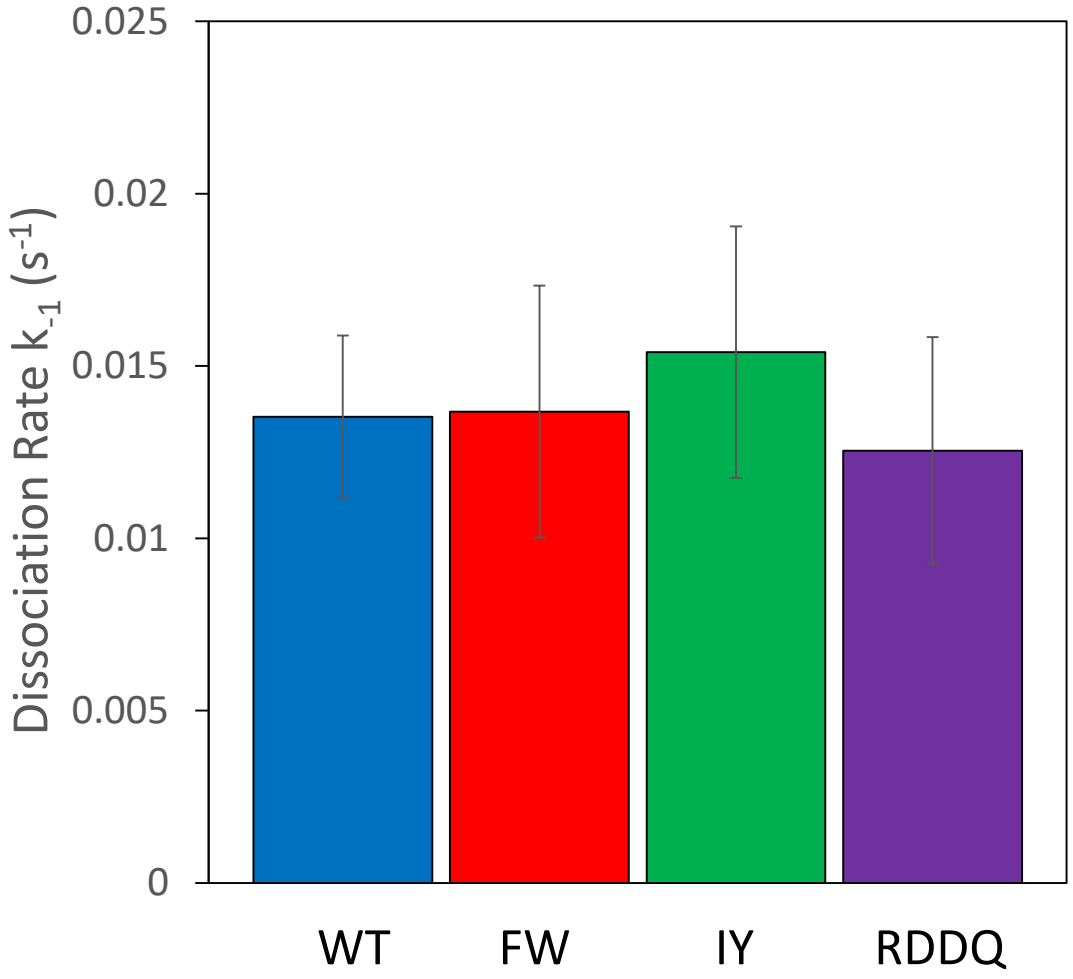


Oligomerization

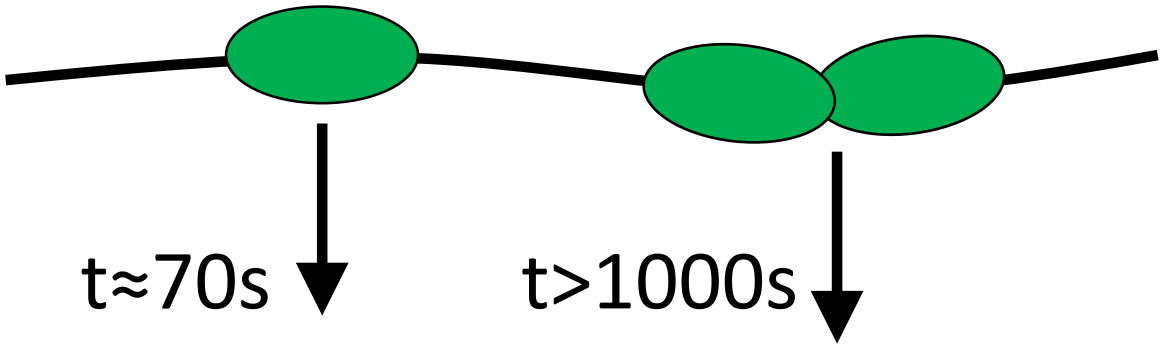


WT and mutant A3G exhibit a single dissociation rate

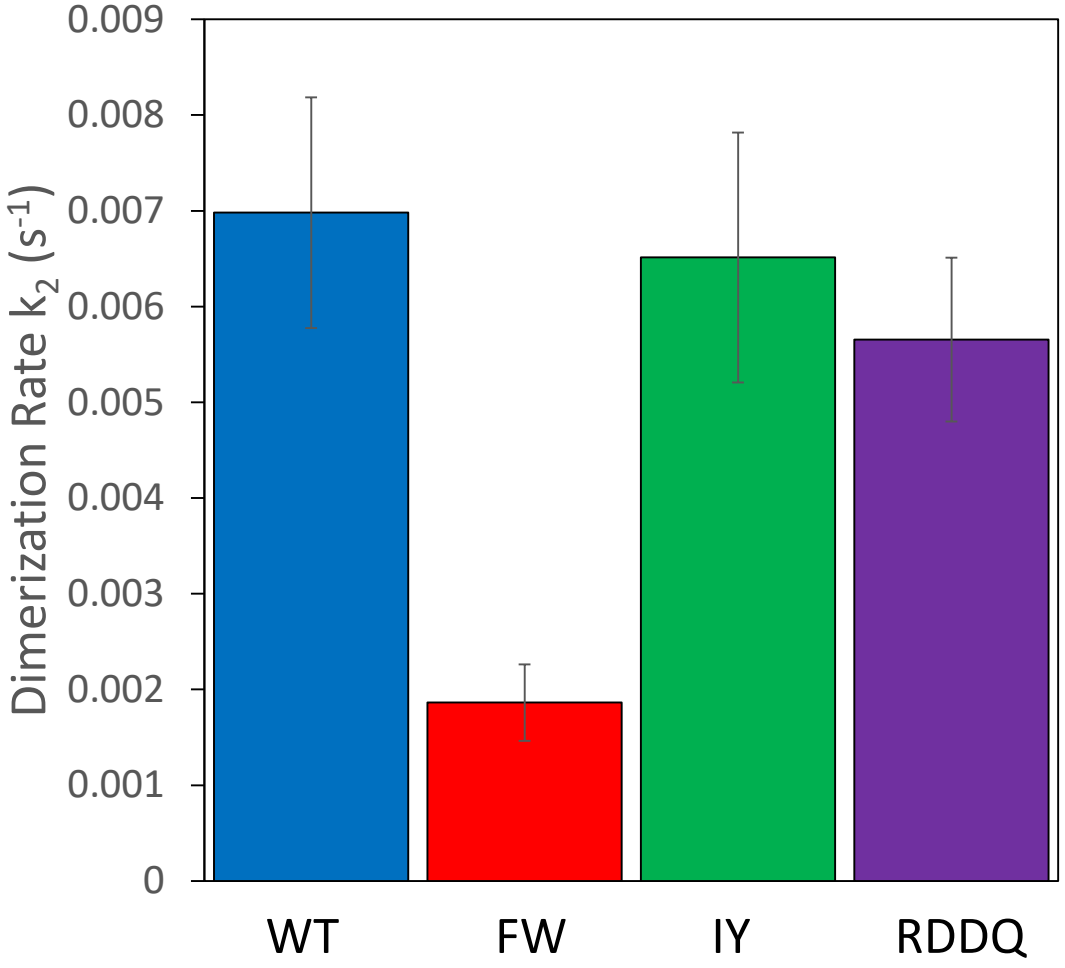
A3G is monomeric in solution



- Agreement between mutants and WT is inconsistent with WT initially binding as dimer and FW initially binding as monomer
- A faster monomer dissociation rate would be clearly visible in dissociation curves

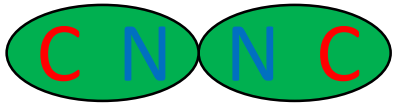


Dimerization is primarily mediated by NTD interaction

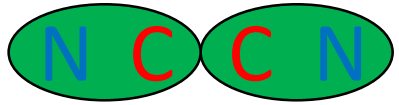


- Disrupting CTD dimerization interface results in statistically insignificant reduction in oligomerization
- Dimerization through NTD is at least 3 times faster than dimerization through CTD

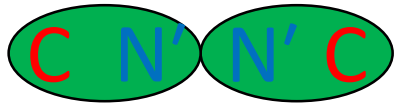
t≈150s



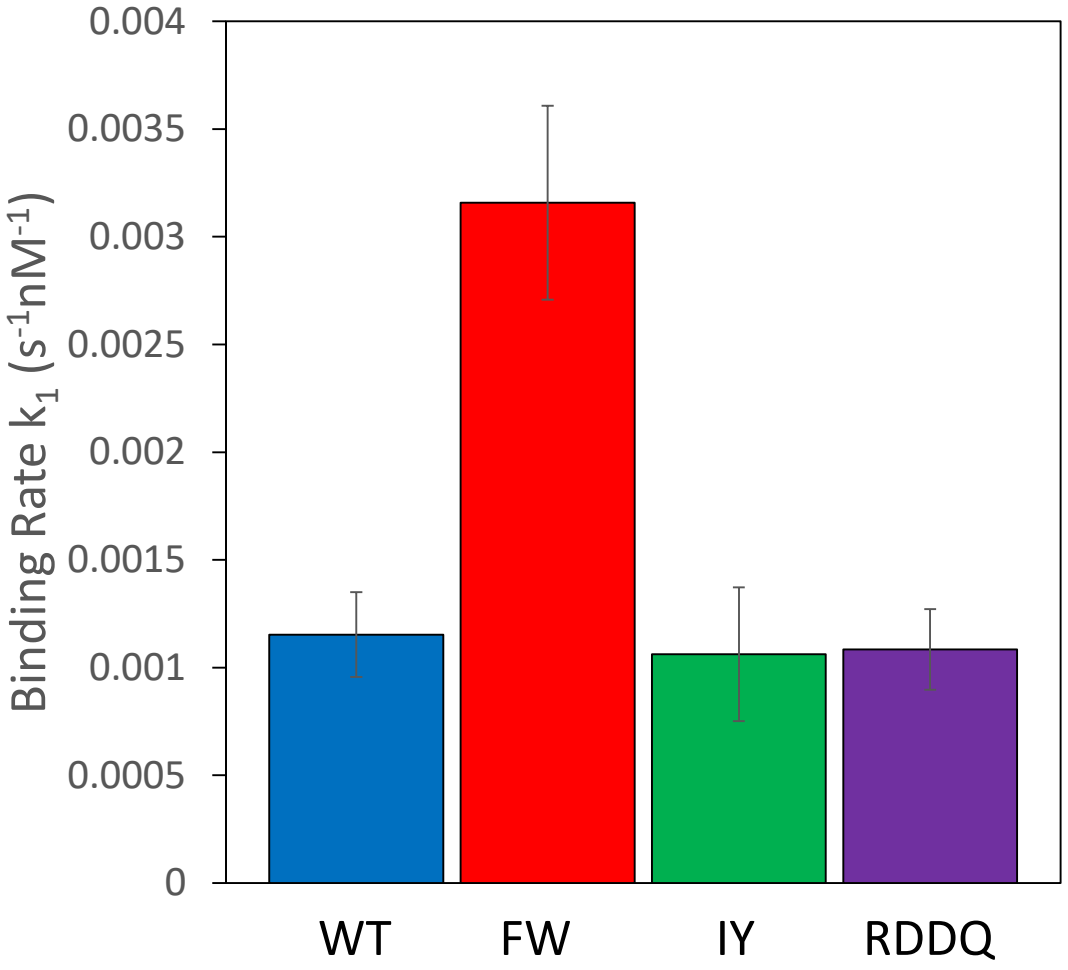
t≈500s



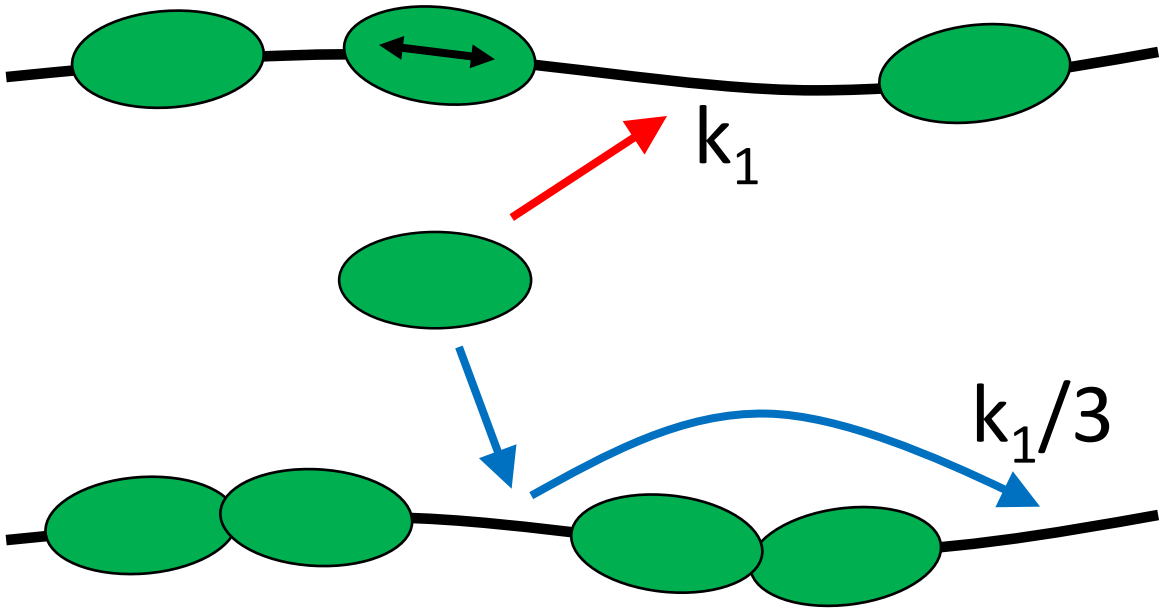
or



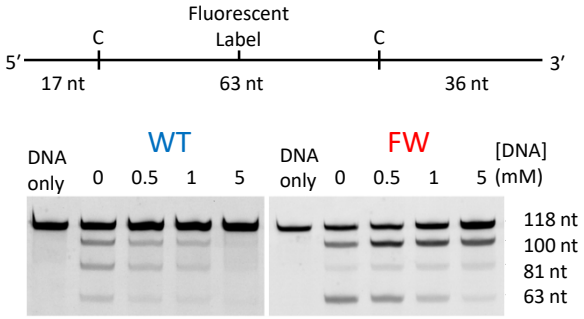
Dimerization slows binding rate

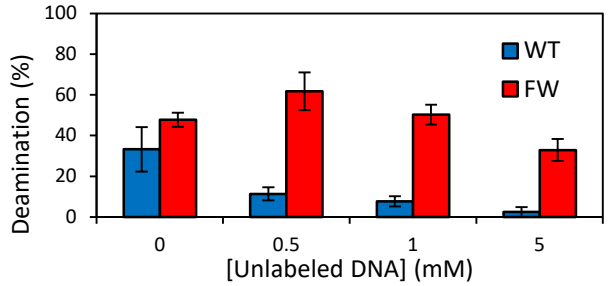


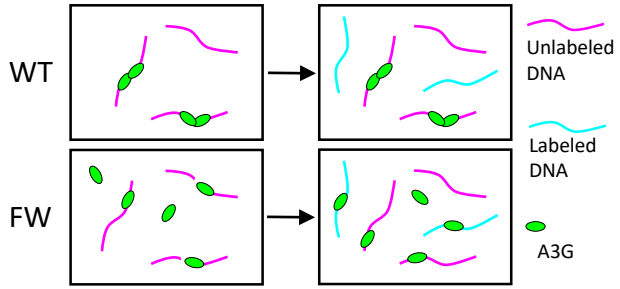
- Monomers slide to accommodate additional A3G binding
- Dimers are stationary and occlude ssDNA binding sites



A3G dimerization inhibits deamination







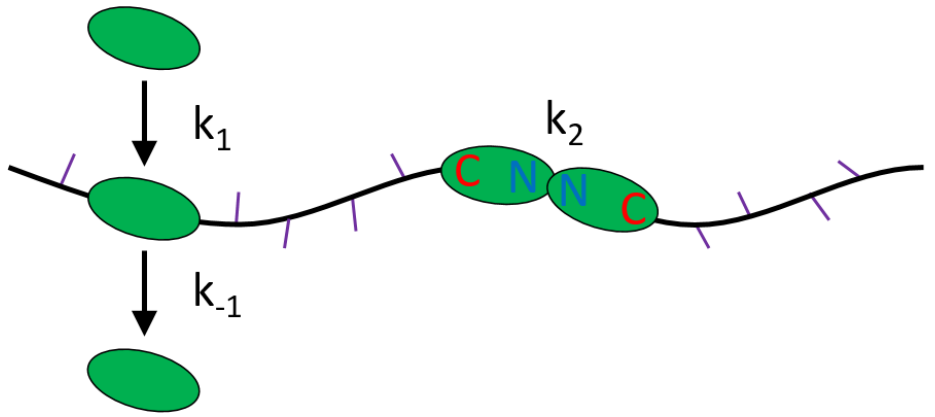
- A3G is first incubated with unlabeled DNA for 3 min, then labeled DNA is added and deamination is measured after 10 min by Uracil DNA glycosylase cleavage.
- Without incubation, WT and FW A3G both deaminate at a similar rate
- WT A3G deamination decreases with unlabeled DNA concentration as oligomerized A3G is unavailable
- Given timescale for dimerization, results show that dimerization inhibits deamination

Significant new findings

- Transiently bound A3G dissociates at a single rate (not multiple rates for monomers, dimers, etc.) for both WT and CD domain mutants
- Mutating the NTD dimerization interface drastically reduces dimerization
- Mutating the CTD dimerization interface does not significantly slow dimerization
- Deamination by A3G decreases when A3G is pre-incubated with ssDNA

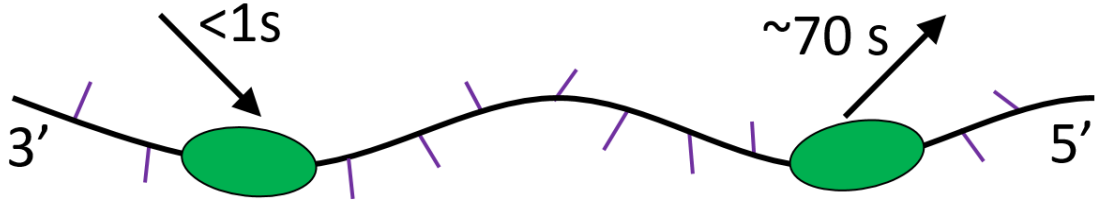
Implications:

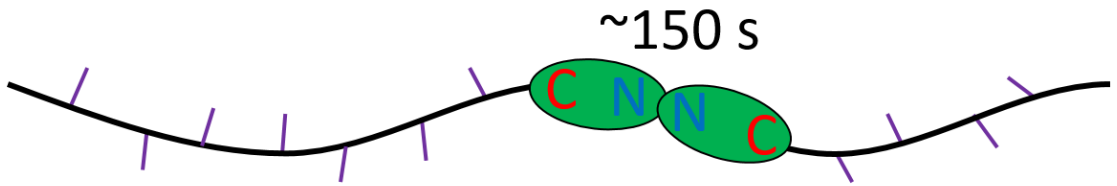
1. WT A3G exists as a monomer in the absence of RNA/ssDNA and during deaminase activity
2. Dimerization mediated by NTD interactions is sufficient to significantly slow A3G binding kinetics
3. Dimerization inhibits deaminase activity



Updated A3G in virio model

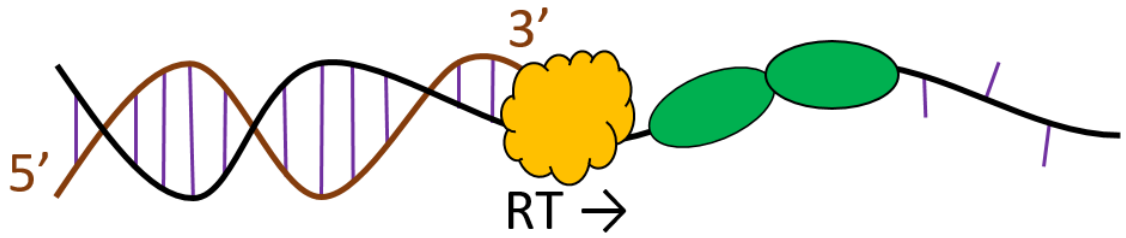
- **Deamination:** A3G monomers bind minus strand viral DNA and deaminate processively 3′ to 5′





- **Dimerization:** Bound monomers form dimers through NTD interactions, transitioning from transient to stable binding

- **Roadblock:** Dimers (and possibly larger oligomers) can remain bound, unable to deaminate ssDNA, acting as a roadblock to RT-catalyzed elongation



Michael Morse, Ran Huo, Yuqing Feng, Ioulia Rouzina, Linda Chelico, and Mark C. Williams. Dimerization regulates both deaminase-dependent and deaminase-independent HIV-1 restriction by APOBEC3G. *Nature Communications* **8**: 597 (2017)

A3G conclusions

- At intravirion concentrations, A3G monomers bind transiently to ssDNA nearly instantly (<<1s). Monomers are highly mobile and rapidly deaminate while bound for ~70s.
- Bound monomers form dimers after ~150s through NTD-NTD interactions. Dimers do not slide along ssDNA and A3G becomes a slow ssDNA binder that acts as a roadblock for reverse transcription
- Correlation between replication studies and DNA binding measurements suggest that A3G dimerization is responsible for deamination-independent inhibition of HIV-1 replication
- With only ~10 A3G subunits in virion, the ability of monomers to deaminate and of dimers to form roadblocks would greatly enhance A3G function

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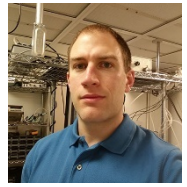

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