Transducing oxidative stress to death signals in neurons

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Accumulation of reactive oxygen species (ROS) has been associated with aging and neurodegenerative diseases. Nevertheless, how elevated ROS levels cause neurodegeneration is unclear. In this issue, Wakatsuki et al. (2015. *J. Cell Biol.* http://dx.doi.org/10.1083/jcb.201506102) delineate how oxidative stress is transduced into death signals, leading to neuronal apoptosis and axonal degeneration.

The human brain consumes ∼20% of the body's energy in the resting state. Oxygen metabolism produces reactive oxygen species (ROS) as a byproduct. Under normal conditions, ROS regulate redox homeostasis and serve as important messengers in cell signaling. However, when environmental stressors exacerbate ROS generation or when detoxification mechanisms fail to remove excessive ROS, the imbalance results in abnormally high levels of ROS that become toxic to cells (referred to as oxidative stress). Neurons have high energy–demanding activities, which cause significant challenges for ROS detoxification, especially in highly specialized cellular compartments such as branchy dendrites and lengthy axons. In addition, because neurons are postmitotic cells and have limited capacity to regenerate in the adult central nervous system, they are especially prone to oxidative stress and its consequences (Mattson and Magnus, 2006).

Oxidative stress has long been associated with human neurological disorders such as Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, and Friedreich ataxia (Andersen, 2004; Calabrese et al., 2006). For example, mutations of known Parkinson's disease–associated genes, including *PAR7*, *PINK1*, *PARK2*, *SNCA* (encoding α-synuclein), and *LRRK2*, can directly or indirectly impair mitochondrial functions and lead to increased ROS levels as well as vulnerability to oxidative stress (Dias et al., 2013). Similarly, mutations in the gene encoding superoxide dismutase 1 (SOD1), a key antioxidative enzyme in the cell, account for ∼20% of familial amyotrophic lateral sclerosis cases (Barber and Shaw, 2010). Oxidative stress can exert cytotoxic effects through the generation of peroxides and free radicals that damage DNA, proteins, and lipids. However, the pathogenic mechanisms and the exact signaling pathways by which oxidative stress causes neurodegeneration are elusive. In this issue, Wakatsuki et al. show that the E3 ubiquitin ligase zinc and ring finger 1 (ZNRF1) plays a critical role in mediating oxidative stress–induced neuronal cell death and axonal degeneration.

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In a previous study by the Araki group, Wakatsuki et al. (2011) demonstrated that ZNRF1 targets AKT for ubiquitin proteasome system (UPS)–dependent degradation in Wallerian degeneration, the progressive degeneration of the distal axonal segment that is separated from the neuronal cell body in nerve injury. Removal of AKT releases phosphorylation suppression on glycogen synthase kinase 3β (GSK3β), which subsequently phosphorylates and induces collapsin response mediator protein 2 (CRMP2) degradation. CRMP2 is required for microtubule stabilization. Thus, the ZNRF1–AKT–GSK3β–CRMP2 pathway mediates axon destruction in Wallerian degeneration (Wakatsuki et al., 2011). As ZNRF1 functions as a key mediator for axonal degeneration, it is reasonable to hypothesize that this pathway may also participate in another major form of neurodegeneration—neuronal cell death. With a particular interest in oxidative stress–induced neurodegeneration, in this study, the authors first used a mouse model of focal cerebral ischemia to demonstrate that CRMP2 phosphorylation is increased in ischemic neurons. They then applied 6-hydroxydopamine (6OHDA) and H_2O_2 (both are frequently used to induce cellular oxidative stress) in primary cultured cortical neurons. They found that oxidative stress triggers AKT ubiquitination and degradation, which is prevented by overexpressing a dominant-negative form of ZNRF1 or by RNAi knockdown of endogenous ZNRF1. These results indicated that the ZNRF1–AKT–GSK3β–CRMP2 pathway is indeed activated in neurons upon oxidative stress.

How then is ZNRF1 activated by oxidative stress? An important clue came from the observation that ZNRF1 is highly phosphorylated in SH-SY5Y neuroblastoma cells when treated with 6OHDA (Wakatsuki et al., 2015). A web-based program predicted that ZNRF1 may be phosphorylated at the tyrosine residue Y103 by EGF receptor (EGFR) tyrosine kinase. Using a combination of primary neuronal culture, in vivo mouse models of cerebral ischemia and 6OHDA-induced brain lesions, and in vitro kinase assay, Wakatsuki et al. (2015) demonstrated that ZNRF1 is specifically phosphorylated at Y103 by EGFR in response to oxidative stress. This activation of ZNRF1 resulted in neuronal apoptosis, as evidenced by increases in caspase 3 cleavage, annexin V–positive staining, and lactate dehydrogenase release. Moreover, the authors found that application of antioxidants such as *N*-acetyl-L-cysteine and curcumin, down-regulation of EGFR activity by siRNA or via the EGFR inhibitor C56, and expression of the dominant-negative form ZNRF1 C184A or the phosphorylation-resistant mutant

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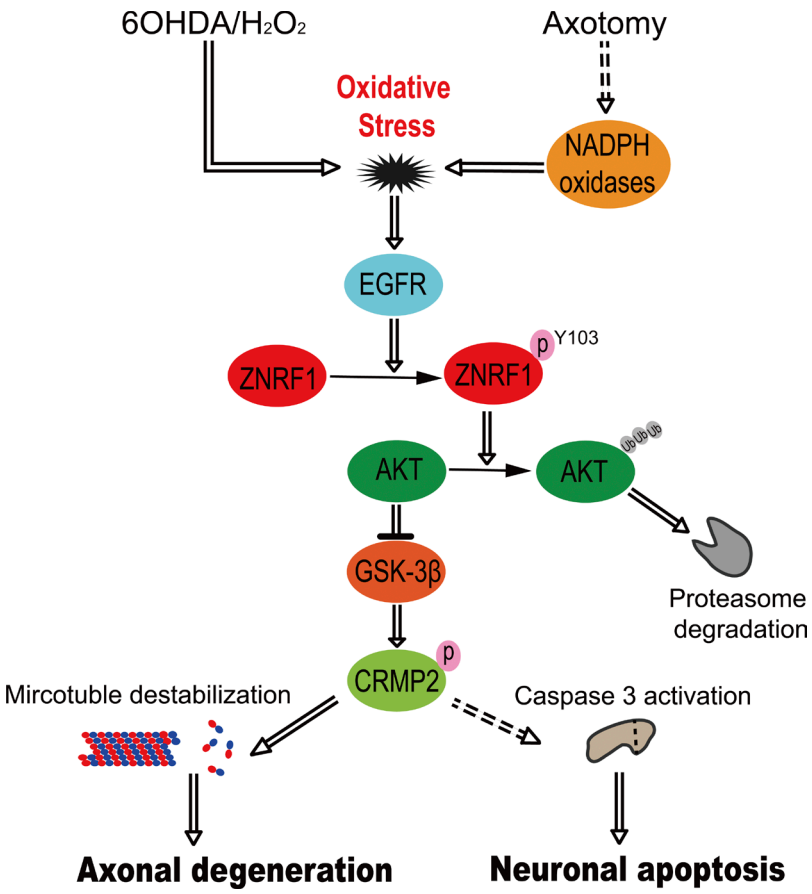

Figure 1. **The ZNRF1 signaling pathway mediates oxidative stress–induced neuronal apoptosis and axonal degeneration.** Oxidative stress induced by treatment with 6OHDA or H_2O_2 or generated by NADPH oxidases in axons upon traumatic injury activates EGFR tyrosine kinase activity and leads to ZNRF1 phosphorylation at Y103. This stimulates the E3 ubiquitin ligase activity of ZNRF1, which ubiquitinates and targets AKT for degradation via the UPS. Degradation of AKT relieves the inhibitory phosphorylation of GSK3β, which then phosphorylates CRMP2 and subjects it to degradation. In axons, CRMP2 is required for microtubule stabilization, whose disassembly results in axonal degeneration. In soma, oxidative stress activates this same ZNRF1 signaling pathway, which causes cleavage and activation of caspase 3, leading to neuronal apoptosis.

ZNRF1 Y103F all prevented 6OHDA-induced neuronal apoptosis. Furthermore, Wakatsuki et al. (2015) characterized the cellular signaling downstream of ZNRF1 and showed that EGFR-dependent ZNRF1 phosphorylation stimulated AKT ubiquitination and degradation. Expression of either a constitutively active form of AKT or a kinase-dead form of GSK3β potently suppressed 6OHDA-induced neuronal cell death (Wakatsuki et al., 2015). Together, these results demonstrate that EGFR-dependent phosphorylation of ZNRF1 at Y103 promotes degradation of AKT and resultant activation of GSK3β, which mediates oxidative stress–induced neuronal apoptosis (Fig. 1).

Does axonal degeneration also use oxidative stress–induced activation of ZNRF1 signaling? If so, where does oxidative stress come from in the first place? Another important finding of this study is that traumatic neural injury induces oxidative stress in axons by NADPH oxidases (Wakatsuki et al., 2015). Using an in vitro model of Wallerian degeneration by primary cultured dorsal root ganglion (DRG) neurons, Wakatsuki et al. (2015) showed that the levels of oxidative stress, the activity of the endogenous EGFR kinase, and the EGFR-dependent phosphorylation of ZNRF1 at Y103 in injured neurites are all robustly increased as early as 3 h after transection. In an attempt to identify the generators of oxidative stress in injured axons, Wakatsuki et al. (2015) examined the effects of inhibiting NAD PH oxidase activity. They found that the NADPH oxidase inhibitors not only prevented injury-elicited elevation of oxidative stress, but also remarkably suppressed Wallerian degeneration. Knockdown of the NADPH oxidase catalytic subunits by RNAi in cultured DRG neurons confirmed these results and further pointed out that the NADPH oxidases NOX2, 3, 4, and DUOX2 may be particularly involved in this process (Wakatsuki et al., 2015). Consistent with their previous study (Wakatsuki et al., 2011) and similar to oxidative stress–induced neuronal apoptosis, interruption of the ZNRF1 signaling pathway at each step significantly delayed Wallerian degeneration of injured axons in cultured DRG neurons (Fig. 1). Finally, Wakatsuki et al. (2015) generated transgenic mice expressing the dominant-negative mutant ZNRF1 C184A and validated the hypothesis that blocking the ZNRF1 signaling cascade protects neurons from oxidative stress–induced cell death and axonal degeneration in vivo.

Wakatsuki et al. (2015) used a combination of pharmacological, genetic, biochemical, immunohistological, and other approaches to unveil the involvement of the EGFR–ZNRF1–AKT–GSK3β–CRMP2 pathway in oxidative stress–induced neurodegeneration. This is a Herculean task, especially given that this is a multistep signaling cascade, and the authors made tremendous efforts to confirm their results in various experimental setups from both in vitro and in vivo models, which establish the oxidative stress–induced, EGFR-dependent activation of the ZNRF1 E3 ligase activity as a common signaling mechanism in both of the two major neurodegeneration forms—neuronal apoptosis and axonal degeneration (Fig. 1).

The ubiquitin–proteasome-mediated degradation system plays an important role in regulating protein homeostasis and is involved in neurodegenerative diseases (McKinnon and Tabrizi, 2014; Zheng et al., 2014). In addition, emerging evidence has linked the UPS to Wallerian degeneration: inhibition of the UPS activity by both pharmacological and genetic methods remarkably suppressed axonal degeneration both in vitro and in vivo (Zhai et al., 2003). A recent study in *Drosophila melanogaster* revealed that *highwire*, an E3 ubiquitin ligase, promotes Wallerian degeneration by targeting the NAD^+ biosynthetic enzyme

nicotinamide mononucleotide adenyltransferase (Nmnat) for degradation (Xiong et al., 2012). And now, the study by Wakatsuki et al. (2015) exemplifies a mechanism by which E3 ligase ZNRF1 acts as a mediator to transduce oxidative stress to death signals in neurons. It should be noted, however, that the ZNRF1 signaling pathway is unlikely to be the only mechanism promoting neurodegeneration. For example, inhibition of ZNRF1 signaling offers axon protection for up to 48 h, whereas expression of the neuroprotective Wld^S protein (Lunn et al., 1989; Coleman and Freeman, 2010; Fang and Bonini, 2012) or downregulation of the prodegenerative *SARM1* gene (Osterloh et al., 2012; Gerdts et al., 2015; Yang et al., 2015) protects injured axons for up to 72 h (Wakatsuki et al., 2015). These results strongly suggest that there are other mechanisms transducing neural injury and oxidative stress to degenerative signals in neurons.

The exciting findings by Wakatsuki et al. (2015) have raised a number of further questions. One immediate question is how EGFR senses oxidative stress. EGFR exists on the cell surface and dimerizes upon binding to its specific ligands that activate its intrinsic intracellular protein–tyrosine kinase activity (Herbst, 2004). In the context of cellular oxidative stress, how is EGFR activated? A second question is, in addition to the ZNRF1–AKT–GSK3β–CRMP2 pathway, is any other signaling pathway downstream of EGFR also activated? A recent study reported that the MAPK cascade is activated in the early response of axon injury (Yang et al., 2015). MAPK pathway activation is another well-known outcome of EGFR signaling (Nguyen et al., 2013). Of note, although Wakatsuki et al. (2011, 2015) argue that AKT phosphorylates and inhibits GSK3β, which subsequently stabilizes CRMP2 and microtubules to prevent axonal degeneration, Yang et al. (2015) claim that AKT promotes axonal survival by phosphorylation of MKK4 at serine 78, which suppresses MKK4-mediated activation of prodegenerative JNK signaling. Further study of EGFR signaling in oxidative stress and neural injury is needed to provide a better understanding of the regulatory mechanisms at different stages of the degenerative process. Third, although NADPH oxidases are involved in the elevation of oxidative stress in injured axons (Wakatsuki et al., 2015), it remains unclear whether this is because axon injury promotes the activity of NADPH oxidases or because the ROS detoxification system is impaired in injured axons and the NADPH oxidase activity is merely required to maintain a steady-state level of ROS. Fourth, what is the relationship between the ZNRF1 signaling pathway, Wld^S/Nmnat, and SARM1 in axonal degeneration? Does Wld^S/Nmnat or loss of function of SARM1 manifest axonal protection by blocking a step in the EGFR–ZNRF1–AKT–GSK3β–CRMP2 axis? Finally, because EGFR has been successfully targeted in the development of antitumor drugs, identification of specific inhibitors of the EGFR–ZNRF1 signaling cascade holds a high hope for the development of effective therapeutics treating neuronal and axonal degeneration in diseases and traumatic injury. And the ultimate question is, when?

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