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## Review

# Ubiquitin-independent p53 proteasomal degradation

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**The mechanism of p53 proteasomal degradation through polyubiquitination is well characterized. The basic assumption behind this mechanism is that p53 is inherently stable unless sensitized to degradation by polyubiquitination. However, a number of studies provide evidence for p53 to be naturally unstable. Consistent with this attribute is the fact that both p53 N- and C-termini are intrinsically unstructured. Recent findings provide evidence for p53 to be degraded by the 20S proteasome by default unless it escapes this process. A number of mechanisms were demonstrated and proposed to play a role in rescuing p53 from default degradation. These mechanisms, their biological implications, and relevance to cancer are reviewed in this article.**

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Regulation of p53 stability is a central process in controlling p53 function. The process of p53 degradation has been intensively investigated and p53 has become a model in studying ubiquitin-dependent 26S proteasomal degradation. Several specific E3 ubiquitin ligases were reported to bind and polyubiquitinate p53, marking it for degradation by the 26S proteasomes.[1–4] However, a number of recent studies from different laboratories provided evidence for p53 proteasomal degradation regardless of its ubiquitination status, and, in fact, no other types of covalent modifications are involved in regulating this process. Thus, the current notion that proteins in general are stable unless destabilized by covalent modification is incomplete in the case of p53. These findings established a new principle in protein level kinetics that we termed 'degradation by default' or 'default degradation'. The emerging new principle is that intrinsically unstructured proteins (IUP) are inherently unstable and have to be stabilized during the course of their synthesis to prevent degradation by default. The degradation of the stabilized protein requires the active process of polyubiquitination to become susceptible to proteasomal degradation.[5]

### Ubiquitin-Independent p53 Proteasomal Degradation

The process of p53 degradation by default, a ubiquitin-independent process, was overlooked simply because it is a passive process. A breakthrough in the field came from the discovery that certain proteins stabilize p53. The first and the most studied one is NADH quinone oxidoreductase 1 (NQO1). NQO1, or DT-diaphorase, is a flavin-containing quinone reductase with a broad substrate specificity.[6] NQO1 catalyzes the reduction in various quinones through a two-electron reduction mechanism using either NADH or NADPH as a reducing cofactor, and it is inhibited by the competitive inhibitor dicoumarol.[7] This two-electron reduction prevents the formation of free radicals (semiquinones) and highly reactive oxygen species (ROS), thus protecting cells against quinones and their derivatives that are by large carcinogens. NQO1 can be induced by various stimuli including phenolic antioxidants, azo dyes, and oxidative stress.[8] Induction of NQO1 is considered to occur through both ARE and XRE elements in the NQO1 promoter[9,10] and regulated mainly by the transcription factor Nrf2.[11] As such NQO1 is considered to be an important defense against cancer.[11] In addition to this detoxifying role, NQO1 regulates p53 stability *in vitro* and in living cells. Human colon carcinoma cells that overexpress NQO1 accumulate elevated level of p53.[12] Accordingly, knockdown of NQO1 reduces the basal p53 level.[13] Furthermore, NQO1 null mice exhibit reduced p53 protein levels and decreased apoptosis in the bone marrow.[14]

NQO1 binds to p53 in an NADH-dependent manner.[15–17] Interestingly, dicoumarol and other competitive inhibitors of NQO1 compete with NADH for the binding to NQO1, resulting in the dissociation of the NQO1-p53 complex. Remarkably, p53 becomes highly unstable and prone to proteasomal degradation in the presence of these inhibitors. This process of p53 degradation is Mdm2 independent. Furthermore, the degradation takes place even under conditions whereby the pathway of protein ubiquitination is completely inhibited.[18] These findings and some others, as detailed below, argue for an alternative pathway of p53 degradation that takes place by default. Cells that are dicoumarol treated to induce p53 degradation by default escape DNA damage-induced apoptosis.[19,20] Another inhibitor of NQO1, which was recently identified, is curcumin. When normal T cells are exposed to DNA damage in the presence of curcumin, p53 accumulation is not induced, and T-cell apoptosis is significantly decreased.[21]

## Mechanisms of Ubiquitin-Independent p53 Degradation

The proteasome is a large, multi-catalytic protease that degrades proteins to small peptides. The 26S proteasome is composed of a core 20S catalytic chamber, capped at both ends with 19S regulatory units. The 19S regulatory particles are responsible for recognizing polyubiquitinated proteins, unfolding them, and opening an orifice into the 20S core catalytic chamber.[22,23] Free 20S core particles constitute a major portion of the total amount of proteasomes and are present both in the nucleus and cytoplasm of the cell.[24] Interestingly, NQO1 co-fractionates with the 20S core particle but not the 26S proteasome.[17] As NQO1 inhibits p53 degradation by default, this finding provided the first evidence that the 20S but not the 26S proteasome regulates this process. The 20S core of the 26S proteasome digests unfolded protein substrates, and the unfolding step is executed by the 19S regulatory particle in a process that is ATP dependent.[25] Given this reverse chaperon activity of the 19S that is important in denaturing the substrate to fit the 20S chamber, it was initially rather puzzling that the ubiquitin-independent process does not require the 19S particle. A number of in vitro studies showed that certain proteins that are naturally unfolded, such as proteins that are fully or regionally intrinsically unstructured, undergo degradation by the 20S proteasome. Recently, it was suggested that as much as 20% of all cellular proteins can be degraded or cleaved by the 20S proteasome specifically at unstructured domains.[26] In fact, the susceptibility to the 20S proteasome may be used as an operational definition approach to determine whether a given protein is unstructured.[27] Consistently, p53 that is unstructured at both N- and C-termini[28] undergoes 20S proteasomal degradation in vitro.[17]

The findings that NQO1 associates with the 20S proteasome, and that it prevents the degradation of proteins with unstructured regions, such as p53, p73,[17] and ODC,[29] is consistent with a model in which NQO1 plays the role of 'gatekeeper' of the 20S proteasome (Figure 1). NADH regulates the association of NQO1 with the potential 20S proteasome substrates,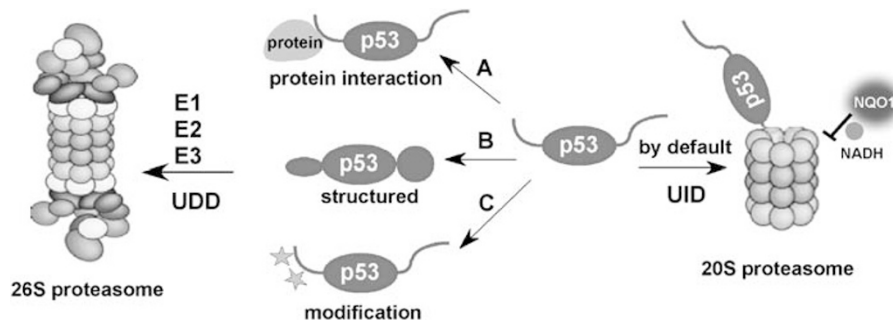 but does not control NQO1 association with the 20S proteasome.[17] At high levels of NADH the substrates are protected and do not enter the 20S catalytic chamber. At low levels of NADH the substrates are not effectively protected and are degraded by the proteasome. This model explains how certain small drugs that compete with NADH, such as dicoumarol, sensitize p53 to degradation.

Interestingly, a similar molecular mechanism was recently described in yeast in the context of the transcription factor Yap4 protein.[30] Lot6 is the NQO1 ortholog in yeast[31] and binds to the 20S proteasome. It was suggested that like p53 in mammalian cells Yap4 in yeast becomes associated with the Lot6–proteasome complex in the presence of NADH. NADH is needed because Lot6 must be reduced to bind Yap4. Remarkably, the binding of Yap4 to the Lot6–proteasome complex protects it from the ubiquitin-independent proteasomal degradation.

## Blocking Ubiquitin-Independent p53 Degradation

Protection by NQO1 is not the exclusive mechanism of controlling ubiquitin-independent p53 degradation. A second proposed mechanism is by formation of protein–protein complexes (Figure 2 option A).[27] Flexibility of structure, in general, has been shown to be associated with binding diversity,[32] and sites of enzyme-catalyzed posttranslational modifications,[33] specifically phosphorylation.[34] There are many proteins that interact with p53.[35,36] According to Genecards (http://www.genecards.org), there are more than 639 p53 interacting proteins. Most of the interacting proteins of p53 bind the unstructured N- or C-terminus. Usually the binding serves as a preliminary step of complex functionality or p53 modification. On binding, the unstructured termini of p53 are expected to acquire a specific structure, therefore preventing the 20S proteasomal-mediated degradation. For example, the SV40 Large T-antigen (LT) binds p53 and inhibits its degradation by default.[37] In other cases it has been documented that the interacting proteins such as Hif-1α, E2F-1, and WT1 stabilize p53.[38–40] The mechanism of this process of p53 stabilization has not yet been resolved but at least in the case of E2F the process does not involve Mdm2.[38] An additional example is the co-repressor Sin3a. The proline-rich region of p53 interacts with Sin3a in the process of p53-mediated transcription repression. Interestingly, expression of Sin3 results in posttranslational stabilization of both exogenous and endogenous p53, by inhibiting proteasome-mediated degradation.[41] Stabilization of p53 by Sin3 requires the proline-rich region of p53, and the Sin3-binding domain, correlating Sin3 binding to stabilization. Sin3 stabilizes p53 in an Mdm2-independent manner. It is therefore very likely that Sin3a, by binding p53, prevents p53 degradation by default.

A third mechanism is p53 conformational changes (Figure 2 option B). The DNA-binding domain is a structured segment of p53. There are well-established 'hot-spot' mutants of p53, overrepresented in the DNA-binding domain. The hot-spot mutants are thermodynamically less stable in structure than the wt p53[42] and accumulate in cells. The mutants may accumulate because unlike wild-type p53 they are poor in inducing Mdm2 expression and therefore escape Mdm2-dependent degradation.[43] We have earlier shown that some of the hot-spot mutants are less susceptible to degradation by



**Figure 1** Metabolic regulation of the degradation by default of p53. NQO1 can prevent the ubiquitin-independent degradation of p53 by the 20S proteasome. This process is active at high levels of NADH suggesting that the NAD$^+$/NADH ratio in the cells is crucial in regulating this process. Competitive inhibitors such as dicoumarol prevent the stabilizing effect of NQO1. Strong alterations in the NAD$^+$/NADH ratio in the cells are expected to affect the degradation by default of p53

**Figure 2** p53 degradation by default and escape mechanisms. p53 with exposed unstructured N- and C- termini is degraded by the 20S proteasome in a ubiquitin-independent manner (UID) by the 20S proteasome, a process that is regulated by NQO1 and NADH. p53 can also possibly escape the UID by binding to functional partners that mask the unstructured termini of p53 (A), by conformational mutations that make the unstructured domains inaccessible to the 20S proteasome (B) or by covalent modifications that enhance binding to other proteins and may also give rise to structuring of the unstructured domains (C). When p53 escapes degradation by default (options A, B, and C) it can be degraded by the second and well-characterized ubiquitin-dependent 26S proteasomal degradation pathway (UDD)

default in cells as they bind NQO1 with higher affinity.[16] Given the fact that some of the mutations strongly disrupt p53 conformation, they may fold into structures that resist degradation by the 20S proteasome. The loss of zinc binding to the DNA-binding domain of p53 has also been shown to increase thermodynamic instability, aggregation, and decrease specific DNA binding *in vitro*.[44,45] Zinc supplementation on the other hand restores wt p53 conformation to misfolded p53 because of HipK2 knockdown in the cells,[46,47] suggesting that Zn also affects p53 conformation and stability in cells, possibly by the mechanism of degradation by default.

A fourth mechanism involves protein modification (Figure 2 option C). Modification of an unstructured protein triggers changes that lead to coupled folding and binding to a target, possibly by formation of folded structures, as shown for the pKID domain of CREB.[48] Most of the p53 posttranslational modifications are at the N- and C-termini. The N-terminus of p53 is highly subjected to phosphorylation, consistent with the finding that overall unstructured domains are more susceptible to phosphorylations.[34] The C-terminus of p53 is subjected to many posttranslational modifications such as phosphorylations, acetylation, methylation, neddylation, ubiquitination, and sumolation, as reviewed.[49–51] These modifications can alter the ability of p53 to bind to different partners, or DNA, and thus result in either transcriptional regulation or de/stabilization depending on the residues modified. What still needs to be elucidated is the effect of these modifications on the structure of p53. It is very likely that phosphate or acetyl moieties can lower the degrees of freedom of the segment, giving rise to a more structured protein. If indeed, like binding to a protein, the posttranslational modifications themselves can induce p53 to adopt a distinct structure, this could strongly affect not only the specificity of interaction but also the ability to be degraded by the 20S proteasome.

### Two Pathways, Same Regulators

The discrimination between the two distinct proteasomal degradation pathways, ubiquitin dependent and ubiquitin independent, can be a difficult task as the two pathways can

be responsive to the same regulators. One example is the tumor suppressor p14[ARF] that inhibits both p53 ubiquitin-dependent degradation and p53 degradation by default. Consequently, the adenovirus E1A oncogene that stabilizes p53 by inducing p14[ARF] also inhibits p53 ubiquitin-independent degradation.[13] Thus, p14[ARF] exhibits a double lock activity by negating the two p53-degradation pathways, ensuring maximal p53 accumulation on demand. The viral oncogene SV40 LT also exhibits double lock activity by binding to p53 and protecting it from both Mdm2-mediated as well as ubiquitin-independent degradation.[12,19]

Conversely, there are regulators that are able to induce both UI and UD degradation. The human papilloma virus (HPV) E6 protein interacts with two regions of p53, the DNA-binding region and the C-terminus unstructured region. Binding of E6 to the DNA-binding region enhances p53 degradation through the ubiquitin pathway, whereas binding to the C-terminus enhances UI degradation that is likely to be 20S proteasomal default degradation.[52] Consistent with this possibility is the finding that the HPV E6 protein is ineffective in destabilizing p53 under overexpression of NQO1, which blocks degradation by default.[37]

### The Biological Meaning of p53 Degradation by Default

The p53 protein accumulates in response to various types of stress. On $\gamma$-irradiation (IR) p53 undergoes modifications and escapes Mdm2 and possibly other specific E3 ligase-mediated degradation.[53] Interestingly, exposure to IR increases the binding of p53 to NQO1, resulting in inhibition of degradation by default and p53 accumulation. NQO1 knockdown, or its inhibition by dicoumarol, increase p53 degradation by default.[17] Under these conditions p53 does not accumulate and p53-dependent apoptosis is compromised. NQO1 null mice show increased sensitivity to benzo(a)pyrene and DMBA-induced skin cancer.[54,55] Studies performed with these mice revealed that treatment with benzo(a)pyrene fails to significantly increase p53 protein level and apoptosis in the skin of NQO1 null mice compared with wild-type mice.[55]

Inhibition of ubiquitin-independent degradation of p53 by NQO1 plays a role in p53 accumulation under oxidative stress as well.[12] Recently, it was shown that mitochondrial

respiration is affected by p53 (discussed below).[56] In this case a regulatory loop is created, whereby ROS, produced by aerobic respiration, are likely to induce Nrf2 nuclear translocation. Nrf2 binds the NQO1 promoter to increase NQO1 production.[57] NQO1 in turn stabilizes p53 but also through the consumption of NADH blocks the formation of ROS, a process that can be blocked by NQO1 inhibitors such as dicoumarol and curcumin (Figure 3).
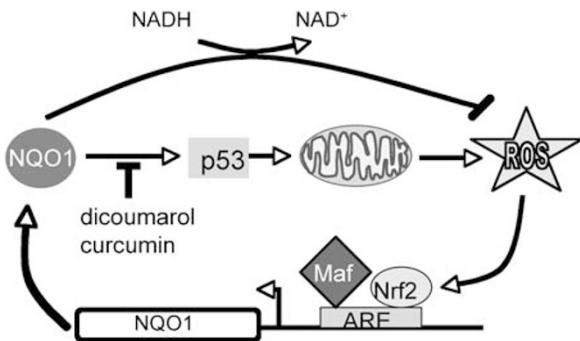


**Figure 3** NQO1 regulation under oxidative stress. p53 plays an important role in the process of aerobic repiration[56] by upregulating mitochondrial activity. The by-product of mitochondrial respiration is ROS that increase cellular oxidative stress. On oxidative stress, Nrf2 is activated and together with Maf basic leucine zipper transcription factor binds the ARE of the NQO1 promoter and induces its expression. Accumulated NQO1 may further support p53 expression but also through the consumption of NADH blocks the formation of ROS to decrease intracellular oxidative stress. Dicoumarol and curcumin are two small molecules that inhibit NQO1 enzymatic activity and hence increasing p53 degradation by default

## p53–NQO1 and Metabolism

It has been postulated that a fundamental cause for cancer is a drastic change in the cell metabolism, known as the Warburg effect. This was based on the observation that many cancer cells predominantly produce energy by the process of glycolysis rather than oxidative phosphorylation even when oxygen is abundant.[58] The association of p53 mutation with cancer is well established, but only recently a number of studies have linked p53 to metabolism.

P53 was shown to influence mitochondrial activity. Using a mouse model, it was shown that the loss of p53 is correlated with less oxygen consumption suggesting that mitochondrial respiration is affected by p53. P53 upregulates SCO2, a gene required for the assembly of a critical component of the mitochondrial COXII complex, which is required for aerobic respiration.[56] Others have also shown that in p53 null mice there is a decrease in basal mitochondrial function, which might be explained by the decrease in the mitochondrial synthesis regulator PGC-1$\alpha$.[59]

On the other hand, p53 inhibits glycolysis by inducing TIGAR (TP53-induced glycolysis and apoptosis regulator) expression.[60] This gives rise to the activation of pentose phosphate pathway (Figure 4). This pathway is one of the three main ways the body creates molecules with reducing power, accounting for approximately 60% of NADPH production in humans. NADPH is required for reduction in oxidized glutathione (GSSG) to GSH that in turn can reduce hydrogen peroxide. In addition, high NADPH activates NQO1 to protect p53. In this pathway the level of NQO1 is downregulated by
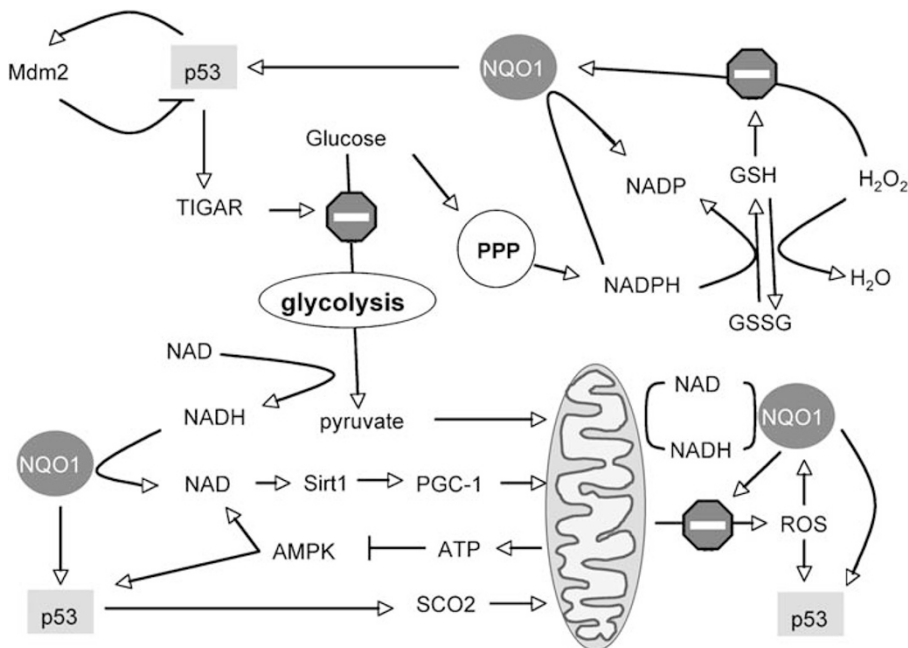


**Figure 4** p53–NQO1 cross talk and metabolism. An overview of the key points in which p53 and NQO1 are in direct cross talk to regulate metabolism and oxidative stress. As explained in the text, p53 through TIGAR regulates glucose catabolism to generate high levels of NADPH, which in turn reduces the cellular oxidative stress. Under this condition, NQO1 level and activity are regulated in harmony with the NADPH level. Another pathway of glucose consumption is glycolysis, to generate pyruvate, which might be further processed in the mitochondria. These steps are accompanied with NADH formation that is crucial to the process of oxidative phosphorylation to generate ATP. NADH is consumed by NQO1 in the process of reduction of quinones, to form NAD$^+$. NAD$^+$ in turn regulates mitochondrial activity through Sirt1 and PGC-1. AMPK activates p53 and stimulates NADH consumption to form NAD$^+$. AMPK activity is blocked when cells reach high ATP levels. Active mitochondria generate ROS that are partially neutralized by NQO1

preventing the activation of NQO1 because of the antioxidant activity of GSSG reduction (Figure 4).

An important value in sensing the metabolism dynamics in the cell is the $NAD^+$/NADH ratio. For example, if the aerobic energy production is high and glycolysis is low this ratio is expected to increase. NQO1 is an oxidoreductase that uses NAD(P)H as a cofactor reducing it to $NAD(P)^+$. Therefore, active NQO1 decreases the level of NAD(P)H and increases the level of $NAD^+$, strongly affecting the $NAD^+$/NADH ratio. $NAD^+$ is a cofactor of Sirt1 and at desired $NAD^+$/NADH ratio, Sirt1 is activated to increase PGC-1 activity, which in turn increases mitochondria level and activity.[61] Thus, at high glycolysis levels when NAD(P)H is high, NQO1 not only can stabilize p53, a process that is NADH dependent, but also can elevate $NAD^+$/NADH levels. Both processes support 'normal' aerobic mitochondrial energy production (Figure 4). Furthermore, $NAD^+$ at high concentrations has been shown to bind p53 and modulate its binding to DNA.[62] The emerging picture is that the cross talk between p53 and NQO1 is important to regulate not only the p53 protein levels but also the metabolic state of a cell. On one hand, p53/NQO1 increase aerobic respiration and energy production and on the other hand effectively reduce ROS, the hazardous by-product of high aerobic metabolism (Figure 4).

### Relevance to Cancer

p53 is widely mutated in more than 50% of human cancers.[63,64] Most p53 mutant proteins accumulate to relatively high steady-state levels. This behavior is typically explained by the fact that a majority of p53 mutant proteins are defective in inducing Mdm2 expression and therefore escape degradation. This has implications for cancer, as many of the p53 mutants display 'gain of function' activities that are tumorigenic. As described above, some of the p53 mutants are more resistant to the ubiquitin-independent pathway simply by binding NQO1 with higher affinity.[16] This provides another mechanism for relatively high steady-state expression levels of these mutant proteins in cancer cells.[65]

By virtue of its ability to protect p53 from ubiquitin-independent degradation, NQO1 can be considered a tumor suppressor. Interestingly, this role was confirmed by a bioinformatics search for tumor suppressors, which studied contributions of the different types of genetic alterations to loss of function: amino-acid substitutions, frame-shifts, and gene deletions. One hundred and fifty-four candidate recessive cancer genes were identified, and among them is NQO1.[66] Consistent with this study is the finding that in 50% of liver cancer cases the NQO1 gene is heavily methylated and poorly expressed.[67] In addition, there is considerable supportive evidence from human epidemiology studies showing that NQO1 protects against tumorigenesis.

A genetically polymorphic C609T NQO1 gene encoding a biologically inactive and unstable NQO1 P187S enzyme was detected in humans.[68,69] The product of this NQO1 genotype is less active in stabilizing p53.[18] Several studies have found that the C609T allele is associated with increased risk of developing different types of tumors such as urothelial tumors, basal cell carcinoma, pediatric leukemia, colorectal cancer, esophageal squamous cell carcinoma, and gastric carcinoma.[70]

A recent paper reports that this polymorphism is a strong prognostic and predictive factor in breast cancer.[71] This paper shows that the NQO1 polymorphism leads to lower basal levels of p53, and also has an effect on the accumulation of p53 in response to epirubicin, in addition to p53-independent effects. These studies support the hypothesis that ubiquitin-independent degradation of p53 is a significant process and NQO1, an enzyme that inhibits this process, is important to keep the tumor suppressor function of p53 effective. NQO1, thus, plays a dual role in protection from carcinogenesis; its anti-oxidative properties protect the cell from carcinogenic oxidative damage, whereas its ability to stabilize the tumor suppressor p53 is important for eliminating damaged cells that are prone to develop cancer.

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