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What a Difference a Phosphate Makes: Life or Death Decided by a Single Amino Acid in MDM2

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In this issue of *Cancer Cell*, Gannon and colleagues create genetically engineered mice to test the role phosphorylation plays in the modification of one serine long thought to play a critical role in controlling the activity of MDM2, one of p53's main negative regulators.

The tumor suppressor p53 is activated by numerous stressors and results in expression or repression of hundreds of genes that elicit a broad range of biological responses culminating in effective tumor suppression. However, p53 activation must be controlled with exquisite care because as little as a 2-fold reduction in its activity can cause radio resistance and increased tumorigenicity (Bond et al., 2004; Wang et al., 2009). Conversely, a 2-fold increase in p53 activity such as in $p53^{7KR/7KR}$ mice (a knock-in model in which seven conserved C-terminal lysine residues were replaced by arginine) can lead to myeloblation and death by heart failure (Wang et al., 2011).

Ubiquitin-mediated proteolysis is central to controlling p53's protein level and activity. In unstressed cells, the E3 ubiquitin-ligase MDM2 recruits E2 ubiquitin-conjugating enzymes to transfer ubiquitins onto p53 and MDM2 itself, resulting in proteasomal degradation of both proteins. MDM4 (also known as MDMX), a protein related to MDM2 but lacking intrinsic E3 ubiquitin-ligase activity, hetero-oligomerizes with MDM2 to modulate MDM2's E3 ligase activity (Wade et al., 2010). MDM2 and MDM4 play non-overlapping and tissue-specific roles to precisely control p53 levels and activity (Wade et al., 2010). Deleting *Mdm2* typically elicits a more extreme phenotype than deleting *Mdm4*, but eliminating p53 rescues both. This demonstrates that both MDM2 and MDM4 are critical nodes in p53 regulation. *MDM2* is a p53-induced gene, and in vitro studies show that increasing MDM2 abundance can attenuate p53 activation, leading p53 to return to low basal levels upon resolution of the inducing stress. The importance of this negative feedback loop for p53 regulation in vivo in different tissues remains unclear.

Correct temporal control of p53 responses is critical, but how this is achieved in vivo remains to be resolved. Posttranslational modifications play critical roles in p53 regulation, so the residues of p53, MDM2, and MDM4 that are modified by damage-activated kinases, phosphatases, and other modifying enzymes are prime candidates for temporal regulators. The acceptable thresholds for p53 regulation have been dramatically revealed by studies showing that mice heterozygous for *Mdm2* or *Mdm4*, with reduced expression of *Mdm2* or *Mdm4*, or with blocked posttranslational modification at damage-modifiable residues have profoundly altered radiation responses (Bondar and Medzhitov, 2010; Wang et al., 2009). Ionizing radiation activates DNA damage-activated kinases such as ATM and CHK2, resulting in phosphorylation of multiple residues on MDM2, MDM4, and p53 (Wade et al., 2010). Studies in human cancer cell lines first suggested that preventing MDM2 Ser395 (mouse Ser394) phosphorylation could impair its damage-dependent degradation and consequently attenuate p53 activation (Maya et al., 2001). DNA damage also induces MDM4 phosphorylation at serine 341,367, and 402, resulting in its MDM2-dependent degradation. Mice expressing Mdm4 3SA, an MDM4 mutant with alanine substitutions at these three positions, are remarkably resistant to ionizing radiation due to attenuated radiation-induced p53 responses in the hematopoietic system but are very sensitive to c-Myc induced lymphomagenesis (Wang et al., 2009). Both phenotypes result from a modest 2-fold reduction in p53 basal and induced activity. These data suggest the importance of regulating MDM4 stability in vivo for controlling p53 activity.

In this issue of *Cancer Cell*, Gannon et al. (2012) demonstrate the importance of MDM2 Ser394 phosphorylation in regulating the responses of mice to irradiation by making S394A (non-phosphorylatable) and S394D (phosphomimetic) mutations. They show that this amino acid can swing the pendulum

from exquisite radio-sensitivity to extraordinary radio resistance. Thus, just as shown for $Mdm4^{3SA/3SA}$ and $p53^{7KR/7KR}$ mice, it is not DNA damage caused by irradiation that induces lethality; rather, it is the p53 response that is critical. Importantly, and in contrast to $Mdm4^{3SA/3SA}$ mice that do not display increased lymphomagenesis following irradiation, 65% of $Mdm2^{S394A/S394A}$ mice developed spontaneous lymphomas in 24 months, a rate similar to that in $p53^{+/-}$ animals. These data indicate that proper MDM2 Ser394 phosphorylation contributes to effective p53-mediated tumor suppression. The mechanistic differences underlying the variance in lymphomagenesis remain unclear, though it appears that p53 levels are reduced significantly more in $Mdm2^{S394A/S394A}$ mice than in $Mdm4^{3SA/3SA}$ mice. Interestingly, the phenotype observed by Gannon et al. (2012) were not associated with differences in MDM2 stability, suggesting that another mechanism may be at play.

Given the attenuated p53 responses in $Mdm2^{S394A/S394A}$ mutants, would constitutive phosphorylation of this residue cause persistent p53 activation and consequent embryonic lethality similar to *Mdm2* and *Mdm4* null mice? Gannon et al. (2012) address this question by substituting Ser394 with aspartic acid (S394D) to create a phosphomimetic mutation. Surprisingly, $Mdm2^{S394D/S394D}$ mice were born at Mendelian ratios, and p53 baseline level and activity were similar to those in wild-type mice. The only difference appeared to be that the duration of irradiation-induced p53 activation was extended in $Mdm2^{S394D/S394D}$ cells. The authors posit that removal of damage-induced Ser394 phosphorylation may be critical for correct temporal regulation of p53 responses to radiation. Interestingly, the p53-induced phosphatase WIP1 can dephosphorylate MDM2 Ser395 and is overexpressed in breast and other cancers with wild-type p53, so WIP1 may play a role in the attenuation response (Lu et al., 2007). Further studies are needed to investigate this intriguing idea.

The surprisingly modest phenotype of $Mdm2^{S394D/S394D}$ mice could be explained in several ways. First, p53 activation may require modification of more MDM2 residues than just Ser394 as suggested by a recent study in human cancer cells (Cheng et al., 2011). A second possibility is that replacing Ser by Asp does not provide a faithful mimic of phosphorylation. The side-chain carboxyl groups in Asp and glutamic acid (Glu) exhibit a −1 charge at physiologic pH, while a phosphate group carries −1.5 charges. Furthermore, the atomic radius of a phosphate group is about three times that of the carboxyl side-chain of Asp or Glu. These differences may be critical when considering the mechanisms by which MDM2 Ser394 may actually impact MDM2 E3 ubiquitin-ligase activity.

How could substitutions of the single C-terminal residue Ser394 cause such dramatic phenotypes? Recent biochemical studies in human cancer cell lines suggest that mutations in six MDM2 C-terminal phosphorylation sites (S386, S395, S407, T419, S425, and S429; MDM2 6A) may impact MDM2 oligomerization and affect E3 ligase function (Cheng et al., 2011). Consistent with the $Mdm2^{S394A/S394A}$ results, the MDM2 6A mutant also exhibited increased E3 ubiquitin-ligase activity toward p53. These observations may be reconciled by a phosphorylation-induced structural alteration model, as observed recently for the E3 ubiquitin-ligase CBL (Dou et al., 2012). CBL regulates the stability of growth factor-activated receptor tyrosine kinases. In the absence of growth factor, CBL adopts an auto-inhibited conformation. However, upon growth factor signaling, CBL undergoes a conformational change that is initiated by phosphorylation of a single tyrosine residue (Y371). This enables CBL to better bind and position an E2 so that it can ubiquitylate its receptor tyrosine kinase substrate, resulting in a 1,400-fold increase in ubiquitylation activity (Dou et al., 2012). However, for MDM2, perhaps lack of phosphorylation enables E2 recruitment for the purpose of p53 degradation whereas phosphorylation may create a conformational change that prevents this. Alternatively, MDM2 S395 phosphorylation by ATM may activate p53 by increasing MDM2 binding of p53 mRNA which reportedly increases p53 mRNA translation (Gajjar et al., 2012). The importance of good hypotheses is that they suggest experiments to test them, and we suspect these will constitute the next phase of deciphering mechanisms of p53 regulation.

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