

other reports collectively suggest that a majority of the chromatin-remodeling complexes may function in or around the NFR.

Until recently, work on transcriptional regulation focused on *cis*-elements. The ability to map nucleosomes provides a new vantage point from which to study transcription, and great strides are being made by investigating how *cis*-elements work in the context of nucleosomes. The new papers show the power of these approaches and indicate that much effort should be spent to understand formation, maintenance, regulation, and the biological functions of NFRs.

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HIV Entry Revisited

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HIV has long served as a model for viruses that enter cells by direct fusion at the plasma membrane. Miyauchi et al. (2009) now provide compelling evidence that HIV enters cells primarily by endocytosis.

Human immunodeficiency virus (HIV), the causative agent of AIDS, was discovered over 25 years ago. Paradoxically, despite being extensively studied, we still do not understand how HIV enters cells to establish infection. In general, enveloped viruses enter cells by one of two modes, direct fusion at the plasma membrane or endocytosis followed by fusion in an endosome, the latter route of entry being dependent on the low pH. HIV is commonly viewed as a prototypical example of a virus that enters cells at neutral pH by fusion at the plasma membrane (Marsh and Helenius, 2006). Yet, in this issue, Miyauchi et al. (2009) present compelling evidence that HIV enters cells primarily by endocytosis. To understand this fusion confusion, let's revisit the evidence.

After the discovery of HIV and its primary receptor on host cells, CD4, it quickly emerged that HIV entry into

host cells does not depend on low pH, suggesting that entry does not involve endocytosis. HIV entry is not inhibited by lysomotropic reagents that completely block the entry of pH-dependent viruses by dissipating the low pH within endosomes (Stein et al., 1987). In fact, interfering with lysosome acidification enhances the efficiency of HIV entry. It has also been shown in a heterologous system that cell-cell fusion can be triggered at neutral pH between cells expressing HIV envelope glycoprotein (Env) and cells expressing CD4 and a coreceptor. Finally, the interaction of Env with CD4 and coreceptor mimetics induces conformational changes in the Env protein that are consistent with the notion that entry can occur at the plasma membrane.

Although these experiments clearly demonstrate that HIV entry is pH independent, the possibility that HIV virions

could use endosomes to enter cells cannot be completely excluded given the limitations of the assays used. For instance, the cell-cell fusion experiments are not necessarily a good predictor of the behavior of a small virus particle that carries only a few Env proteins. In addition, HIV is endocytosed efficiently and can readily infect cells when decorated with an unrelated envelope protein that forces it into an endocytic entry pathway. Thus, despite HIV being a prototypical example of a virus that enters cells at the plasma membrane, there are sufficient reasons to revisit the topic.

Miyauchi and colleagues address the three major limitations of prior work. First, they use a direct assay to analyze entry. The classical experiments for HIV entry are based on indirect assays such as viral gene expression, which occurs many hours after the virus enters the cell. The risks associated with interpreting

such assays are illustrated by the entry mechanism of the avian leukosis virus, which depends on low pH subsequent to the interaction between the virus and its receptor (Mothes et al., 2000). Second, Miyauchi et al. track single virus particles using live cell imaging. Live cell imaging has opened up new avenues to study the multistep entry process of viruses (Campbell and Hope, 2008). Incorporating membrane fusion and the mixing of viral contents with cytoplasm (referred to as content mixing) into the visual read-out for viral entry increases our confidence that viruses are being imaged en route to infecting the cell (Lakadamyali et al., 2003; Melikyan et al., 2005). Third, the authors directly examine the role of endocytosis in HIV entry.

In their new work, Miyauchi et al. (2009) use an assay for HIV entry that measures the delivery of capsid-associated β -lactamase directly into the cytosol of the host cell. In the cytosol, this enzyme shifts the emission wavelength of a fluorescent reagent, making content mixing easy to measure (Cavrois et al., 2002). Using this assay, the authors observe that HIV becomes rapidly resistant to entry inhibitors that are membrane impermeable, but continues to be vulnerable to a temperature-dependent block in membrane fusion. These data suggest that although HIV is rapidly endocytosed, fusion and content mixing occur more slowly. In this assay, HIV behaves the same way as any virus with a pH-dependent envelope glycoprotein that enters cells via endocytosis prior to fusion and content mixing.

Miyauchi and colleagues use live cell imaging to monitor the progression of lipid mixing and content mixing for each individual HIV particle. They label HIV particles with two fluorescent dyes. The lipophilic fluorescent dye DiD labels the lipid bilayer of the viral envelope. In addition, the virus is filled with soluble green fluorescent protein (GFP) molecules that are released during content mixing. In these assays, the loss of the lipid label indicates fusion with the plasma membrane. In contrast, fusion within an endosome does not result in the immediate disappearance of the label because the lipid dye simply flows from the viral particle into the surrounding endosomal vesicle. Due to the low resolution of con-

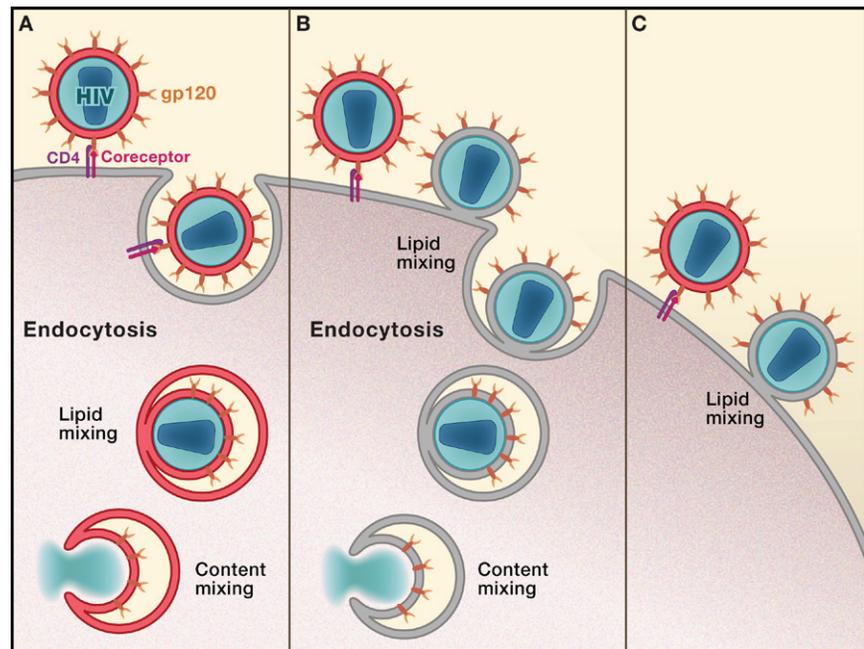


Figure 1. Entry Pathways for HIV

By visualizing the mixing of viral lipids (red) and contents (blue) with host cell membranes and cytosol, respectively, Miyauchi et al. (2009) observe three distinct routes for entry of host cells by HIV. These include endocytic events in which two-colored HIV particles are internalized, undergo lipid mixing with the vesicle membrane, and deliver their contents into the cytoplasm (A). There are fusion events that occur at the plasma membrane and proceed at least to the stage of hemifusion (B). These are followed by subsequent endocytosis and content mixing. There are also fusion events at the plasma membrane that do not result in any subsequent content mixing (C).

ventional fluorescence microscopy, the lipid dye appears to stay at the site. Content mixing, on the other hand, results in the loss of soluble GFP irrespective of the location in the cell.

Applying this assay to the study of HIV entry, the authors observe three different outcomes (Figure 1). First, they observe bona fide endocytic events whereby particles are internalized, move toward the perinuclear area of the cell, and lose their content markers. Because the lipid dye is simply transferred from the particle to the surrounding endosome, its fluorescence is not lost (Figure 1). These events are indistinguishable from the entry pathway used by HIV viruses engineered to express the fusion protein of the Semliki Forest virus, which depends on low pH for function. Second, HIV virions can undergo fusion at the plasma membrane, but complete fusion only occurs after endocytosis and the onset of movement toward a perinuclear location. Finally, a third group of particles never undergoes content mixing despite the occurrence of lipid mixing at

the plasma membrane. These particles show limited movement and are likely to be immobilized at the surface. Interestingly, Semliki Forest virus and avian leukosis virus, which depend on low pH for entry, show similar phenotypes when forced to fuse at the plasma membrane by the lowering of the culture pH (Marsh and Helenius, 2006; Mothes et al., 2000). These data indicate that content mixing of HIV is blocked when fusion is induced at the plasma membrane.

Finally, Miyauchi and colleagues address whether HIV entry requires endocytosis. They confirm earlier data by Daecke et al. (2005) showing that dynamin is required for HIV entry. Dynamin is a GTPase that promotes the scission of endocytic vesicles from the plasma membrane. Intriguingly, dynamin is required for both internalization and content mixing, and is potentially required for fusion pore formation or enlargement. Thus, the work by Miyauchi and colleagues presents the most comprehensive analysis of HIV entry to date and demonstrates that

it does depend on endocytosis. Their findings show that HIV predominantly uses existing cellular endosomes as transport carriers to gain access to the cytoplasm. When fusion is induced at the plasma membrane, content mixing is not observed. This block in virus entry at the plasma membrane cannot be overcome by depolymerization of the actin cytoskeleton. A better understanding of this block will likely explain why the virus has a preference for endocytosis. Given that HIV appears to use an endocytic pathway for entry, less well-studied viruses whose entry mechanisms are also pH independent deserve a second look.

Is this the end of the story on HIV entry? Certainly not. As technologies improve, the HIV entry pathway will likely be revisited. Super-resolution fluorescence microscopy will likely allow direct visualization of the proposed entry intermediates, such as lipid mixing within endosomes. Future imaging technologies may also connect fusion and content mixing to downstream

events such as uncoating, reverse transcription, and nuclear import, thereby providing insights into the temporal and spatial coordination of HIV entry. Finally, HIV entry needs to be reanalyzed in the context of cell-to-cell transmission, its primary mode of spread. Recent progress in this area has captured HIV in the act of transmission and also points to a role for endocytosis in the target cell, but a detailed analysis of the entry mechanism has not been performed (Hubner et al., 2009). The comprehensive study of Miyauchi et al. may be just one step in our journey to understand this virus that still kills millions of people each year. Although basic science may sometimes appear slow, the elegant experimental design applied by Miyauchi et al. provides insights that may facilitate the rational design of effective new therapies to combat AIDS.

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Ure(k)a! Sirtuins Regulate Mitochondria

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Increasing evidence suggests that multiple metabolic pathways are regulated by sirtuin-dependent protein deacetylation in the mitochondria. In this issue, Nakagawa et al. (2009) show that the sirtuin SIRT5 deacetylates and activates a mitochondrial enzyme, carbamoyl phosphate synthetase 1, which mediates the first step in the urea cycle.

Mitochondria are key players in metabolism, energy maintenance, and apoptosis. Disruption of mitochondrial pathways can lead to metabolic disease, oxidative damage, and cancer. Therefore, elucidating how these pathways are regulated has therapeutic implications. There is compelling evidence that reversible acetylation of mitochondrial proteins is a key mechanism of metabolic regulation. In this issue of *Cell*,

Nakagawa et al. (2009) describe the regulation of the first step of the urea cycle by a previously uncharacterized mitochondrial sirtuin, SIRT5, through deacetylation of the mitochondrial enzyme carbamoyl phosphate synthetase 1 (CPS1). A related study by Yu et al. (2009) describes the regulated acetylation of ornithine carbamoyltransferase (OTC), which catalyzes the second step of the urea cycle. These studies

reveal that reversible protein acetylation is a major mechanism for regulating the urea cycle in mitochondria.

Conserved from bacteria to humans, sirtuins catalyze NAD⁺-dependent protein deacetylation. With some sirtuins, the ability to promote protein ADP-ribosylation has been reported (reviewed in Smith et al., 2008). In humans, there are seven sirtuins (SIRT1-SIRT7), three of which are localized to mitochondria (SIRT3, SIRT4,