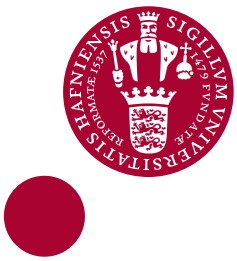

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The class III histone deacetylase sirtuin 6 (SIRT6) modulates numerous functions in the cell by deacetylating histone lysine residues. Interestingly, SIRT6's efficiency in *in vitro* experiments is far greater against substrates carrying long-chain fatty acyl modifications such as myristoylated lysine compared with acetylated counterparts, but the deacetylase activity can be stimulated by fatty acids and small-molecule allosteric modulators. A new study helps to explain this puzzling activation using a novel activator, thorough kinetic investigation, and mutagenesis studies. These data help elucidate the molecular requirements for activation of SIRT6 and provide a foundation for development of activators for therapeutic purposes.

The sirtuins comprise a family of seven NAD^+-dependent lysine deacylase enzyme isoforms (SIRT1–7). Each family member has distinct cellular roles and subcellular localization, with SIRT6 primarily located to the nucleus, where it participates in chromatin remodeling, cleaving acetyl groups from the side chains of acetylated lysine residues in histone proteins. However, it has been shown that SIRT6 can also exhibit ADP-ribosyl transferase activity as well as deacetylase activity against nonchromatin targets that are involved in a plethora of biological functions, such as DNA repair, glucose homeostasis, and aging (1, 2). Overexpression of SIRT6 has beneficial health outcomes, suggesting that a greater understanding of its role and function—and the corresponding ability to modulate those functions—would be useful. A new study by Klein *et al.* (3) provides important advances on these fronts, developing new small molecules and mutated variants to explore and manipulate SIRT6 activity, establishing new steps in SIRT6's catalytic mechanism, and identifying unique aspects of this mechanism that delineate it from other sirtuin isoforms.

Investigating SIRT6 has been challenging in part because its activity *in vitro* does not mirror known cellular functions: In enzyme assays, long-chain acyl substrates (*e.g.* ϵ-*N*-myristoyllysine, Kmyr) are deacylated >100-fold faster than analogous acetylated substrates (4, 5). SIRT6 protein structures have shown that this is due to the existence of a hydrophobic cavity that extends the binding site, matching well with the longer-chain molecules (Fig. 1). However, Denu and co-workers (5) previously discovered that fatty acids can boost the deacetylase activity of SIRT6 by occupying the hydrophobic cavity. This suggested that endogenous activators of SIRT6 might increase its deacetylase activity *in vivo* and also that it might be possible to identify novel SIRT6 activators to further investigate or modulate SIRT6. Recently, small-molecule activators have also been developed by the groups of Steegborn (6) and Zhang (7). The compound developed in the latter mentioned study was shown to bind in an allosteric pocket above the distal part of the hydrophobic cavity (Fig. 1). Additionally, a prodrug version of the compound was found to regulate SIRT6 function in cells and is now commercially available as a probe compound. However, the mechanism and kinetic parameters of SIRT6 activation were not investigated in great detail in those reports and remain poorly understood.

To gain further insight into these questions, Denu and co-workers (3) report a deep dive into the mechanism of SIRT6, enabled by the discovery of novel SIRT6 activators and a critical mutant construct. The authors first use activity-based screening of several compound libraries of fatty acids and aromatic carboxylates to look for novel activators that could enhance SIRT6 deacetylation, identifying several hits that increased activity up to 312-fold. Subsequent chemical derivatization of one of the hit compounds furnished the design of a highly potent activator, CL5D. Also, CL5D failed to increase long-chain deacylase activity, but was rather found to competitively inhibit SIRT6 demyristoylation, suggesting that it binds in the same hydrophobic pocket as observed for fatty acid activators (5).

In efforts to identify the crucial residues that are critical for the catalytic enhancement of SIRT6 deacetylation activity, a number of SIRT6 variants with mutated arginine or lysine residues positioned at diverse locations in the protein were expressed and evaluated. The mutation of Arg-65 to Ala (R65A) resulted in an enzyme that retained base-level deacetylase activity but failed to be activated by CL5D and exhibited drastically reduced demyristoylase activity. Elaborate kinetic profiling of both WT enzyme and R65A mutant uncovered that SIRT6 activators enhance deacetylation by boosting a rate-determining catalytic step after substrate binding but prior to the release of nicotinamide (the first irreversible step in SIRT-mediated hydrolysis), which the authors attribute to a slow conformational change after ruling out known chemical steps. The R65A mutant was previously found to hamper SIRT6 deacetylation of cellular targets (8), but the mechanism was not known. In the context of the new data from Klein *et al.*, this result strongly suggests that Arg-65 assists in the *in vivo* efficacy of SIRT6 and that endogenous modulators likely serve an important role in activating the enzyme in a cellular context.

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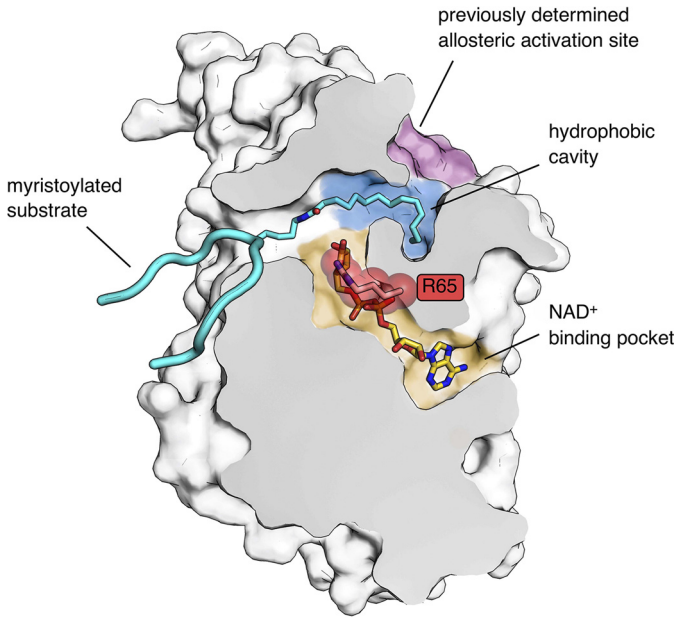

Figure 1. *Slice view* of SIRT6 co-crystalized with myristoylated histone H3 Lys-9–derived substrate (*cyan*) and ADP-ribose (*yellow*) (Protein Data Bank entry 3ZG6). The additional *color coding* highlights the hydrophobic cavity of the substrate-binding site (*blue*), the NAD^+-binding pocket (*beige*), the allosteric activation site (*magenta*), and the Arg-65 (*R65*; in *red space-filling format*). This residue is positioned *above* the NAD^+-binding pocket and makes extensive polar and electrostatic interactions with the ribose and pyrophosphate of ADPR in the co-crystal structure.

Overall, this study addresses the possible functional differences between the behavior of an enzyme *in vitro versus* its native environment in the cell. It is likely that other sirtuin enzymes have similar endogenous activation mechanisms. For example, the relatively unexplored family member SIRT7 has been shown to undergo co-activation by oligonucleotides to enhance its activity *in vitro* (9). Recently, it was shown that using entire nucleosome particles as substrates might provide a more accurate measurement of the activity of SIRT7 (10), and, in turn, it was also speculated that the same might be true for SIRT6 (10). Thus, cellular interaction partners might serve a crucial role in activation of sirtuin enzymes. It is possible that endogenous activators may provide regulatory mechanisms. For example, the sirtuin enzymes may act as sensors and only initiate hydrolysis upon certain changes in the cellular environment (*e.g.* by changes in chromatin structure or fatty acid concentration).

In future studies, the use of SIRT6 activators might be implemented and standardized for improved assay conditions so that the conditions used in biochemical assays better resemble a physiologically relevant environment. It is likely that additional endogenous interaction partners and activators will be discovered for sirtuins and histone deacetylases and that the enzymatic activity for other sirtuin enzymes might likewise be greatly impacted by the discovery of novel activators. With the advances in molecular-resolution imaging techniques and in cryo-EM, it might soon be possible to investigate conformational changes during sirtuin enzyme catalysis. Techniques like these might also help reveal additional sirtuin interaction partners and help explore novel endogenous modulators.

Activators of SIRT1, and sirtuin inhibitors in general, have been extensively explored, but the therapeutic potential of SIRT6 activators remains relatively unknown. Development of selective sirtuin inhibitors has been challenging due to the common NAD^+-dependent deacylase mechanism and overlap in substrate recognition between sirtuin isozymes, meaning that activators that bind allosterically might hold more promise for achieving selective perturbation of individual sirtuin subtypes. Additionally, as other SIRT6 activators bind allosterically to the enzyme, it will be interesting to explore whether the use of activator combinations can synergistically enhance deacetylase activity even further. It will be interesting to follow the discovery of novel activators of both SIRT6 and other sirtuins/histone deacetylases in the future, which may lead to deeper understanding of the cellular function of these hydrolases and ultimately form the basis for development of new therapeutics.

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