

# Bending Forks and Wagging Dogs—It's about the DNA 3' Tail

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Protecting, reversing, and remodeling stalled replication forks are critical to genome stability and require coordinating DNA replication, remodeling, and repair. In this issue, [Kile et al. \(2015\)](#) find that unexpected HLTF specificity for DNA's 3'-hydroxyl tail helps control these biological functions.

The discovery that the HIRAN domain of HLTF can use the DNA 3' tail to reverse replication forks, reported in this issue of *Molecular Cell* ([Kile et al., 2015](#)), provides insight into the complex, dynamic, and highly regulated process of fork protection and recovery. In the idiom of the “tail wagging the dog,” a small part is unexpectedly controlling the whole. Likewise, the magician's fork-bending trick implies unexpected control of a bending force. By analogy, HIRAN binding to the DNA 3' tail has exciting implications for a bending mechanism and reversal function of relatively large replication fork assemblies, which are crowded with massive machines that ensure genomic stability and life itself. Not only are there the canonical polymerases, helicases, clamps, clamp loaders, ligases, and endonucleases that ensure standard leading and lagging strand replication, there are “repirosomes” to deal with damaged and stalled replication forks, plus fork protection pathway complexes. With so many DNA-binding proteins, so much DNA and probable non-coding RNAs, the likelihood for traffic jams and mistaken identity is a logistical nightmare. In response to this extreme evolutionary pressure to maintain genome integrity, selection encompasses positive (to stabilize the substrate interaction) and negative (to destabilize the enormous number of off-target DNA sites) aspects of protein design. Thus, for DNA damage responses, negative design is critical to strengthen repulsive interactions with the large excess of non-target DNA that would otherwise overwhelm the intended complex by mass action. Negative design or preventing interactions that can lead to toxic intermediates is therefore as essential as encoding the actual activity. This

negative design is made even more difficult for enzymes that do not recognize a specific base damage but recognize DNA structures. They must recognize junctions of ssDNA and dsDNA in a specific context but not act on ssDNA or dsDNA alone. Due to the strong evolutionary selection for the target and against toxic and mutagenic incisions, this highly specific DNA structural recognition is likely encoded into and regulated by the protein structure. Importantly, [Kile et al. \(2015\)](#) take a detailed structural observation, HLTF binding to the 3' terminus of a ssDNA, and show that this is an essential activity for HLTF function.

HLTF, a RAD5 ortholog and a SWI/SNF2 family member, has two catalytic activities: (1) a ubiquitin ligase ([Unk et al., 2008](#)) and (2) a dsDNA translocase that can remodel stalled replication forks (splayed three-way dsDNA arms) into four-way junctions resembling a Holliday junction ([Blastyák et al., 2010](#)). Knockdown of HLTF leads to increased sensitivity to DNA damaging reagents UV and MMS, implicating HLTF in DNA damage response ([Unk et al., 2008](#)). Kile and co-workers used genetic, biochemical, and structural methods to probe HLTF. They show association of HLTF with active replication forks, with timing resembling RPA, PCNA, and RAD18. They determined that the HLTF HIRAN domain, named from its conservation in the N termini of two SWI/SNF2 translocases, HIP116 and Rad5, selectively binds to ssDNA at the 3' hydroxyl ([Figure 1](#)). The binding pocket resembles a small slot, formed by two tyrosines, and fits two unpaired nucleotides. A histidine packs against the sugar of the 3' terminal nucleotide. An aspartate, at the bottom, interacts with the hydroxyl and electrostatically and sterically pre-

vents any other modification at the 3' terminal, such as a 3' phosphate. Pertinent to negative design, binding to a hydroxyl of a 5' ssDNA terminus would be sterically prevented by the spacing of the slot and the aspartate. The authors identified which mutants in this pocket disrupted binding to ssDNA and examined correlation of ssDNA binding to known HLTF activities. Binding to the 3' ssDNA terminus was not required for splayed-arm DNA-dependent ATPase activity but was required to catalyze fork regression. Surprisingly, the mutants of the tyrosines forming the slot walls and Arg71 interacting with the DNA backbone had the most modest effect on ssDNA binding of the HIRAN residues tested, while also having the strongest effect on fork regression. Mutation of these residues perhaps interfered with the 3' versus 5' ssDNA terminus specificity. Importantly, the authors examined replication fork progression in vivo. Unexpectedly, they found that an HLTF knockout cell line had faster fork progression than WT, suggesting that WT HLTF slows down fork progression. Mutants with reduced affinity for 3' ssDNA termini showed the same phenotype as the null, so binding to the 3' ssDNA is key. Fork reversal and restart is a central mechanism in the protection and accurate processing of stalled replication forks that is paramount to genomic integrity, so HLTF binding and reversal at forks is expected to have broad biological impacts.

Yet, the authors showed that binding to a 3' hydroxyl on ssDNA is not absolutely required for fork regression, as other proteins that can regress forks (SMAR-CAL1, RECG, and UVSW) do not have such a requirement ([Kile et al., 2015](#)). That the first DNA complex of HLTF

showed this previously unrecognized specificity is reminiscent of flap endonuclease (FEN), where the first DNA complex revealed binding of an unpaired nucleotide and 3' hydroxyl into a highly specific pocket (Figure 1) (Chapados et al., 2004). It was later shown that the preferred 5' flap substrate had an unpaired 3' flap (Finger et al., 2009). Indeed, we now know that FEN1 uses the unpaired 3' flap to order a disordered region and help induce efficient incision (Tsutakawa et al., 2011; Tsutakawa and Tainer, 2012). The stimulated activity from binding of an unpaired nucleotide in the 3' hydroxyl pocket enables an end product that is immediately ligatable by replication ligases. Without the unpairing, there would be a 1-nucleotide gap. Although a strong preference for an unpaired 3' flap was not immediately apparent for the biology of a 5' flap-specific endonuclease, it is encoded in the tertiary structure as a key element of negative design. Thus, we postulate that binding to the hydroxyl of an unpaired 3' terminus is likely part of HLTF's functional specificity for reversing stalled replication forks.

These HLTF findings engender new questions and experiments. Might there be as-of-yet unidentified HLTF targets in the cell? Enzymes, whose specificity is for a specific DNA structure and independent of sequence, have a problem. They're extremely nearsighted by the physical chemistry of hydrophobic and electrostatic interactions, and it's not simple to select their target DNA structures over other substrates. So they must accurately sense their low-abundance substrates in the context of vastly greater concentrations of normal DNA and, at the same time, be blocked from binding and incising all incorrect substrates. Thus, HIRAN's specificity provides a clue for HLTF targets. A 3' ssDNA region is not inherently apparent in the model HLTF substrate, the

splayed arm. This lack of recognition also historically occurred with FEN1 and the 5' flap substrate. Would a splayed-arm substrate with two or more unpaired nucleotides at the 3' terminus actually be a better substrate? Why is there a clear specificity encoded structurally in the HIRAN domain slot for two unpaired nucleotides? Could the finding of ssDNA binding be a clue that HLTF can act in RNA binding, as found for TDP2 (Schellenberg et al., 2012; Shi et al., 2012)? What in the structural mechanism is revealed by the observation that the defect levels in ssDNA binding by the HIRAN mutants was not directly correlated to defect levels in the fork remodeling? How does HLTF impact the recently discovered protection pathway for stalled forks, which may involve fork reversal

(Schlacher et al., 2012), along with pathways for replication restart and repair?

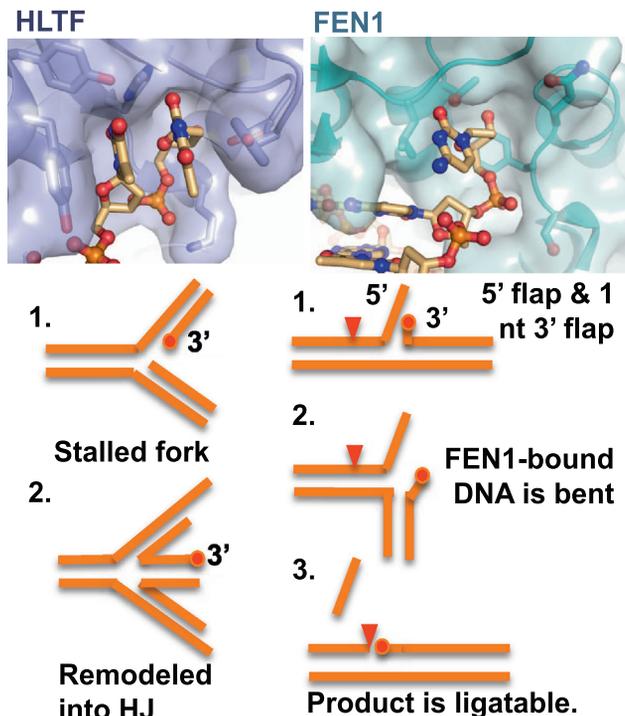
Currently, this integrated study unveils important binding and functional clues as to HLTF target substrates and function in cells. As fork reversal is increasingly recognized as a dynamic and regulated process with key implications for replication completion, genomic stability, and the DNA damage response, what HLTF is doing with the 3' tail promises to reveal fundamental mechanisms in biology. Stay tuned.

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**Figure 1. Pockets for a 3' Hydroxyl on an Unpaired Nucleotide**

Surface representations of FEN1 and HIRAN domain proteins show how the unpaired nucleotide(s) (stick representation) nestle between side chains with hydrogen bonding at the bottom of the pocket that selects for the 3' hydroxyl. The pockets break stacking with contiguous bases, selecting against dsDNA. (A) In the HIRAN domain of HLTF, the two unpaired nucleotides slot in between two tyrosines, with the 3' hydroxyl hydrogen bonded to an aspartate at the bottom of the pocket. The target substrate for HLTF is postulated to be a stalled replication fork with a product that resembles a Holliday junction. (B) FEN1 shows a similar specificity, albeit for one unpaired nucleotide (PDB: 3Q8K). The unpaired nucleotide packs between a leucine and glutamine, with hydrogen bonding of the 3' hydroxyl to a threonine. The reason for activity is that FEN1 incises its 5' flap substrate one nucleotide into the dsDNA. An unpaired nucleotide on the 3' flap is essential for obtaining a product with no gaps and one that is immediately ligatable.