It has been questioned whether Na^+, owing to its positive charge and inability to bind covalently to the Schiff base like a proton, could be pumped with support of the retinal Schiff base and retinal isomerization. In Kr2, subtle differences in the amino acid composition of the environment surrounding the covalent Schiff base link of the retinal and along the transport pathway are responsible for the ability to pump Na^+ against an osmotic gradient. The structure-based model of Na^+ translocation proposed independently by the two groups relies on the deprotonation of the Schiff base after retinal isomerization. The proton is transferred to the neighboring Asp116 and causes it, or nearby Leu120, as shown in Gushchin *et al.*[6], to flip away to enable passage of Na^+ across a neutral Schiff base.

Another interesting region was identified just at the beginning of the ion-translocation path. The ion-uptake cavity (blue cavity in **Fig. 2**) contains residues that affect the selectivity of the pump. Point mutations introduced in this region affect the transport of the ions, with one unusual case leading to preferential K^+ transport, which has not been observed for the wild-type protein. This observation adds another exciting opportunity to use K^+ gradients to hyperpolarize the nerve cells in optogenetics. K^+ is particularly interesting because it is present at high concentration in the cytosol and would potentially be less invasive for the studied system.

Publication of these initial Kr2 structures has answered many questions but also has opened the way for a range of follow-up experiments. For example, it will be interesting to compare the effect of Na^+ currents for the optogenetic control of diverse systems and to verify the extent to which engineered Kr2 variants pumping K^+ will be useful. Engineering of further interesting variants will be facilitated by a more thorough experimental characterization of the pumping mechanism and localization of Na^+-binding sites. Given the impact that structural data has had on unraveling the photocycle of retinal-binding proteins, we are looking forward to seeing structures of photoactivated Kr2 intermediates to provide further mechanistic insights into this promising optogenetic tool.

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1. Deisseroth, K. *Sci. Am.* **303**, 48–55 (2010).
2. Nagel, G. *et al. Science* **296**, 2395–2398 (2002).
3. Nagel, G. *et al. Proc. Natl. Acad. Sci. USA* **100**, 13940–13945 (2003).
4. Schobert, B. & Lanyi, J.K. *J. Biol. Chem.* **257**, 10306–10313 (1982).
5. Inoue, K. *et al. Nat. Commun.* **4**, 1678 (2013).
6. Gushchin, I. *et al. Nat. Struct. Mol. Biol.* **22**, 390–395 (2015).
7. Kato, H.E. *et al. Nature* doi:10.1038/nature14322 (6 April 2015).
8. *Nat. Methods* **8**, 1 (2011).
9. News Staff. *Science* **330**, 1612–1613 (2010).
10. Kravitz, A.V. *et al. Nature* **466**, 622–626 (2010).
11. Häusser, M. *Nat. Methods* **11**, 1012–1014 (2014).
12. Kato, H.E. *et al. Nature* **482**, 369–374 (2012).
13. Lin, J.Y., Knutsen, P.M., Muller, A., Kleinfeld, D. & Tsien, R.Y. *Nat. Neurosci.* **16**, 1499–1508 (2013).
14. Berndt, A., Lee, S.Y., Ramakrishnan, C. & Deisseroth, K. *Science* **344**, 420–424 (2014).
15. Wietek, J. *et al. Science* **344**, 409–412 (2014).
16. Wickstrand, C., Dods, R., Royant, A. & Neutze, R. *Biochim. Biophys. Acta* **1850**, 536–553 (2015).
17. Lodish, H.F. *Molecular Cell Biology* (W.H. Freeman, 2000).
18. Kolbe, M., Besir, H., Essen, L.O. & Oesterhelt, D. *Science* **288**, 1390–1396 (2000).
19. Ho, B.K. & Gruswitz, F. *BMC Struct. Biol.* **8**, 49 (2008).

A bumpy road for RNA polymerase II

Luciana E Giono & Alberto R Kornblihtt

The identification of a second regulatory checkpoint controlling RNA polymerase II elongation near the poly(A) site of protein-coding genes reveals an additional level of complexity in the modulation of eukaryotic transcriptional elongation and termination.

A great deal of the regulation of eukaryotic gene expression relies on the control of transcription performed by RNA polymerase II (RNAPII). This process can be regulated at the level of initiation, elongation and termination, and each of these three enzymatic events is linked to differential phosphorylation of specific amino acid residues within the repeated heptamer of the C-terminal domain (CTD) of RNAPII's largest subunit. Each of the 52 CTD repeats of the mammalian enzyme (or 26 in budding yeast) bears the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, in which all residues except the prolines are subject to regulatory phosphorylation. A commonly accepted paradigm holds that Ser5 phosphorylation by the CDK7 kinase subunit of general transcription factor TFIIH is a hallmark of transcriptional initiation, whereas Ser2 phosphorylation meditated by the CDK9 subunit of the P-TEFb complex is needed for effective elongation. However, this simplistic scheme has been challenged by the subsequent demonstration of important roles for additional kinases such as CDK12 and CDK13 (ref. 1) and for other CTD residues such as Tyr1, Thr4 or Ser7 (ref. 2) as well as by evidence that hyperphosphorylation of both Ser2 and Ser5 after DNA damage can lead to inhibition of elongation[3].

One way to distinguish differential phosphorylation patterns that control polymerase initiation, elongation and termination decisions from those that are merely correlated with these events is to assess the effects of specific pharmacological kinase inhibitors. In a report in this issue, Murphy and colleagues[4] monitor the effects of two CDK9 inhibitors, DRB and KM05382, both genome wide and on individual model genes. Their genome-wide analysis was performed with the powerful global run-on sequencing (GRO-seq) technique, which allows global mapping of RNAPII densities. The authors found that most genes possess a CDK9-dependent checkpoint to elongation within 500 bp downstream of the transcription start site (TSS), regardless of whether RNAPII pauses proximally to the promoter. In other words, when CDK9 kinase activity was inhibited, RNAPII could proceed from the TSS to an early checkpoint but no further. Surprisingly, RNAPII molecules that had passed the early CDK9 checkpoint when the inhibitor was added continued to transcribe through the remainder of the gene body without apparent difficulty. The authors propose that CDK9 activity is necessary to facilitate RNAPII passage through the early checkpoint and that RNAPII molecules that have already negotiated this passage

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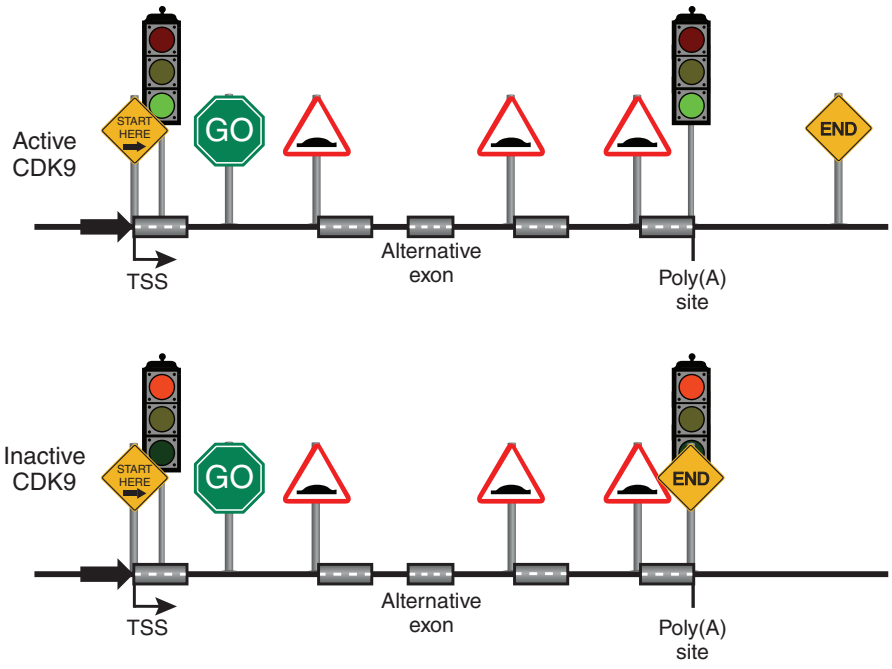

Figure 1 RNA polymerase II on the road. Most eukaryotic protein-coding genes contain an early, CDK9-dependent elongation checkpoint near the transcriptional start site (TSS) (represented by the TSS-proximal traffic light). Once RNAPII negotiates passage through this point, it can resume transcription even in the presence of CDK9 inhibitors (represented by the 'go' sign). The speed bumps represent the transient pauses at the beginning of each constitutive exon (previously reported by Lis and colleagues[11]) that are absent from alternatively spliced exons. Under normal conditions, termination of transcription occurs downstream of the poly(A) site (indicated by the 'end' sign). A new second CDK9-dependent checkpoint near the poly(A) site, identified by Murphy and colleagues[4] (represented by the TSS-distal traffic light) directs premature transcriptional termination when CDK9 activity is inhibited.

are able to continue even in the presence of CDK9 inhibitors, thus creating a wave of transcription toward the 3′ end. Intriguingly, at bidirectional promoters directing divergent transcription, the authors identified early CDK9 checkpoints at similar distances from the TSS in both directions. Although the function of the divergent 'antisense' checkpoints is still elusive, there is an emerging consensus that early, rate-limiting 'sense' checkpoints may facilitate the recruitment of pre-mRNA–processing factors to permit rapid and synchronous induction of certain genes upon external signals that stimulate P-TEFb activation and/or recruitment.

Passport controls operate at borders not only when travelers enter a country but also when they leave it. This serves as a metaphor that applies to the second important finding of the report by Murphy and colleagues[4]: the uncovering of an elongation checkpoint associated with the poly(A) site. The universal poly(A) signal, AAUAAA, within the last exon of eukaryotic genes, marks the cleavage-and-polyadenylation site (also known as the poly(A) site) located 15–17 nucleotides downstream in the pre-mRNA. Cleavage and polyadenylation determine the location of the 3′ end of the mRNA, and its position does not normally coincide with the site at which RNAPII terminates transcription. In fact, unlike the case in bacteria, transcription termination within eukaryotic protein-encoding genes is not directed by universal terminator sequences and instead may occur at multiple positions located hundreds or even thousands of bases downstream of the poly(A) site. Nevertheless, the poly(A) signal and the resultant cleavage at the poly(A) site are strictly necessary for RNAPII to terminate further downstream[5]. One explanation for this requirement is provided by the 'torpedo' model, which holds that cleavage at the poly(A) site generates a free 5′ end on the RNA transcript that is generated by RNAPII 'reading through' the poly(A) signal. This 5′ end may act as an entry site for the 5′-3′ exonuclease Xrn2, which, by 'chewing' the RNA from the 5′ end, 'chases' RNAPII until it bumps into it and thereby destabilizes polymerase interactions with DNA to cause termination[6].

In summary, the poly(A) signal and its neighboring cleavage site are not termination sites under normal conditions. However, the current work demonstrates that, upon CDK9 inhibition, RNAPII terminates transcription prematurely, proximally to the poly(A) site, revealing the existence of a second elongation checkpoint at the end of the last exon. Most interestingly, the patterns of RNAPII CTD phosphorylation at this second checkpoint mimic those of RNAPII at bona fide termination sites, thus suggesting that similar termination mechanisms underlie these events.

In addition to the Ser2 residue of the RNAPII CTD, targets of CDK9 kinase activity include the E subunit of the negative elongation factor NELF and the Spt5 subunit of the DRB-sensitive factor DSIF. DRB is one of the two inhibitors used by the authors to reduce CDK9 activity. Because the levels of Ser2 phosphorylation upstream of the poly(A) site are not affected by CDK9 inhibition, and NELF is not present at the 3′ ends of genes, the authors speculate that phosphorylation of DSIF, which converts the protein into a positive elongation factor, is necessary to pass the poly(A)-site checkpoint. Consistently with this interpretation, the association of Spt5 with the poly(A)-site region of a model gene was lost after CDK9 inhibition. It should be noted that although both the DRB and KM05382 CDK9 inhibitors tested in the present study show some specificity for CDK9, this specificity is not absolute, so a potential role for additional kinases cannot currently be ruled out.

The function of the poly(A)-associated elongation checkpoint is not yet as clear as that of the upstream checkpoint. Murphy and colleagues[4] propose that the poly(A)-site checkpoint may provide a later point for rapid regulation of the synthesis of long transcripts upon specific signals. Indeed, premature transcription termination or transcriptional attenuation is a highly conserved regulatory strategy used by bacteria[7], although it occurs through a different mechanism. It might additionally serve to prevent futile transcription of hundreds of nucleotides downstream of the poly(A) site under conditions in which productive transcription is hindered at the gene promoter.

One of the most interesting conclusions of the study is that in the presence of CDK9 inhibitors RNAPII continues to transcribe normally between the two checkpoints, at a typical overall speed of 2–3 kb/min. This observation complements previous reports by several groups that the path of RNAPII inside genes is not free of 'bumps' that affect elongation rates at a more 'microscopic' level compared with the more conspicuous RNAPII peaks at gene boundaries. This finer internal modulation of RNAPII elongation is mediated primarily by chromatin structure and histone modifications. First, there is evidence that nucleosomes are preferentially positioned in exons[8–10] to create a landscape in which RNAPII transiently stalls before each exon. This was elegantly shown by

Lis and colleagues with PRO-seq, a modification of GRO-seq that allows RNAPII densities to be determined genome wide at a single-nucleotide resolution[11]. This nucleosome-mediated RNAPII delay at each exon facilitates recognition of exons within the nascent pre-mRNA by spliceosomal subunits and promotes their subsequent inclusion in the mature mRNA, a process known as exon definition. Alternative exons are included into mRNA with reduced efficiency and therefore are less defined; this correlates well with the reduced stalling of RNAPII observed at their 5′ ends. Equally elegant single-molecule biophysical studies performed by Bustamante and colleagues have demonstrated that nucleosomes behave as fluctuating barriers that locally increase pause density and reduce the apparent pause-free velocity of RNAPII[12], thus confirming the bumpy nature of elongation through eukaryotic protein-coding genes. A second layer of intragenic elongation control is caused by differential histone marking and DNA methylation that, by either inhibiting or promoting elongation, regulate both constitutive and alternative splicing[13].

In summary, the findings of Murphy and colleagues[4] present important further insights into the complexity of RNAPII elongation (**Fig. 1**), a subject that has been under study for decades and that still continues to provide even more surprises than has the regulation of transcriptional initiation.

COMPETING FINANCIAL INTERESTS
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1. Bartkowiak, B. *et al. Genes Dev.* **24**, 2303–2316 (2010).
2. Heidemann, M., Hintermair, C., Voss, K. & Eick, D. *Biochim. Biophys. Acta* **1829**, 55–62 (2013).
3. Muñoz, M.J. *et al. Cell* **137**, 708–720 (2009).
4. Laitem, C. *et al. Nat. Struct. Mol. Biol.* **22**, 396–403 (2015).
5. Proudfoot, N.J. *Genes Dev.* **25**, 1770–1782 (2011).
6. West, S., Gromak, N. & Proudfoot, N.J. *Nature* **432**, 522–525 (2004).
7. Merino, E. & Yanofsky, C. *Trends Genet.* **21**, 260–264 (2005).
8. Tilgner, H. *et al. Nat. Struct. Mol. Biol.* **16**, 996–1001 (2009).
9. Spies, N., Nielsen, C.B., Padgett, R.A. & Burge, C.B. *Mol. Cell* **36**, 245–254 (2009).
10. Schwartz, S., Meshorer, E. & Ast, G. *Nat. Struct. Mol. Biol.* **16**, 990–995 (2009).
11. Kwak, H., Fuda, N.J., Core, L.J. & Lis, J.T. *Science* **339**, 950–953 (2013).
12. Hodges, C., Bintu, L., Lubkowska, L., Kashlev, M. & Bustamante, C. *Science* **325**, 626–628 (2009).
13. Kornblihtt, A.R. *et al. Nat. Rev. Mol. Cell Biol.* **14**, 153–165 (2013).

Integrin bondage: filamin takes control

Nicola De Franceschi & Johanna Ivaska

Regulation of integrin activity is critical for human health, and the steps mediating integrin activation are well established. In contrast, the counteracting mechanisms of inactivation are less understood. An integrin inhibitor, filamin, is shown to stabilize the integrin resting state by bondage of the cytoplasmic domains of the integrin heterodimer, thus providing evidence of a new mechanism for integrin retention in the inactive state.

More than two decades ago, integrin activity was suggested to be regulated not only by the integrin β-subunit cytoplasmic domain but also by a conserved sequence present in all integrin α subunits[1]. However, the molecular details have remained unclear. Liu *et al.*[2], in this issue, now report that filamin, a known integrin inhibitor, forms a ternary complex engaging the cytoplasmic tails (CTs) of both integrin α_{IIb} and β_3, providing the first structural evidence of such a mechanism. Importantly, the study establishes a key role for the integrin α subunit in filamin-mediated integrin inactivation, because disruption of the α_{IIb}-filamin interaction is sufficient for constitutive integrin activation.

Integrins are a family of heterodimeric transmembrane receptors formed by α and β subunits. They are essential for cell adhesion and spreading, because they provide a link between the extracellular matrix (ECM) and the actin cytoskeleton. They also mediate ECM-triggered signals to cells, thus regulating a vast array of cellular responses. Both integrin subunits are composed of a large extracellular ectodomain, a single transmembrane domain (TMD) and a relatively short intracellular CT. Integrin activation has been intensively studied with increasingly sophisticated techniques, and these studies have established the bidirectional nature of regulation of integrin activity via either extracellular ligands (outside-in activation) or intracellular binding partners (inside-out activation)[3]. This process involves large structural rearrangements of the entire molecule. The current view is that the ectodomains exhibit a switchblade-like extension, which results in increasing affinity for the extracellular ligand and separation of the 'legs' connecting the ectodomain with the TMD[3]. On the cytoplasmic side, the α- and β-subunit TMDs and CTs are clasped together in the inactive receptor by interactions between residues from two regions of the integrin subunits, depicted as the outer-membrane clasp (OMC) and the inner-membrane clasp (IMC)[4].

The OMC comprises a GxxxG motif, present in the α subunit, which confers a tilt angle to the two TMDs[4]. The IMC involves both packing of hydrophobic residues and formation of a salt bridge between α-subunit R995 and β-subunit D723, and it is of particular interest because numerous intracellular proteins interact with the membrane-proximal region of either integrin subunit[5], to regulate the activation or inactivation of the receptor by dynamically acting on the IMC. Although disruption of the IMC by activators such as talin has been studied in great detail[3], the molecular mechanism of the inactivation process is still largely uncharacterized. Yet, the physiological relevance of integrin inactivation is exemplified by the phenotypes of mice lacking integrin inactivators[6]. It has been speculated that one possible mechanism for inactivation could be stabilization of the IMC

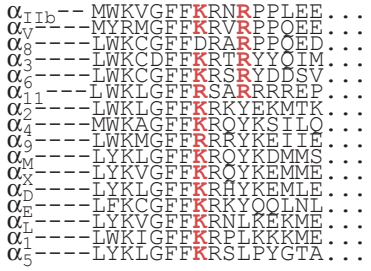

Figure 1 Alignment of the membrane-proximal and cytoplasmic regions of all integrin α chains. The ε-amino group of K994 and the guanidyl group of R997 are important in mediating interaction with filamin via their side chains (residues in red).

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