ROS

REVIEW ARTICLES

Parkin New Cargos: a New ROS Independent Role for Parkin in Regulating Cell Division

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ABSTRACT | Cell cycle progression requires the destruction of key cell cycle regulators by the multi-subunit E3 ligase called the anaphase promoting complex (APC/C). As the cell progresses through the cell cycle, the APC/C is sequentially activated by two highly conserved co-activators called Cdc20 and Cdh1. Importantly, APC/C^{Cdc20} is required to degrade substrates in G2/M whereas APC^{Cdh1} drives the cells into G1. Recently, Parkin, a monomeric E3 ligase that is required for ubiquitin-mediated mitophagy following mitochondrial stress, was shown to both bind and be activated by Cdc20 or Cdh1 during the cell cycle. This mitotic role for Parkin does not require an activating phosphorylation by its usual kinase partner PINK. Rather, mitotic Parkin activity requires phosphorylation on a different serine by the polo-like kinase Plk1. Interestingly, although $Parkin^{Cdc20}$ and $Parkin^{Cdh1}$ activity is independent of the APC/C, it mediates degradation of an overlapping subset of substrates. However, unlike the APC/C, Parkin is not necessary for cell cycle progression. Despite this, loss of Parkin activity accelerates genome instability and tumor growth in xenograft models. These findings provide a mechanism behind the previously described, but poorly understood, tumor suppressor role for Parkin. Taken together, studies suggest that the APC/C and Parkin have similar and unique roles to play in cell division, possibly being dependent upon the different subcellular address of these two ligases.

KEYWORDS | Anaphase promoting complex; Cell cycle; Parkin; Parkinson's disease; Reactive oxygen species; Tumor suppressor

ABBREVIATIONS | APC/C, anaphase promoting complex; CRL, cullin-RING ligase; DA, dopaminergic; MDV, mitochondria-derived vesicle; MEF, mouse embryonic fibroblast; PD, Parkinson's disease; PINK1, PTEN-induced kinase 1; Plk1, polo-like-kinase 1; ROS, reactive oxygen species

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1. INTRODUCTION

It has been known for many years that Parkinson's disease (PD) arises due to premature death of dopamine-containing neurons [1]. Despite the pathology of this disease being clear, the molecular mechanisms driving neuronal cell death are less well understood. Accumulating evidence indicates that neurons from PD patients are less capable of maintaining their redox potential due to decreased ability to detoxify the reactive intermediates of oxidative stress [2]. This results in perilous effects that can trigger neurotoxicity including the production of the H_2O_2 from defective dopamine oxidation, upregulation of iron ions which can then generate reactive oxygen species (ROS) and impaired complex I activity which leads to increased ROS and decreased glutathione (GSH) levels [3]. Thus it is hypothesized that the devastating pathological effects seen in PD are due in part to the inability of neurons to mount an effective adaptive oxidative stress response. Consistent with this model, it has been shown that culling defective mitochondria and regulating mitochondrial movement play a key role in maintaining energy balance [4]. Thus, it is generally accepted that mitochondrial malfunction is likely to be an important component of neuronal death.

A breakthrough into understanding the etiology of neuronal cell death observed in PD patients came when it was discovered that loss of Parkin (PARK2) function contributes to familial PD [5, 6]. Parkin belongs to the RBR (Ring-between-Ring) class of E3 ubiquitin ligases, which utilize both RING and HECT domains to transfer ubiquitin to substrate proteins [7–9]. Parkin polyubiquitylates its substrates through K48 linkages, which lead to destruction by the 26S proteasome [5, 6]. Thus, upon discovering that Parkin is an E3 ligase, it was hypothesized that the death of dopaminergic (DA) neurons observed in PD could be solely due to toxicity associated with accumulation of Parkin substrates [10]. Indeed, mouse models have identified a Parkin substrate protein called PARIS whose upregulation causes DA neuron death [11]. However, this discovery is the exception rather than the rule as most Parkin-knockout mouse models fail to model the human pathophysiology and selective DA neuron loss [12]. Consistent with this, it has now been shown that Parkin can also catalyze mono-ubiquitylation with different lysine linkages (K6, K11, or K63) [13, 14]. These lysine linkages label substrates involved in many fundamental processes including signal transduction and autophagy [7, 13]. The most studied of these roles is the ability of Parkin to remove impaired mitochondria by mitophagy [15] as well as having a role to play in mitochondrial morphology [16]. Thus, Parkin has been implicated in having a neuroprotective role and prevents cell death in various stress paradigms including mitochondrial and ER stress [17]. This pro-life role of Parkin is particularly important in post-mitotic neurons that cannot use established cell cycle mechanisms to remove damaged cells.

In addition to its role in neuron protection (pro-life), Parkin has been earmarked as a tumor suppressor gene since 2003 [18]. However, the molecular mechanism by which Parkin prevents aberrant cell division has until recently remained elusive. PARK2 is located on a common fragile site FRA6E, a mutational hotspot that is frequently deleted in multiple tumor types [19]. Mutated PARK2 is also found in many human cancers as well as in hereditary Parkinson's disease (**Table 1**) [20, 21]. Also, recent studies have revealed that p53 down regulates Parkin expression and this contributes to the role of p53 in regulating glucose metabolism [22]. Although the exact molecular mechanisms are not yet fully understood, Parkin deficiency activates glycolysis and reduces mitochondrial respiration, leading to the Warburg effect in human cancer cells [23]. Further clues as to how PARK2 loss leads to cell cycle deregulation and oncogenesis were provided in 2014 when it was discovered that PARK2 is an important coordinator of G1/S-phase cyclin dependent kinases CDK2, 4, and 6 [24]. These CDKs are commonly deregulated in cancer [25]. In an elegant paper Timothy Chan and colleagues showed that in normal cells Parkin forms complexes with the two cullin-RING ligases (CRLs) that target cyclin D1 and cyclin E1 for degradation via the 26S proteasome. In Parkin-deficient cells, these novel CRLs are non-functional, leading to accumulation of proto-oncogenic cyclin D and cyclin E and eventual tumorigenesis [24].

TABLE 1. List of phenotypes linked to Parkin deficiency in various cell types

Cell Genotype	Effect	References
Parkin-deficient primary neurons and PARK2-deficient cell lines	Cyclin E or CDK4 and cyclin D accumulate	[24, 26]
Ovarian, breast, hepatocellular, bladder squamous cell, and non-small cell lung cancers	6q26, the locus that Parkin lies on, shows loss of heterozygosity	[24, 27]
Acute lymphocytic leukemia (ALL), chronic myeloid leukemia (CML), and some colorectal cancer cells	Abnormal methylation in PARK2 gene results in a decreased expression of Parkin	[28]
Hepatocarcinoma, glioblastoma, lung cancer, breast cancer, and colon cancer cell lines	Overexpression of Parkin represses cell growth	[21, 27]
Pancreatic cancer	Parkin deficiency contributes to pancreatic tumorigenesis by inducing spindle multipolarity and misorientation	[29]
Human cancer cells	Parkin deficiency contributes to the p53-mediated Warburg effect	[23]
Parkin-knockout MEFs	Escape senescence and become transformed	[30]
Parkin-knockout MEFs	More sensitive to BI2536 (a Plk1 inhibitor)	[30]
Parkin$^{-/-}$ mice	Suppression of caspase activity, enhanced hepatocyte proliferation, and resistance to apoptosis, resulting in hepatic tumor development	[31]

Excitingly, Zhenkun Lou and colleagues at the Mayo Clinic in Rochester have now shown that Parkin plays an important role in the degradation of key regulatory proteins controlling the metaphase-anaphase transition and mitotic exit [30]. Briefly, by analyzing Parkin-deficient mouse embryonic fibroblasts (MEFs) and xenograft models, this group revealed that Parkin plays an important role in the degradation of key regulatory proteins controlling the metaphase-anaphase transition and mitotic exit. Previously thought to be the realm of the anaphase promoting complex (APC/C) ubiquitin ligase, Parkin directly mediates the destruction of many APC/C substrates. Similar to APC/C defects that lead to aberrant chromosome segregation and genomic instability [32], this observation coupled with its role in controlling S-phase entry [24] could explain why Parkin-deficient cells are linked to many cancers. Here we summarize the paper by Lee et al. [30] that establishes this exciting new role for Parkin to play and compare the new findings to the more established role of Parkin in mitophagy.

2. NEUROPROTECTIVE ROLE OF PARKIN

One of the main jobs of Parkin in the brain is to effectively negate the harmful effects of ROS. This is important as the brain utilizes about 20% of the oxygen supply of the body, a significant portion of which is converted to ROS during mitochondrial electron transport [33]. High levels of oxidative stress in the brain are linked to many neurodegenerative diseases and can arise from impaired mitochondrial function [34]. Mitophagy in neurons thus plays an important protective role as it is a mechanism by which defective mitochondria can be culled without killing the cell. The role Parkin plays in mitophagy has been the subject of many excellent reviews and is outlined in **Figure 1** [13, 35, 36]. In brief, under physiological conditions most of Parkin is located in the cytosol. Upon sensing depolarized mitochondria Parkin is recruited from the cytosol to the mitochondria to mediate the selective autophagic removal of the damaged mitochondria [37]. This change of subcellular address is dependent upon Parkin being

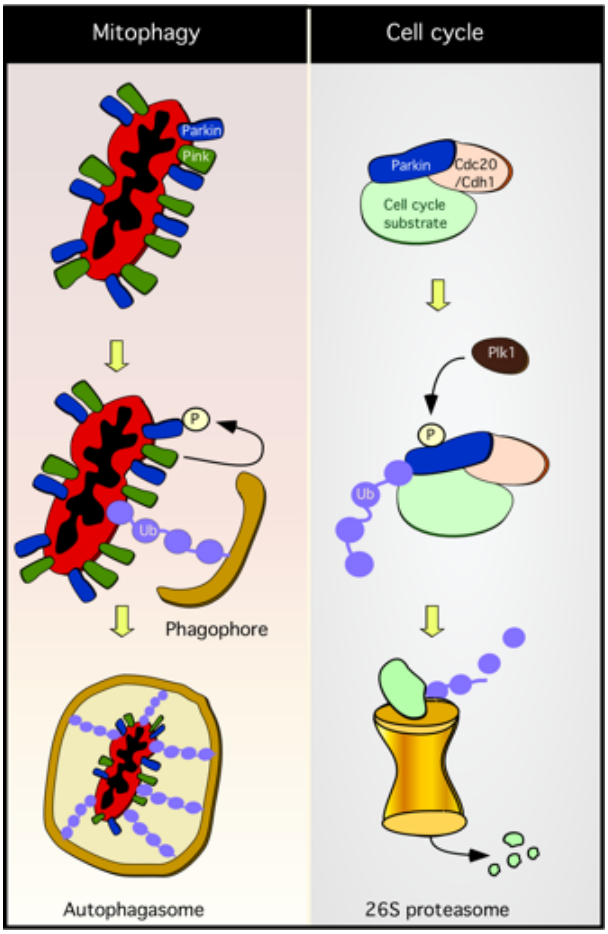

FIGURE 1. Parkin forms complexes with PINK during mitophagy and APC/C activators during cell division. Following mitochondrial depolarization PINK (green) accumulates on the outer mitochondrial membrane and recruits Parkin (blue) from the cytosol. PINK-mediated activation of Parkin on Ser 65 allows the formation of ubiquitin chains that attracts the autophagic machinery. Damaged mitochondria are engulfed by autophagosomes, which fuse with lysosomes and mature into autophagolysosomes. During cell division Parkin binds to Cdc20 or Cdh1 and, upon activation by Plk1, mediates the 26S proteasome-bound destruction of substrates controlled by these two APC/C activators.

phosphorylated at Ser 65 by PINK1 (PTEN-induced kinase 1) [38]. Not surprisingly, recessive PD is also associated with mutations in PINK [39] as the accumulation of PINK1 and its kinase activity are both necessary for Parkin translocation to the mitochondria and subsequent mitophagy [40–42]. Once activated Parkin ubiquitylates multiple mitochondrial substrates, including Mfn1 and Mfn2, proteins that mediate mitochondrial fusion [43–45]. Through a process not yet fully understood, the ubiquitylation of the mitochondria substrates designates the mitochondria for clearance by the autophagy machinery.

To complete mitophagy, these autophagasomes fuse to lysosomes. The adaptor protein p62, which is found on the outer membrane of newly formed autophagosomes, has recently been shown to be a key component in linking these two pathways [46]. Also, peroxiredoxin 6, a unique member of the ubiquitous PRDX family, is recruited to depolarized mitochondria and is required for translocation of Parkin to the mitochondria [47]. In addition, it was recently shown that following mild oxidative stress, the PINK/Parkin protein degradation system also generates mitochondria-derived vesicles (MDVs). MDVs are small vesicles that remove dysfunctional mitochondrial proteins by transporting them to the lysosomes [48]. This mechanism is a less drastic way to preserve mitochondrial quality and does not rely upon mitochondrial depolarization (as does mitophagy) to initiate the process. It remains to be seen if defects in this pathway are linked to the neurodegeneration seen in PD patients.

The in vivo signal that initiates mitophagy in cells is currently unclear, but one possible candidate is mitochondrial ROS. Consistent with this, only ROS originating from mitochondria, the major intracellular source of ROS [49], can induce the Parkin mitochondrial recruitment [50]. Likewise this translocation only occurs in cultured neurons in the absence of antioxidants, implicating a key role for ROS in this response [51]. Mitochondrial fission, mediated by the GTPase Drp1, may also be required for Parkin-mediated elimination of defective mitochondria as both Pink and Parkin mutants in *Drosophila* can be partly rescued by increased Drp1 activity [16]. Besides its interaction with Parkin, it has been proposed that PINK acts as a neuroprotective protein against oxidative stress through its interaction with DJ-1 [52]. DJ-1 is a conserved antioxidant protein that scavenges H_2O_2 through self-oxidation. Following oxidative stress, DJ-1 localizes to the outer mitochondrial membrane, but the molecular details of its relationship with PINK remain ob-

scure [51, 53]. Parkin also has additional neuroprotective roles. Significantly, it protects neurons against alpha-synuclein toxicity which occurs in PD patients [54]. While mechanisms remain unclear in dopaminergic neurons, alpha-synuclein phosphorylation is increased in Parkin loss of function mutants and decreased when Parkin is overexpressed [55, 56]. Taken together, Parkin has many neuroprotective roles to play which are both dependent on and independent of its association with PINK.

3. THE ROLE OF PARKIN IN CELL CYCLE PROGRESSION

As discussed above, Parkin plays an important role in maintaining the redox status of the cell. In recent years, it has become apparent that redox signaling plays an important role in cell cycle progression [57]. In eukaryotes, if ROS levels exceed the detoxifying ability of the cells, a stress signal is generated resulting in cell cycle arrest. In contrast, low levels of ROS promote cell growth by acting as secondary messengers in signaling cascades [58]. More recently, ROS have been shown to regulate cell cycle decisions by switching the redox state of certain cysteine residues in key cell cycle proteins [59]. For example oxidation of Cdc25, an essential cell cycle phosphatase [60] as well as components of the ubiquitin machinery [61] inhibits cell cycle progression. Hence, cell division is highly regulated by the cellular redox environment, with ROS concentrations being a determining factor. Until recently, Parkin's role in regulating cell division was only thought to be linked to its role in maintaining cellular redox potential. Now Parkin has a second function in maintaining genome integrity independent of its role in mitophagy [30].

4. PARKIN IS REGULATED BY CDC20 AND CDH1—CO-ACTIVATORS OF THE APC/C

The anaphase promoting complex/cyclosome (APC/C) is a multisubunit E3 ubiquitin ligase that ensures accurate chromosome segregation by degrading key regulatory proteins [32, 62, 63]. Thus, proper APC/C function is critical in maintaining genomic integrity. For example, APC/C-mediated proteolysis of securin and aurora kinases drives the cell from G2 through M phase into G1, respectively [32]. Accordingly, APC/C activity itself is strictly controlled so these targets are destroyed in the correct order. For example, the presence of co-activators, inhibition by the spindle checkpoint pathways, post-translational modifications, and subcellular localization all impinge on APC/C function. Importantly, two highly conserved co-activator proteins called Cdc20 and Cdh1 sequentially stimulate the APC/C to drive the cell through the metaphase-anaphase and M-G1 transitions, respectively. Interestingly, there is also a third APC/C activator (Ama1) that works specifically during meiosis in yeast [64, 65] although no mammalian homologues have been found as yet.

It is easy to understand how APC/C dysregulation leads to mitotic catastrophe, chromosome instability, or uncontrolled cell proliferation [62]. Consistent with these changes, the levels of many APC/C^{Cdh1} or APC/C^{Cdc20} substrates including Plk1, aurora A, aurora B, cyclin B1, and securin are elevated in many cancers [66, 67]. Given the essential role of the APC/C in mitotic cell division, it is surprising that very few APC/C subunit mutations have been associated with disease states. Paradoxically, Cdc20 is overexpressed in many cancers [67], indicating that other mechanisms might regulate the degradation of key cell cycle proteins. This is not a new concept as others have shown in yeast that Cdc20 and Cdh1 have functions that are independent of the APC/C [68, 69]. Consistent with this possibility, Lee et al. [30] have shown that Parkin can associate with either Cdc20 or Cdh1 in a complex that is distinct from APC/C^{Cdc20} or APC/C^{Cdh1}. This study demonstrated that $Parkin^{Cdc20}$ and $Parkin^{Cdh1}$ ubiquitylate many cell cycle regulators leading to the notion that these complexes function in parallel with the APC/C. As would be anticipated from this model, Parkin-deficient MEF cells show increased levels of mitotic proteins such as Plk1, aurora A, aurora B, cyclin B1, Cdc20, and UbcH10. They also result in multiple mitotic defects including chromosome misalignment, chromosome lagging, chromosome bridge formation, prometaphase-like arrest, anaphase, and cytokinesis failure [30].

5. PLK1 BUT NOT PINK ACTIVATES PARKIN DURING CELL DIVISION

Following mitochondrial depolarization, Parkin activation requires PINK phosphorylation on Ser 65.

However, PINK is not needed for Parkin to target two mitotic substrates Plk1 and cyclin B1 [30]. Instead, Parkin activation is accomplished by the polo-like-kinase 1 (Plk1). Plk1 has multiple roles at different stages of cell division including mitotic entry, the G2/checkpoint, centrosome maturation, spindle assembly, chromosome segregation, and cytokinesis [70]. Importantly, Plk1 phosphorylates the APC/C inhibitor Emi1 leading to Cdc20 activation [71, 72]. Lee et al. [30] discovered that Plk1 phosphorylates Parkin on a serine residue (S378) within the RBR domain. Consistent with this, treating cells with a Plk1 inhibitor or Plk1 shRNA results in blocked Parkin S378 phosphorylation. Likewise, mutation of S378 to A378 abolishes Parkin's ability to trigger destruction or aurora A, aurora B, and cyclin B1. Consistent with this observation, S378 phosphorylation is essential for Parkin to bind to Cdc20 and consequently required for destruction of its cell cycle substrates. Taken together, these results build upon the model that Plk1 phosphorylation of S378 is required for Parkin activation. Importantly, Parkin and APC/C work independently as mitotic abnormalities induced by inactivation of APC/C were rescued by ectopic expression of Parkin. Although the APC/C and Parkin share cell cycle substrates, Parkin cannot substitute for the APC/C activity during embryonic development [73]. These results indicate that the critical functions of APC/C cannot be accomplished by endogenous Parkin. Interestingly, Parkin overexpression suppresses the chromosome segregation defects in cell lines depleted of APC/C function. These results are perhaps explained by the differences in subcellular localization observed between Parkin and the APC/C. While Parkin localizes to the centrosome, the APC/C is found in the kinetochore. Therefore, Parkin overexpression could allow mislocalization resulting in acquisition of new functions. If correct, these results would suggest that subcellular localization, in addition to substrate specificity, is a major regulatory force governing Parkin activity.

6. PARKIN'S ROLE AS A TUMOR SUPPRESSOR

Maintaining the correct order of events is critical for the proper duplication and segregation of the genome. Genomic instability is a major initiating factor for many disease states including developmental abnormalities and cancers. Importantly, Cdh1 mutation has been associated with several tumor types including non-Hodgkin lymphomas [74, 75]. Similarly, loss of Parkin activity elevates the levels of several cell cycle substrates resulting in aberrant chromosome segregation. This places Parkin in a double-edged sword with respect to tumor initiation and progression. As described by Lee et al. loss of Parkin induces checkpoint pathways that prevent cell division [30]. However, this same phenotype can also provide the mutagenic motor to drive a cell out of senescence. Consistent with this observation (**Table 1**), Parkin-knockout MEFs readily escape senescence and become tumorigenic in vivo. Lee et al. also showed that Parkin overexpression in lung cancer cells results in elevated cyclin B ubiquitylation and tumor growth suppression in vivo. These effects only occurred when either wild-type Parkin or the activated S378D derivative was expressed, but not with the defective S378A mutant. These results suggest that tumorigenicity can be suppressed by Parkin expression. Interestingly, Parkin-depleted tumor cell lines and tumor xenografts were exquisitely sensitive to inhibitors of polo-like kinase 1. Therefore, their addiction to high levels of mitotic kinases may potentially be exploited as a promising therapeutic tool in Parkin-deficient tumors.

7. CONCLUDING REMARKS

The discovery of Parkin's APC/C independent function in cell cycle control sheds lights on a more complicated picture for mitotic regulation than previously appreciated. By incorporating another ubiquitin ligase into this control pathway, the cell is able to more precisely execute the destruction of key regulatory proteins at different cellular addresses. In addition, the use for the APC/C activating proteins Cdc20 or Cdh1, as well as requiring activation by the mitotic kinase Plk1, places Parkin under the control of well-established regulators that are plugged into both cell cycle progression cues as well as checkpoint pathways. Finally, although Parkin is not required for cell division or development like the APC/C, its function is still important for maintaining genomic stability. This role is critical for normal cell growth characteristics in a number of contexts including neoplasia and neuron health. Now the door has been opened wider, new research may identify

additional roles for Parkin in protecting us from cellular dysfunction.

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Parkin new cargos | The E3 ligase Parkin (represented by the blue and orange cars) can recognize different substrates (represented by the luggage). Activation by the kinase PINK at Serine 65 directs Parkin to degrade mitochondrial proteins and execute mitophagy. In contrast, activation by the kinase Plk1 at Serine 378 allows Parkin to associate with either Cdh1 or Cdc20 which in turn mediates the degradation of cell cycle proteins.