

Figure 3: Forest plot of protective LRRK2 haplotype N551K-R1398H-K1423K in individuals with versus without Parkinson's disease in the white, Asian, and Arab-Berber series

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median age of the eight control carriers of G2019S was 64 years (range 48–76 years). Due to the strong confounding potential of these three variants on disease-association analyses, any patient with a copy of these risk alleles was excluded from the analysis. Other possible rare risk variants (E334K, R1325Q, and T1410M) and protective variants (A221V and A1151T) with differences in frequency between patients with PD and controls were noted. When data for all rare variants were combined, the presence of any rare variant was not associated with PD in the white series (OR 1.01, 95% CI 0.81–1.25; $p=0.95$), Asian series (1.03, 0.57–1.85; $p=0.92$), or Arab-Berber series (0.78, 0.28–2.20; $p=0.64$). Additionally, no association was noted in the white series (0.89, 0.55–1.43; $p=0.62$), Asian series (1.05, 0.37–2.99; $p=0.93$), or Arab-Berber series (no PD cases, two [$<1\%$] controls, Fisher's exact $p=1.00$) when the data were combined only for those variants predicted by use of the SIFT program to be not tolerated.²⁴ Webappendix p 26 provides a summary of variants for which there were no carriers in any of the three series.

Discussion

The results of our study, one of the largest so far of the genetics of PD, show that a single gene, *LRRK2*, harbours many rare and common variants that confer susceptibility to PD in diverse populations (panel). Although population stratification is an inherent caveat

of this type of large-scale collaborative effort (and a potential limitation of the present study in the absence of genome-wide population control markers), these findings exemplify the confluence and independent effects of rare and common variations on gene loci that have a major effect in shaping both familial and sporadic disease.

About a third of variants we assessed were not identified in any study participant. These included four previously documented pathogenic mutations (*LRRK2* N1437H, R1441G, Y1699C, and I2020T), showing that they are rare mutations in the population samples we assessed. 26 variants were recorded at a frequency greater than 0.5% in any of the three series, and only 13 were noted at a frequency greater than 0.5% in all three series. This finding draws attention to the importance of studying genetic variability in large samples and in different ethnic groups, because frequencies and genetic effects might vary substantially.²⁶

The newly identified associations warrant further discussion. M1646T in the COR (C-terminal of Ras) domain of *LRRK2* was identified in the white series, and the effect was consistent in many countries (figure 1). This variant was not identified in participants of Asian descent and was rare in the series of Arab-Berber participants. *LRRK2* A419V was consistently more common in patients than in controls in Asian sites (figure 2). Although we cannot exclude the possibility of a non-coding element in linkage disequilibrium, the N-terminal region of the protein seems functionally relevant to disease development. *LRRK2* M1646T is the first common-risk factor to have been identified in white populations, whereas A419V is now the third risk factor reported to be specific to individuals of Asian ancestry, along with R1628P and G2385R.^{12,14,15} *LRRK2* R1628P was not significantly associated with risk in our Asian series. This variant was common only in the Taiwanese series, in which a non-significant protective effect was noted. Our inability to replicate the previously reported risk effect of R1628P is likely to be due to a combination of the low frequency of this variant, natural sampling variation, and population heterogeneity, in view of the results of previous studies of ethnic Han Chinese populations (of note, G2385R did show association).^{14,15}

The identification of a common three-variant haplotype (N551K-R1398H-K1423K) that seems to act in a protective manner (figure 3) is also important. It suggests that the reduced penetrance that is noted in patients with *LRRK2*-associated parkinsonism might be due to variants acting in cis or trans with the pathogenic variant and that *LRRK2* activity can be exploited to modify symptom onset in patients. Any future therapeutic strategies that lower risk in *LRRK2*-associated parkinsonism might protect against symptomatic onset in idiopathic PD.^{14,27} The previous report¹⁴ of a protective effect with N551K and R1398H showed a reduced kinase

	Aminoacid	White series		Asian series		Arab-Berber series	
		Patients (n=6995)	Controls (n=5595)	Patients (n=1376)	Controls (n=962)	Patients (n=240)	Controls (n=372)
rs2256408	R50H	7 (0.10%)	1 (0.02%)	+	+
rs75054132	A75A	0	1 (0.27%)
rs33995463	L119P	21 (0.31%)	23 (0.44%)	0	2 (0.55%)
rs41286468	L122L	5 (0.08%)	7 (0.13%)
rs112794616	A211V	4 (0.06%)	11 (0.21%)	0	1 (0.27%)
rs56108242	C228S	2 (0.03%)	2 (0.04%)
rs28365216	N238I	3 (0.22%)	2 (0.22%)
rs72546315	H275H	3 (0.04%)	2 (0.04%)	1 (0.43%)	0
rs17490713	N289N	1 (0.01%)	2 (0.04%)	NA	NA
rs41286466	A312A	26 (0.38%)	15 (0.28%)	1 (0.7%)	0	0	4 (1.10%)
rs78501232	E334K	14 (0.21%)	4 (0.07%)
rs113065049	V366M	1 (0.02%)	0
rs34594498	A419V	5 (0.07%)	3 (0.06%)	+	+
rs35847451	S416S	12 (0.18%)	16 (0.29%)
rs75711334	L488L	1 (0.01%)	0
rs79996249	K544E	2 (0.03%)	2 (0.04%)
rs78154388	S663P	2 (0.03%)	2 (0.04%)
rs72546319	V674V	0	2 (0.04%)	0	1 (0.27%)
rs58559150	Q923H	1 (0.01%)	2 (0.04%)	+	+
rs75148313	S973N	1 (0.01%)	2 (0.04%)
rs113217062	I1006M	1 (0.01%)	0
rs76535406	S1096C	0	2 (0.04%)
rs35808389	L1114L	5 (0.07%)	1 (0.02%)
rs74985840	A1151T	1 (0.01%)	5 (0.09%)
rs80179604	S1228T	5 (0.07%)	4 (0.07%)
rs4640000	P1262A	1 (0.01%)	1 (0.02%)	NA	NA
rs72546338	R1325Q	10 (0.15%)	3 (0.06%)	4 (0.29%)	1 (0.11%)
rs17466213	I1371V	7 (0.10%)	4 (0.07%)	1 (0.07%)	0	+	+
rs72546327	T1410M	5 (0.07%)	1 (0.02%)
rs113589830	D1420N	1 (0.01%)	0
rs111435410	A1430A	2 (0.03%)	1 (0.02%)
rs112998035	R1441R	1 (0.07%)	0
rs33939927*	R1441C	10 (0.15%)	0
rs34995376*	R1441H	1 (0.07%)	0
rs74681492	P1446L	10 (0.74%)	6 (0.62%)
rs111501952	V1450I	2 (0.15%)	1 (0.11%)
rs113431708	R1483Q	1 (0.01%)	0
rs35507033	R1514Q	+	+	0	1 (0.27%)
rs33949390	R1628P	7 (0.10%)	0	+	+
rs35303786	M1646T	+	+	3 (1.25%)	2 (0.54%)
rs111503579	L1653L	2 (0.03%)	1 (0.02%)	4 (0.30%)	9 (0.93%)
rs79909111	S1721S	1 (0.02%)	1 (0.02%)
rs263192805	R1728H	1 (0.01%)	3 (0.05%)
rs35602796	M1869T	5 (0.07%)	2 (0.04%)
rs77428810	R1941H	2 (0.03%)	1 (0.02%)
rs34637584*	G2019S	48 (0.71%)	3 (0.06%)	1 (0.07%)	1 (0.11%)	72 (30.25%)	4 (1.10%)
rs111739194	L2063STOP	1 (0.02%)	2 (0.04%)
rs33995883	N2081D	+	+	2 (0.15%)	0	+	+
rs34869625	G2170G	20 (0.30%)	21 (0.39%)	1 (0.60%)	0
rs35658131	Y2189C	1 (0.01%)	2 (0.04%)	+	+
rs113511708	T2356I	7 (0.1%)	5 (0.09%)

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	Aminoacid	White series		Asian series		Arab-Berber series	
		Patients (n=6995)	Controls (n=5595)	Patients (n=1376)	Controls (n=962)	Patients (n=240)	Controls (n=372)
(Continued from previous page)							
rs79546190	V2390M	1 (0.01%)	1 (0.02%)
rs78964014	E2395K	1 (0.01%)	0
rs60545352	M2408I	1 (0.01%)	0	0	2 (0.54%)
Data are number (%). PD=Parkinson's disease. +=a variant that was noted with a minor allele frequency of at least 0.5% and as such was analysed as a common variant. ..=a variant that was not noted in the series. NA=a variant that was out of the Hardy-Weinberg equilibrium in the specific series. *Pathogenic variants for which the number (%) of carriers is summarised for the entire sample; any carriers of these pathogenic variants were removed from the summaries provided for each of the remaining non-pathogenic variants.							
Table 4: LRRK2 rare variants							

activity for the R1398H variant, suggesting this Roc domain substitution might be the most likely functional allele on the haplotype.

Although the results of our study have identified an association of PD only with common variants, they also draw attention to the many rare variants in *LRRK2* that could contribute to disease risk. Genetic loci that contribute to disease risk might do so through variants that span the whole range of minor allele frequencies, from rare mutations to frequent single nucleotide polymorphisms.²⁸ Despite the very large sample size, we noted only three of seven previously described pathogenic *LRRK2* mutations. Hence, the search for mutations contributing to familial PD should include an analysis of single pedigrees, with further assessment in very large population studies. Single pedigrees might result in some false-positive results, which can be filtered out with large population samples. For example, two variants (I1371V and T2356I) have been proposed as pathogenic and to account for the clinical and functional features of *LRRK2*-associated parkinsonism.^{29,30} However, in our study, both variants were noted in patients and controls at the same frequency (table 4). Conversely, we noted other possible rare risk (E334K, R1325Q, and T1410M) and protective (A211V and A1151T) variants; however, because of their low frequency, large meta-analytical approaches are necessary to define their roles fully.

In this study, we focused on exonic variants because all pathogenic variants identified in *LRRK2* so far have been single nucleotide missense changes. However, silent, synonymous variants were also included because they can result in alternative splicing and, since protein translation is a function of codon use and transfer RNA abundance, could affect the rate of protein domain folding and secondary modifications.³¹ Neither copy number variants nor other risk factors in non-coding regions that regulate *LRRK2* expression or alter splicing were assessed in our study.

As new loci for susceptibility to diverse diseases are continuously being discovered in genome-wide association and whole-genome sequencing studies, the results of our study show the importance of revisiting loci at which rare or common variants have been

identified, since they could harbour many more independent signals of genetic risk in different populations.^{25,32,33} Furthermore, *LRRK2* sequencing studies in under-represented populations (eg, from South America, sub-Saharan Africa, Middle East, and western Asia) will undoubtedly show novel ethnic-group-specific risk variants and could clarify the role of variants that were rare or absent in our study. *LRRK2* variants, including novel exonic variants, were reported as part of the 1000 Genome Project, lending support to this hypothesis.³⁴

Large-scale parallel resequencing (targeted genomic capture of the specific regions—eg, gene-specific, exome, transcriptome, and whole-genome sequencing) is likely

Panel: Research in context

Systematic review

We searched PubMed with the terms "*LRRK2*" and "Genetics Parkinson's disease" and identified all *LRRK2* coding variations reported up until April 1, 2010. We also contacted our global network of collaborators and the members of the Genetic Epidemiology Of Parkinson's Disease (PD) Consortium for unreported variants.

Interpretation

By focusing on the role of *LRRK2* variation in PD, we have identified a common risk factor in the white population (M1646T), the third common risk factor in Asian populations (A419V), and a common global protective haplotype (N551K-R1398H-K1423K). This work complements the meta-analysis of PD genome-wide association,²⁵ which suggests a possible association at the *LRRK2* locus. We define some of the genetic variation that is likely to be contributing to the association noted in recent genome-wide association efforts and nominate potential functionally and clinically relevant variants. We show modulation of the underlying toxic effect is possible because of the protective nature of the N551K-R1398H-K1423K haplotype. The identification of common variants that affect risk clearly shows a greater role for *LRRK2* in idiopathic disease than previously thought.

to identify many more variants in candidate genes that might predispose to PD. Characterisation of each variant will require this type of collaborative international effort to define their pathogenicity, frequency in different populations, and contribution to disease pathogenesis through genotype–phenotype assessment.

Contributors

OAR and MJF were the principal investigators and were responsible for the concept and design of the study. AIS-O, JAB, OAR, and CVG were responsible for the technical aspects of the study. MGH and NND were responsible for all the analyses; OAR and MJF were responsible for drafting the report. All authors participated in study design and approach, sample collection, data acquisition, and critical revision and final approval of the report.

Conflicts of interest

JOA, MJF, and ZKW report holding a patent on *LRRK2* genetic variability and MJF has received royalties for licensing of genetically modified *LRRK2* mouse models. DMM declares a patent pending entitled *Methods to treat PD*. CK and RK declare receiving payment in their role as consultants for Centogene and Takeda Pharmaceutical, respectively. All other authors declare that they have no conflicts of interest.

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Caffeine induces apoptosis by enhancement of autophagy via PI3K/Akt/mTOR/p70S6K inhibition

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Key words: apoptosis, autophagy, PI3K/Akt/mTOR/p70S6K, ERK1/2, caffeine

Abbreviations: PI3K, phosphoinositide-3 kinase; 4E-BP1, eukaryotic initiation factor 4-binding protein 1; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; 3-MA, 3-methyladenine; MEFs, mouse embryonic fibroblasts; p70S6K, 70-kDa ribosomal protein S6 kinase; PI, propidium iodide; MPP⁺, 1-methyl-4-phenylpyridinium

Caffeine is one of the most frequently ingested neuroactive compounds. All known mechanisms of apoptosis induced by caffeine act through cell cycle modulation or p53 induction. It is currently unknown whether caffeine-induced apoptosis is associated with other cell death mechanisms, such as autophagy. Herein we show that caffeine increases both the levels of microtubule-associated protein 1 light chain 3-II and the number of autophagosomes, through the use of western blotting, electron microscopy and immunocytochemistry techniques. Phosphorylated p70 ribosomal protein S6 kinase (Thr389), S6 ribosomal protein (Ser235/236), 4E-BP1 (Thr37/46) and Akt (Ser473) were significantly decreased by caffeine. In contrast, ERK1/2 (Thr202/204) was increased by caffeine, suggesting an inhibition of the Akt/mTOR/p70S6K pathway and activation of the ERK1/2 pathway. Although insulin treatment phosphorylated Akt (Ser473) and led to autophagy suppression, the effect of insulin treatment was completely abolished by caffeine addition. Caffeine-induced autophagy was not completely blocked by inhibition of ERK1/2 by U0126. Caffeine induced reduction of mitochondrial membrane potentials and apoptosis in a dose-dependent manner, which was further attenuated by the inhibition of autophagy with 3-methyladenine or *Atg7* siRNA knockdown. Furthermore, there was a reduced number of early apoptotic cells (annexin V positive, propidium iodide negative) among autophagy-deficient mouse embryonic fibroblasts treated with caffeine than in their wild-type counterparts. These results support previous studies on the use of caffeine in the treatment of human tumors and indicate a potential new target in the regulation of apoptosis.

Introduction

Caffeine has a diverse range of pharmacological effects.¹ In addition to its various effects on the cell cycle and growth arrest, higher (4–10 mM) concentrations of caffeine can induce apoptosis in several cell lines, such as 10 mM caffeine in human neuroblastoma cells,² 4 mM caffeine in human pancreatic adenocarcinoma cells³ and 5 mM caffeine in human A549 lung adenocarcinoma cells.⁴ Although caffeine has been reported to modulate cell cycle checkpoints and perturb molecular targets of the cell cycle, the exact mechanism of caffeine-induced apoptosis remains unclear.¹

Autophagy is a key mechanism in various physiopathological processes, including tumorigenesis, development, cell death and survival.^{5,6} It has also been shown to have a complex relationship with apoptosis, especially in tumor cell lines.⁷ Several reports

have shown that autophagy not only enhances caspase-dependent cell death, but is also required for it.⁸ In contrast, it has also been shown that autophagy plays an important role in promoting cell survival against apoptosis.⁷ Caffeine has been reported to inhibit some kinase activities, including various forms of phosphoinositol-3 kinase and mammalian target of rapamycin (mTOR).^{9,10} Recently, in food spoilage studies involving yeast, caffeine has been shown to induce a starvation response,¹¹ which is a key regulator of autophagy causing its induction. However, the exact mechanism by which caffeine induces autophagy is still unknown.

Here we report that higher concentrations of caffeine enhance autophagic flux in a dose-dependent manner in various cell lines. Furthermore, we show that caffeine-induced autophagy is mainly dependent on PI3K/Akt/mTOR/p70S6 signaling and eventually results in apoptosis.

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Results and Discussion

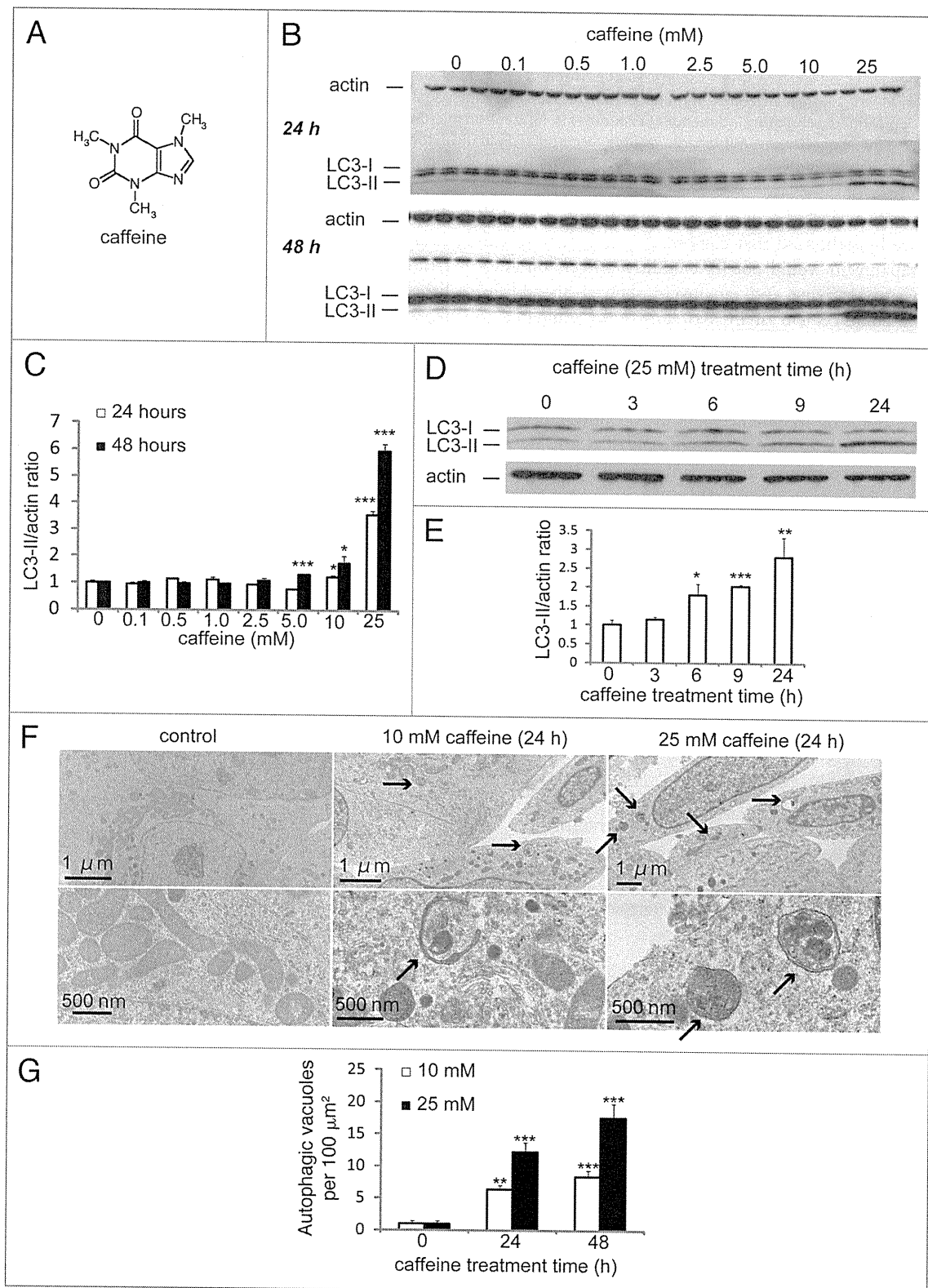
Caffeine (Fig. 1A) is a widely used psychoactive drug that has been used for centuries to increase alertness and energy. It has been reported that caffeine induces autophagy in *Zygosaccharomyces bailii* in association with a starvation response, caused by an unknown mechanism.¹¹ However, it remains unknown whether caffeine affects autophagy in mammalian cells. To determine if caffeine regulates autophagy at a steady state, we first examined levels of the microtubule-associated protein 1 light chain 3 (LC3)-II, which is an LC3-phosphatidyl-ethanolamine conjugate and a promising autophagosomal marker.¹² LC3-II levels (compared to actin loading controls) increased with 5–25 mM caffeine treatment over 48 hours in SH-SY5Y (Fig. 1B and C), PC12D and HeLa cells (Suppl. Fig. S1A and B). The LC3-II/actin ratio also increased in a time-dependent manner in SH-SY5Y (Fig. 1D and E) and HeLa cells (data not shown). Using an electron microscopy technique, the numbers of autophagic vacuoles (AVs) were markedly increased in SH-SY5Y cells treated with 10 or 25 mM caffeine, but not in the control (Fig. 1F and G). Morphometric analysis revealed that the number of AVs per 100 μm^2 of SH-SY5Y cytoplasm in control (Mean \pm standard deviation: 1.3 ± 0.50), whereas that in caffeine-treated cells (10 mM: 8.0 ± 0.82 ; 25 mM: 15 ± 1.9) for 24 hours. Expression levels of p62, a well-known autophagic substrate, were also decreased by caffeine treatment in SH-SY5Y (Fig. 1H and I) and HeLa cells (Suppl. Fig. S1C and D). Furthermore, 10 mM caffeine treatment markedly increased the number of EGFP-LC3-positive vesicles in SH-SY5Y cells transiently transfected with EGFP-LC3 (data not shown) and HeLa cells stably expressing EGFP-LC3 (Figs. 1J and K).^{12,13} This effect was confirmed by the observation that caffeine administration also increased the number of vesicles positive to endogenous LC3 (Suppl. Fig. S1E).

Endogenous LC3 is post-transcriptionally processed into LC3-I, which is found in the cytosol. LC3-I is in turn lipidated to LC3-II, which then associates with autophagosome membranes.¹⁴ LC3-II can accumulate due to increased upstream autophagosome formation or impaired downstream autophagosome-lysosome fusion. To distinguish between these two possibilities, we assayed LC3-II in the presence of E64D plus pepstatin A or bafilomycin A1, which inhibits lysosomal proteases or blocks downstream autophagosome-lysosome fusion and lysosomal proteases, respectively.^{15,16} Caffeine significantly increased LC3-II levels in the presence of E64d plus pepstatin A or bafilomycin compared to E64d plus pepstatin A or bafilomycin alone in (Fig. 2A and B; Suppl. Fig. S1F and G) and HeLa cells (Fig. 2C and D; Suppl. Fig. S1H and I). A saturating dosage of bafilomycin A1 was used in this assay and no further increases in LC3-II levels were observed when cells were treated with higher concentrations. Similar results were observed in PC12D cell lines (data not shown). To confirm the caffeine effect on autophagic flux, we assessed the numbers of autolysosomes and autophagosomes in HeLa cells. The ratio of the numbers of autolysosomes (positive to both LC3 and LAMP2) to autophagosomes (positive to LC3) was increased by 10 mM caffeine treatment for 48 hours (Fig. 2E). Quantification data using ImageJ also showed significant

increase of the ratio (Fig. 2F). These results strongly indicate that high concentration of caffeine treatment enhances autophagic flux.

The class I phosphatidylinositol 3-phosphate kinase (PI3K)/Akt/mTOR/p70ribosomal protein S6 kinase (p70S6K) signaling pathway and the Ras/Raf-1/mitogen-activated protein kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway are two well-known pathways involved in the regulation of autophagy. Both are associated with tumorigenesis and often activated in numerous types of tumors.¹⁷ Therefore, we examined the effect of caffeine on both of these pathways, using western blotting, according to the protocol by Inoki and colleagues.¹⁸ After a 24 hour treatment with caffeine, there was a significant decrease in the levels of phosphorylated p70 S6 kinase, S6 ribosomal protein and 4E-BP1, compared with total normal levels in SH-SY5Y (Fig. 3A), HeLa and PC12D cells (data not shown). Consistent with these results, nonphosphorylated 4E-BP1 proteins were increased by caffeine treatment (Fig. 3A). To further investigate the upstream inhibition of mTOR by caffeine, we examined Ser473 phosphorylation of Akt, which measures both Akt/mTOR and mTORC2 activity. As shown in Figure 3B, treatment with caffeine also decreased the level of phosphorylated Akt in SH-SY5Y cells, which was consistent with a previous report.¹⁹ Similar findings were obtained in HeLa (Suppl. Fig. S2A) and PC12D cells (data not shown). Subsequently, we examined whether caffeine increases the phosphorylation of ERK1/2, a key regulator of autophagy downstream of Akt. As shown in Figure 3C, treatment with caffeine increased phosphorylated ERK1/2. The effects of caffeine on mTOR inhibition were initially detected 3 hours after the addition of caffeine and reached a maximal level after 6 hours in SH-SY5Y (Fig. 3D) and 9 hours in HeLa cells (Suppl. Fig. S2B and C).

Caffeine has been shown to inhibit PI3K and components of the PI3K/Akt pathway.^{9,20} Next, we performed experiments to confirm whether caffeine-induced autophagy is activated through the PI3K/Akt pathway. Insulin or insulin-like growth factor upregulates PI3K and its downstream targets including Akt and mTOR, resulting in the inactivation of autophagy.²¹⁻²³ As shown in Figure 4A and B, insulin treatment for 30 minutes significantly phosphorylated Akt at Ser473, whereas the phosphorylation was completely abolished by additional treatment with caffeine. No significant differences of the LC3-II/actin ratio between caffeine treatment and caffeine treatment with insulin were observed. Also, caffeine and Akt1/2 inhibitors did not have additive effects on the levels of LC3-II/actin ratio compared to the single treatment of caffeine or Akt inhibitors (Fig. 4C and D). To further confirm the caffeine effects on this pathway, cells were transiently transfected with myristoylated Akt (myr-Akt), a constitutively active form of Akt.²⁴ Caffeine treatment of both cells transfected with control vector and myr-Akt markedly decreased the levels of the phosphorylated Akt (Fig. 3E), indicating that caffeine directly inhibits the Akt phosphorylation. If caffeine facilitates autophagy through PI3K/Akt and ERK1/2 signalings, the autophagy should be partially blocked by ERK1/2 inhibition using the mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor, U0126. U0126 significantly but mildly



reversed the levels of LC3-II/actin ratio (Fig. 4F and G). The failure of U1026 to reverse completely the caffeine effect can be explained by the autophagy induction through Akt/mTOR signaling. In addition, only Akt knockdown with inducible short

hairpin RNAs (shRNAs) to specifically and stably knock down all three Akt isoforms sufficiently increases autophagic flux.²⁵ Therefore, we concluded that the caffeine-induced autophagy is mainly dependent on the PI3K/Akt/mTOR pathway.

Figure 1A–G (See opposite page). Caffeine increases autophagic flux in various cell lines. (A) Structural formula of caffeine. (B and C) SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours were analyzed by immunoblotting (B) with antibodies against LC3 and actin. Densitometry analysis of LC3-II levels relative to actin (C) was performed using three independent experiments. (D and E) SH-SY5Y cells treated with 25 mM caffeine for 3–24 hours were analyzed by immunoblotting (D) with antibodies against LC3 and actin. Densitometry analysis of LC3-II levels relative to actin (E) was performed using three independent experiments. (F) Electron microscopic examination of SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours. Autophagic vacuoles accumulating in the cytoplasm are shown by arrows. (G) Morphometric analysis of autophagic vacuoles was performed with 30 different areas of the cytoplasm of control and caffeine-treated cells.

Because caffeine induces autophagy dependently of mTOR inhibition, we hypothesized that combination treatment of caffeine with rapamycin would not have additive effects on autophagy. However, caffeine and rapamycin showed an additive effect on the enhancement of LC3-II/actin ratio compared to the single treatment of caffeine or rapamycin (Fig. 5A and B). Several lines of evidences support the hypothesis that resistance to rapamycin results from a positive feedback loop from mTOR/S6K1 to Akt, resulting in enhancement of Akt phosphorylation at Ser 473.^{26–28} Recently, mutual suppression of the PI3K/Akt/mTOR pathway by combination of rapamycin with perifosine, an Akt inhibitor, induces synergistic effects on autophagy-induced apoptosis as well as enhancement of autophagy, suggesting that dual inhibition of the PI3K/Akt/mTOR by rapamycin with caffeine would be also a rational treatment for cancer.²⁹

Several anti-cancer agents are known to inhibit the PI3K/Akt/mTOR/p70S6K pathway and simultaneously activate ERK1/2, resulting in induction of autophagy in tumor cell lines.^{30,31} The upregulation of this process has beneficial effects in neurodegenerative diseases, such as Parkinson and Huntington diseases, whereas an excess of autophagy can lead to cell death.^{32,33}

Therefore, we decided to investigate whether caffeine-induced autophagy rescues or induces cell death. Using PC12D cells treated with 1-methyl-4-phenylpyridinium (MPP⁺), a well-established Parkinson disease model,³⁴ we determined that 1 mM caffeine treatment was not sufficient for the induction of autophagy (Suppl. Fig. S4 and B) and promoted increased cell viability, whereas >2.5 mM caffeine decreased cell viability (Fig. 6A). In addition, a significant decrease in cell viability was noted in cells treated with >2.5 mM caffeine without MPP⁺. Also, mitochondrial membrane potentials assessed by JC-1 were significantly preserved by 1 mM caffeine treatment compared to the control with MPP⁺, while those were lost by >5 mM caffeine treatment (Fig. 6B and Suppl. Fig. S5A). These data suggest that caffeine-induced autophagy is not protective in these cell lines and leads to cell death.

Autophagy and apoptosis may act independently in parallel pathways or may influence one another.⁷ To confirm the relationship between these pathways in cells treated with caffeine, we examined caffeine effects on the cell cycle with a propidium iodide (PI) staining assay. Treatment with 2.5–10 mM caffeine increased the percentage of cells in the sub-G₁ peak, which is indicative of

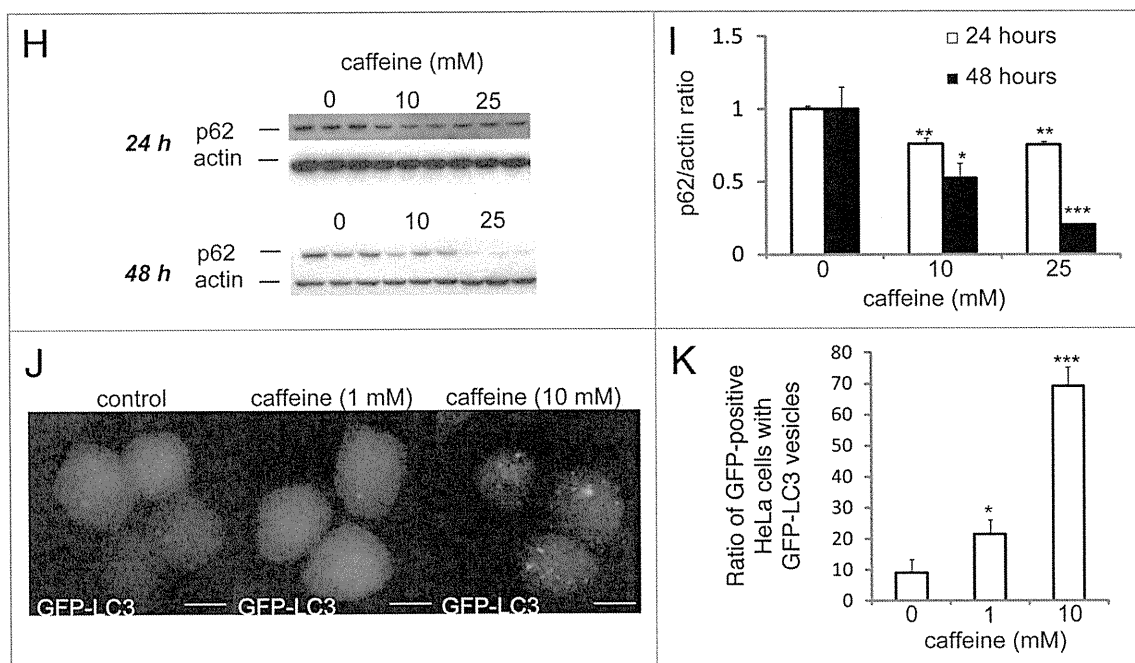


Figure 1H–K. Caffeine increases autophagic flux in various cell lines. (H and I) SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours were analyzed by immunoblotting with antibodies against p62 and actin. Densitometry analysis of p62 levels relative to actin (I) was performed using three independent experiments. (J and K) HeLa cells stably expressing EGFP-LC3 were treated with various concentrations of caffeine for 24 hours and analyzed using confocal microscopy. The percentage of EGFP-positive HeLa cells with >5 EGFP-LC3 vesicles was assessed (K) described previously in reference 43. Error bars, S.D.; *p < 0.05; **p < 0.01.

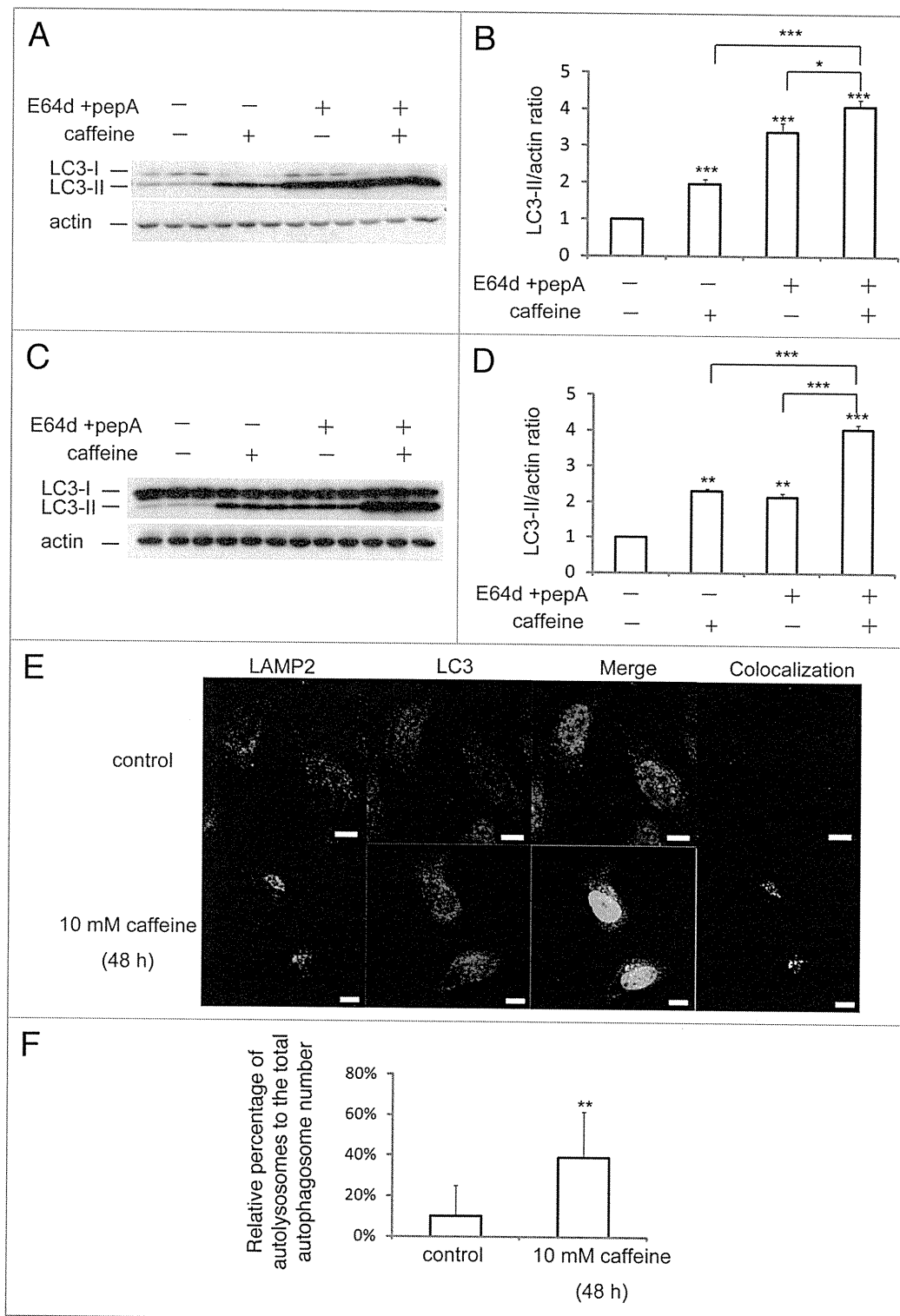


Figure 2. Caffeine does not block autophagosome-lysosome fusion. (A–D) SH-SY5Y (A) or HeLa (C) cells treated with 10 mM caffeine with or without E64d (10 μ g/ml) and pepstatin A (10 μ g/ml) were analyzed by immunoblotting with antibodies against LC3 and actin. Densitometry analysis of LC3 levels relative to actin in SH-SY5Y (B) and HeLa (D) cells was performed using three independent experiments. (E and F) HeLa cells treated with various concentrations of caffeine for 48 hours were analyzed using confocal microscopy (E). Number of the autolysosomes and autophagosomes were automatically counted using ImageJ “Colocalization” Plugin and the ratios were calculated (F).

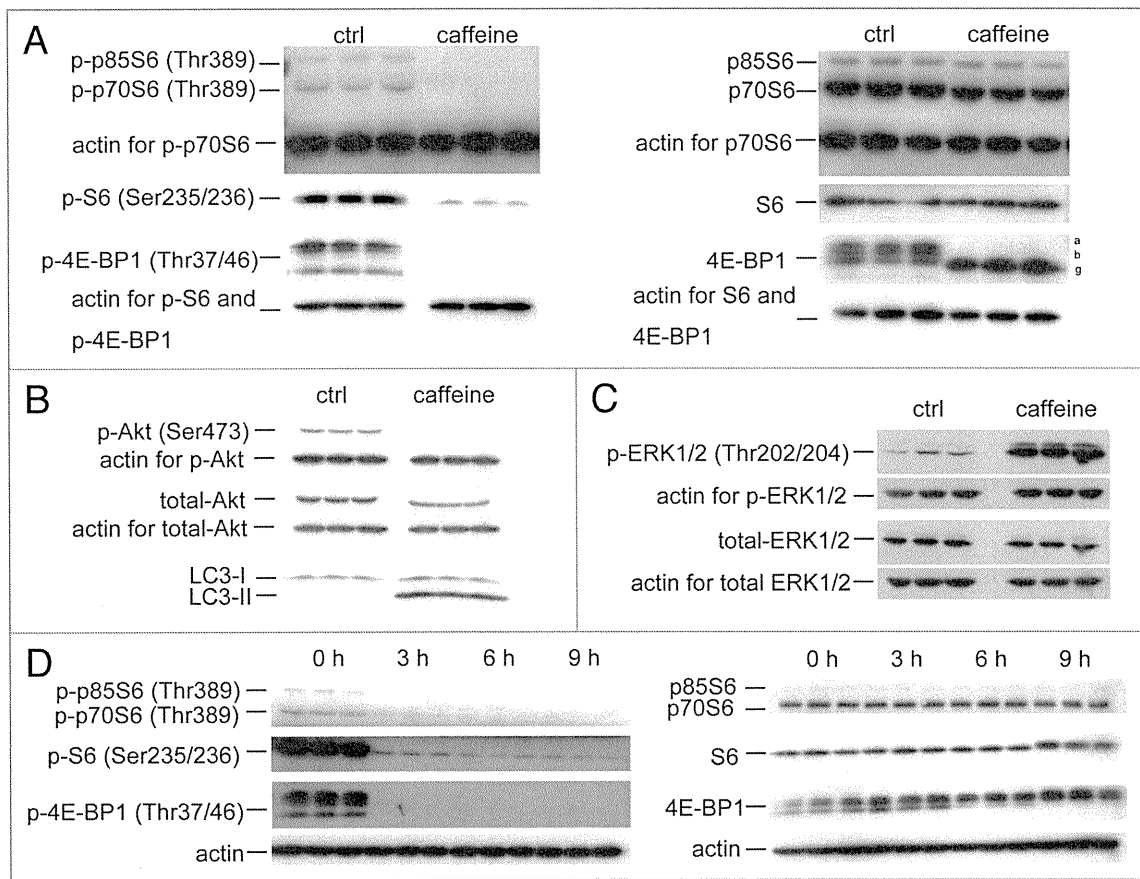


Figure 3. Caffeine inhibits the Akt/mTOR/p70S6 signaling pathway and activates ERK1/2 signaling. (A and B) SH-SY5Y cells treated with or without 10 mM caffeine for 24 hours were analyzed for mTOR activity by immunoblotting for levels of phosphor- and total p70 ribosomal S6 protein, S6, 4E-BP1 (A), Akt (B) and actin. (C) SH-SY5Y cells treated with or without 10 mM caffeine for 0, 3, 6 or 9 hours were analyzed by immunoblotting for levels of phosphor- and total ERK1/2 and actin. (D) SH-SY5Y cells treated with 10 mM caffeine for various time periods were analyzed by immunoblotting for levels of phosphor- and total p70 ribosomal S6 protein, S6, 4E-BP1 and actin.

apoptosis (Fig. 6C). To confirm whether caffeine-induced cell death is apoptotic, we examined the activity of caspase-3, a well-known inducer of apoptosis. Treatment with 10 mM caffeine markedly increased levels of cleaved caspase-3 and decreased full-length caspase-3 in PC12D cells (Fig. 6D), consistent with previous reports on the induction of apoptosis by caffeine.³⁵⁻³⁷

To test whether caffeine-induced apoptosis is dependent on autophagy, we determined whether the inhibition of autophagy by 3-methyladenine (3-MA) or Atg7 siRNA knockdown affects caffeine-induced cytotoxicity in PC12D cells. Treatment with 1 or 5 mM 3MA or Atg7 knockdown significantly decreased the percentage of cell death or cells with reduced mitochondrial membrane potentials caused by caffeine treatment (5 or 10 mM) (Fig. 6E and F and Suppl. Fig. S6B). As can be seen from the increased caffeine-induced apoptosis shown in Figure 6A and C, our data suggests that caffeine-induced autophagy is necessary for apoptotic cell death. To further confirm this, we compared autophagy-deficient mouse embryonic fibroblasts (MEFs), lacking the *Atg7* gene (*Atg7*^{-/-}), without LC3-II expression (Suppl. Fig. S4E), and matched wild-type (*Atg7*^{+/+}) MEFs, in which autophagy is induced by caffeine in a dose-dependent

manner (Suppl. Fig. S4C and D). As expected, the level of caffeine-induced cell death (positive to trypan blue staining) in *Atg7*^{-/-} MEFs was less than that in *Atg7*^{+/+} MEFs (Fig. 7A). The numbers of early apoptotic cells (annexin V positive, PI negative) were significantly increased in both a time-dependent and dose-dependent manner by caffeine treatment of *Atg7*^{+/+} MEFs compared to *Atg7*^{-/-} MEFs (Fig. 7B–D). Also, apoptotic or necrotic cells (annexin V positive) were significantly increased by caffeine treatment of *Atg7*^{+/+} MEFs compared to *Atg7*^{-/-} MEFs (Suppl. Fig. S6). Together, these results indicate that caffeine-induced autophagy partly occurs upstream of apoptosis and is not a protective response to caffeine.

In various tumor cell lines, higher concentrations of caffeine alone induce p53-dependent G₁ phase arrest and under certain conditions apoptosis can also occur in a p53-independent manner.¹ Furthermore, disruption at the G₂/M checkpoint by caffeine allows cells time to repair DNA damage by driving them through mitosis, eventually resulting in apoptosis.^{36,38,39} Consistent with these reports, the results of our study indicate that increased concentrations of caffeine treatment cause a dose-dependent increase in apoptosis. More recently, autophagy, a process long known to

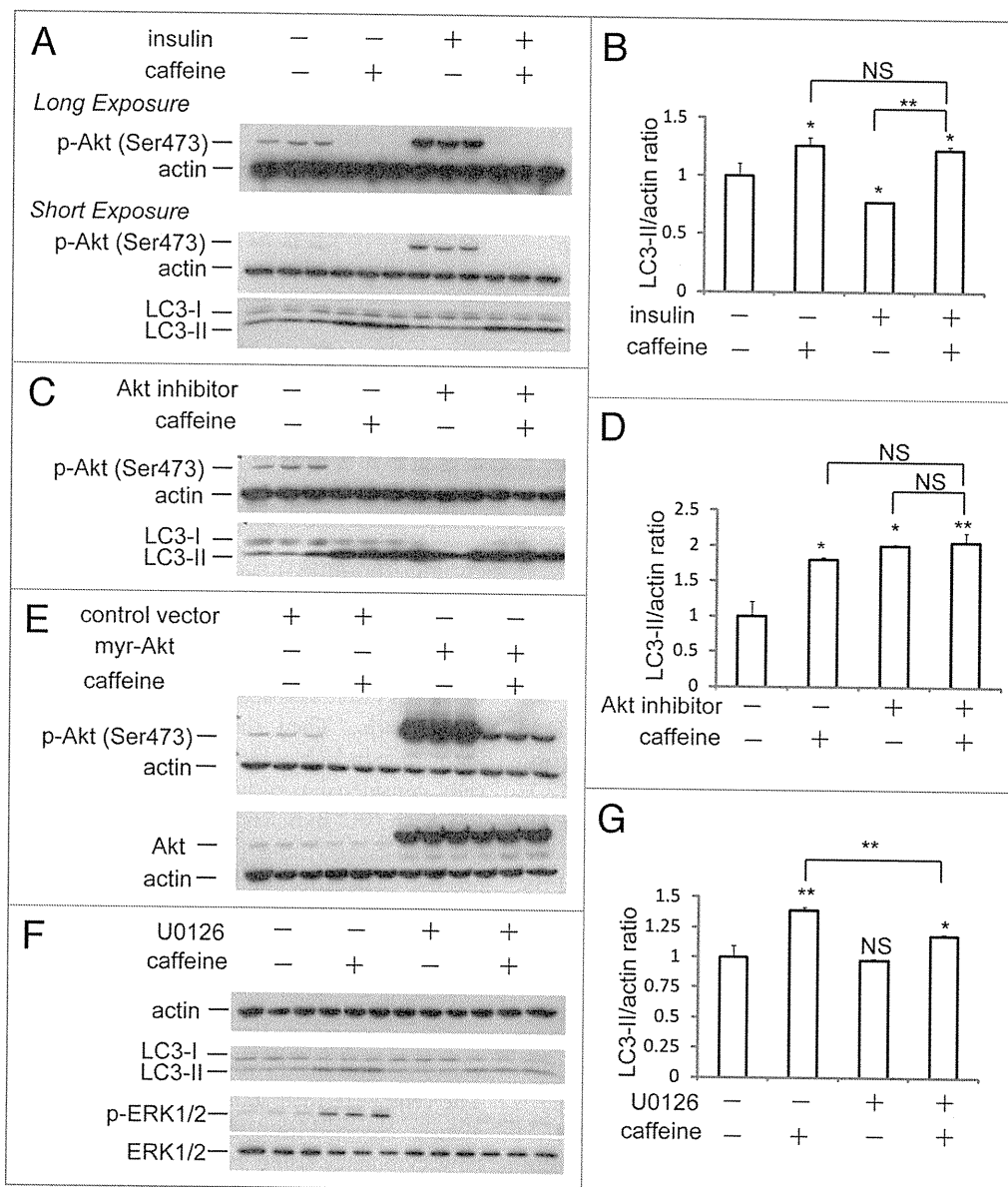
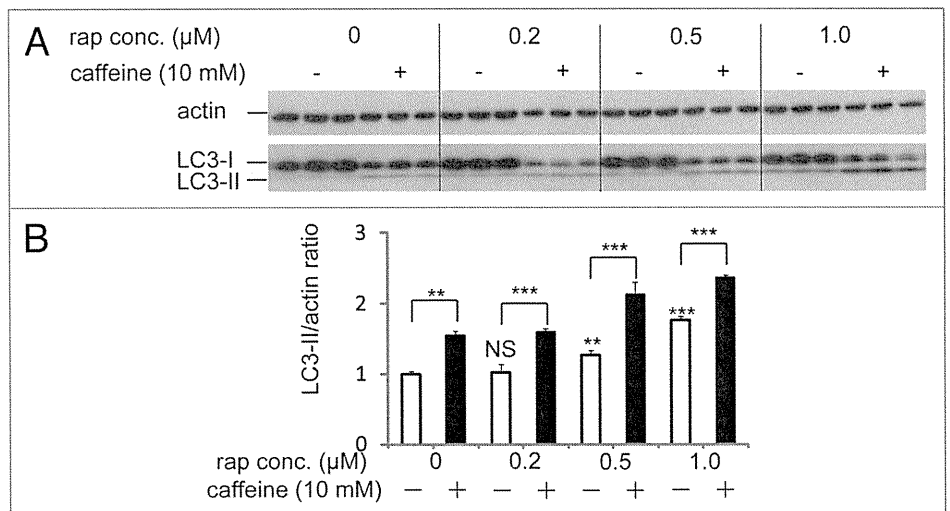


Figure 4. Caffeine-induced autophagy is dependent on PI3K/Akt/mTOR pathway. (A) SH-SY5Y cells treated with 25 mM caffeine for 3 hours followed by treatment with or without 200 nM insulin for 30 minutes were analyzed by immunoblotting. (B) Densitometry analysis of LC3-II levels relative to actin was performed using three independent experiments. (C) SH-SY5Y cells treated with 25 mM caffeine, 50 μ M Akt1/2 inhibitors or 25 mM caffeine with 50 μ M Akt1/2 inhibitors for 6 hours were analyzed by immunoblotting. (D) Densitometry analysis of LC3-II levels relative to actin was performed using three independent experiments. (E) SH-SY5Y cells were transfected for 24 hours with either a control plasmid DNA (pcDNA3.1) or a plasmid encoding constitutively active Akt (myr-Akt), and then treated with H₂O or 10 mM caffeine for 6 hours. Immunoblotting was performed using antibodies against Akt, p-Akt (Ser 473) and actin. (F) SH-SY5Y cells treated with 25 mM caffeine with or without 20 μ M U0126 for 6 hours were analyzed by immunoblotting using antibodies against actin, LC3, p-ERK and ERK. (G) Densitometry analysis was performed using three independent experiments. Error bars, SD; * $p < 0.05$; ** $p < 0.01$; N.S., not significant.

provide a survival advantage to cells undergoing nutrient deprivation and other stresses, has also been linked to the cell death process.⁷ The cross-talk between apoptosis and autophagy is complex and sometimes contradictory; however, it is critical to the overall fate of the cell. In this study, we have shown that autophagy is induced by higher concentrations of caffeine without starvation, mainly via the inhibition of PI3K/Akt/mTOR/p70S6K signaling. Likewise, when caffeine-induced autophagy is blocked by 3-MA

treatment or *Atg7* knockout, apoptosis is partially attenuated, suggesting that caffeine-induced autophagy occurs upstream of caffeine-induced apoptosis. It also indicates the involvement of other pathways in caffeine-induced apoptosis. These results provide new insight into the effects of caffeine on cell death and survival and its use as a possible intervention strategy for the upregulation of apoptosis by a harnessing of its autophagic activity in tumor treatment.

Figure 5. Rapamycin treatment with caffeine has an additive effect on enhancement of autophagy. (A) SH-SY5Y cells treated with various concentrations of rapamycin with or without 10 mM caffeine for 48 hours were analyzed by immunoblotting. (B) Densitometry analysis was performed using three independent experiments. Error bars, SD; * $p < 0.05$; ** $p < 0.01$; N.S., not significant.



Materials and Methods

Cell line. HeLa cells were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 100 U/ml penicillin/streptomycin (Sigma) at 37°C and 5% CO₂. PC12D and SH-SY5Y cells were maintained in DMEM (Sigma) supplemented with 10% FBS (Sigma), 5% horse serum and 100 U/ml penicillin/streptomycin at 37°C and 5% CO₂. All experiments with PC12D were performed after differentiation with NGF treatment for 48 hours. *Atg7*^{+/+} and ^{-/-} MEFs were maintained in DMEM (Sigma) supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 1% sodium pyruvate (Gibco, 11360), 1% non-essential amino acid (NEAA) and 4.2 μl 2% beta-mercaptoethanol at 37°C.

To establish a HeLa GFP-LC3 stable cell line, proliferating HeLa cells were transfected with a GFP-LC3 plasmid.¹⁴ Forty-eight hours post-transfection with Lipofectamine 2000 (Invitrogen), positive stable clones were selected by growing cells with G418 (400 μg/ml) for 2 weeks and maintained in DMEM (Sigma) supplemented with 10% FBS (Sigma), 100 U/ml penicillin/streptomycin and 200 μg/ml G418 at 37°C and 5% CO₂. All cellular experiments were performed with cells cultured in complete medium with FBS as explained above.

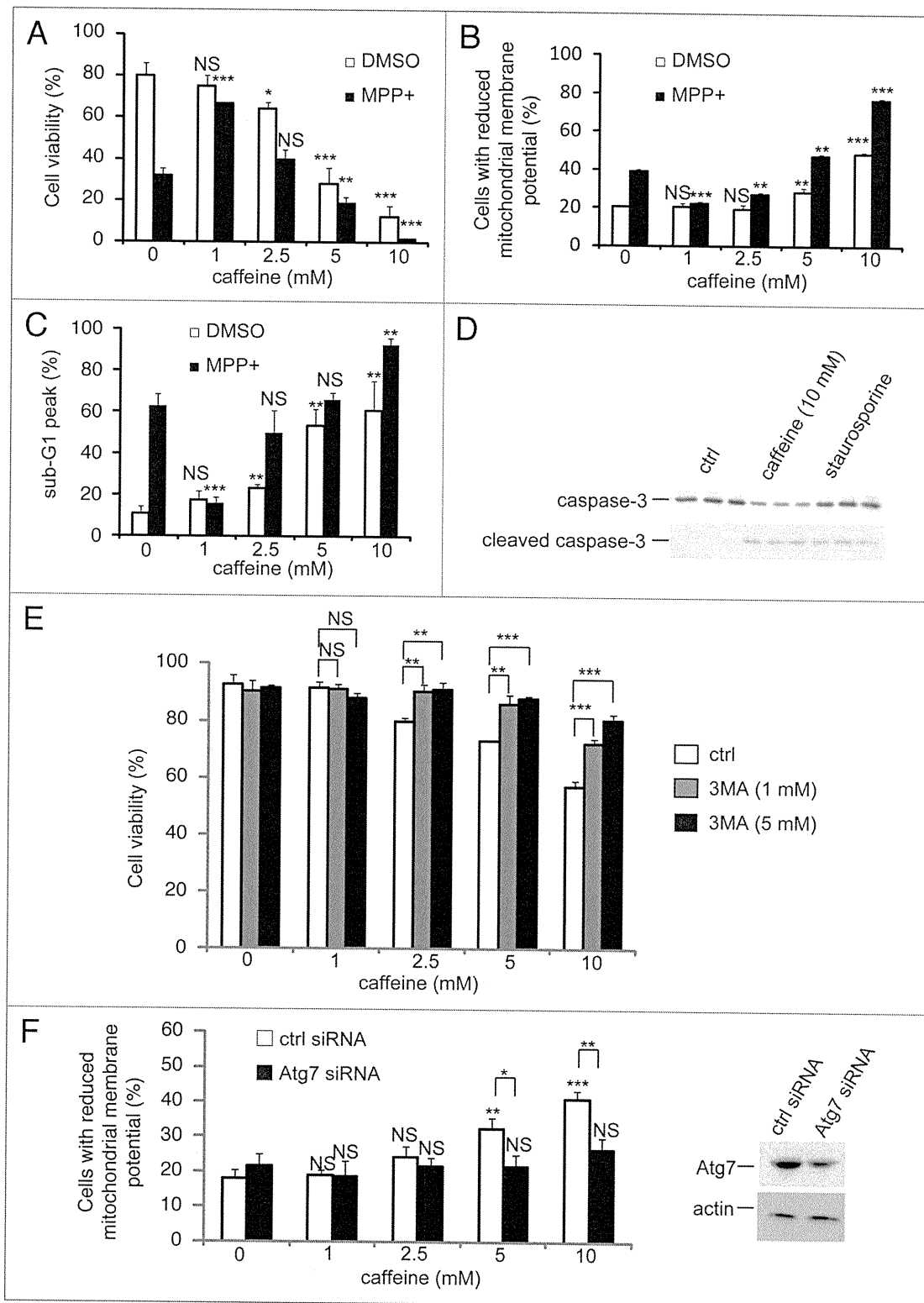
Cell viability assays. A trypan blue dye (Invitrogen, 15250-061) exclusion assay was used to examine cell viability and performed according to previously reported protocols.^{40,41} Changes of mitochondrial membrane potentials were assessed also with the lipophilic cationic membrane potential-sensitive dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide)

(Wako, 106-00131) according to the manufacturer's protocol. Detection of early apoptotic cells was determined using an annexin V/propidium iodide (PI) detection kit (Invitrogen), according to the manufacturer's protocol. Briefly, 0.5×10^6 *Atg7*^{+/+} or ^{-/-} MEFs were exposed to caffeine (0–25 mM) for 24 hours and washed twice. Then, they were incubated at room temperature with annexin V/Alexa488 and PI for 15 minutes. Annexin V-PI cells, considered as early apoptotic cells, were enumerated using FACScan (BD Biosciences). Data were analyzed with CellQuest (BD Biosciences) and FlowJo softwares (Tree Star Inc.). Cells positive or negative for annexin V were regarded as apoptotic or non-apoptotic cells, respectively.

Cell cycle analysis. To examine apoptosis, 1.0×10^4 cells/well PC12D cells were seeded onto 96-well culture plate and incubated for 48 h in DMEM with NGF and treated with caffeine for 72 h. The cells were harvested and washed with PBS and fixed with ice-cold 70% ethanol at 4°C for 2 h. The cells were then stained with PI solution according to previously reported protocol.⁴¹ DNA content was analyzed by flow cytometry using FACScan and CellQuest software (BD Biosciences).

Compounds. Compounds used included caffeine (Wako, 031-06792), E64d (Sigma, E8640), pepstatin A (Sigma, P5318), rapamycin (LC Laboratories, R5000), CCI-779 (Selleck Chemicals, S1044), MPP⁺ (Sigma, M0896), bafilomycin A1 (Sigma, B1793), 3-methyladenine (Sigma, M9281), insulin (Sigma, I0516), U0126 (Sigma, U120), Akt1/2 inhibitors (Sigma, A6730), staurosporine (Cell Signaling Technology, 9953) and DMSO (Sigma, D2650).

Figure 6 (See next page). Caffeine induces apoptosis by enhancement of autophagy. (A) After PC12D cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with DMSO or MPP⁺ for 72 hours, cell viability was measured using trypan blue dye exclusion assay. Data are the means of triplicate experiments. (B) After cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with DMSO or MPP⁺ for 48 hours, mitochondrial membrane potential was analyzed by JC-1 using a flow cytometry. Data are the means of triplicate experiments. (C) After PC12D cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with DMSO or MPP⁺ for 72 hours, caffeine-induced sub G₁ area was analyzed by propidium iodide staining assay using a flow cytometry. Data are the means of triplicate experiments. (D) PC12D cells were treated with H₂O or caffeine for 24 hours or staurosporine (positive control) for 3 hours and analyzed with immunoblotting for levels of caspase-3 and cleaved caspase-3. (E) After PC12D cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with or without 1, 3 or 5 mM 3MA for 24 hours, cell viability was measured by trypan blue dye exclusion assay. (F) PC12D cells were transfected with control siRNA or siRNAs targeting *Atg7*. Forty eight hours later, they were treated with 0, 1, 2.5 or 10 mM caffeine for 24 hours and mitochondrial membrane potential was analyzed using JC-1. The knockdown effects on *Atg7* were confirmed by immunoblotting using antibodies against *Atg7* and actin. Data are the means of triplicate experiments. Error bars, S.D. NS, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Plasmid DNAs. Myristoylated Akt (21–151), a constitutively active form of Akt, was purchased from Millipore.

siRNA knockdown experiments. PC12D cells were transfected with rat Atg7 siRNAs (Invitrogen, 10620318-9) using Lipofectamine RNAiMAX (Invitrogen, 13778-075) according to the manufacturer's protocol.

Western blotting. Cell pellets were lysed on ice in RIPA buffer for 20 minutes in the presence of protease inhibitor (Roche). Western blotting was performed according to a previously published report.⁴² The antibodies used were as follows: anti-p70 ribosomal protein (Cell Signaling Technology, 2708), anti-ribosomal protein (Cell Signaling Technology, 2217), anti-4E-BP1

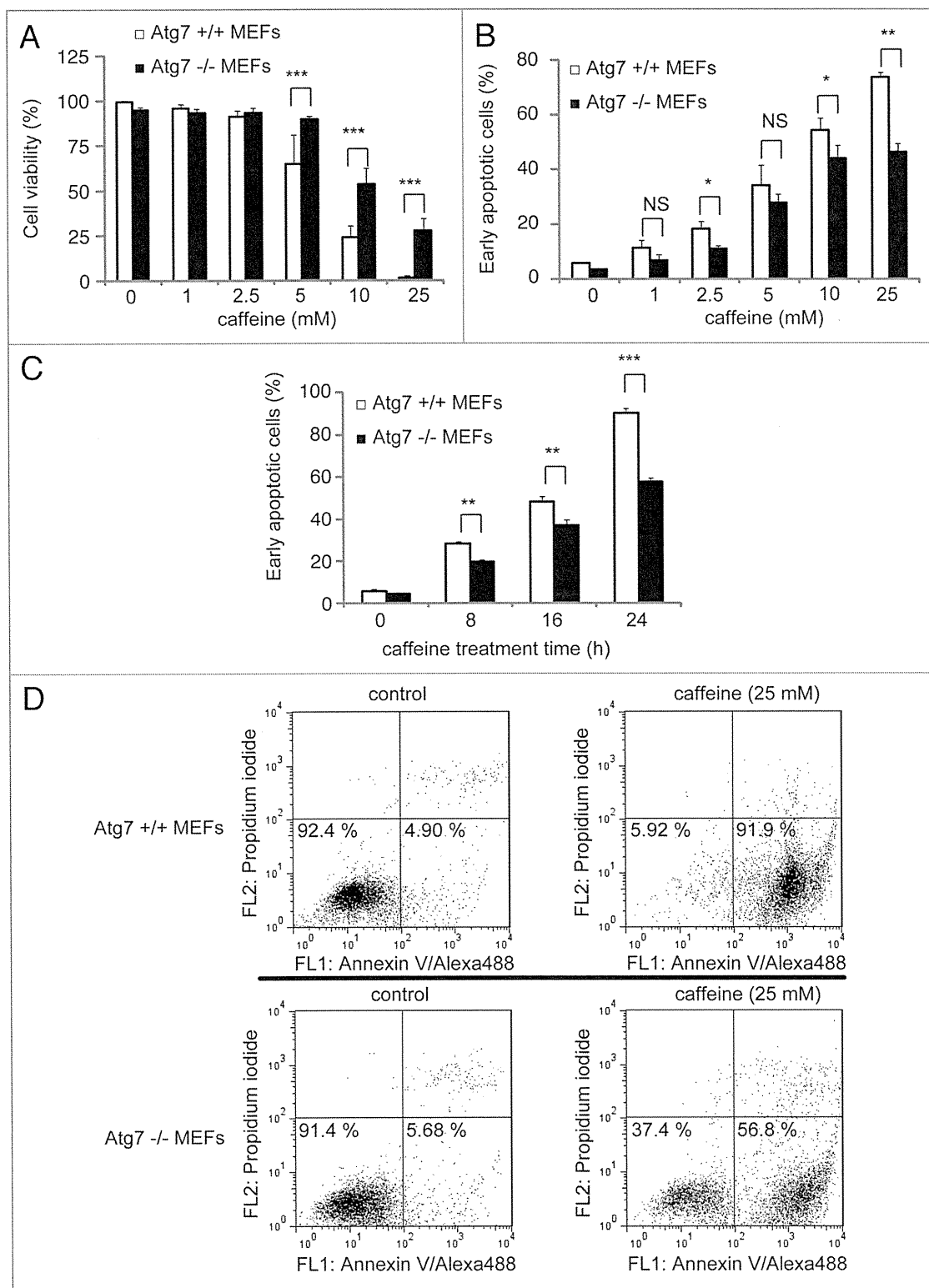


Figure 7. Cells without *Atg7* expression are more resistant to caffeine-induced apoptosis. (A) After *Atg7*^{+/+} or ^{-/-} mouse embryonic fibroblasts (MEFs) were treated with 0, 1, 2.5, 5, 10, 25 mM caffeine for 24 hours, the cell viability was measured by trypan blue dye exclusion assay. Data are the means of triplicate experiments. (B–D) Fluorescence-activated cell-sorting analysis for annexin V/propidium iodide (PI). *Atg7*^{+/+} or ^{-/-} MEFs were cultured with various concentrations of caffeine for 24 hours (B) or with 25 mM caffeine for various times (0, 8, 16 or 24 hours) (C and D). Annexin V/PI staining was subsequently performed to assess early or late apoptosis and necrosis. 5×10^3 cells were analyzed by flow cytometry and the percentage of early apoptotic cells (annexin V-positive and PI-negative cells, the lower right region in (D)) was determined. Data are the means of triplicate experiments. Error bars, SD. NS, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(Cell Signaling Technology, 9452), anti-Akt (Cell Signaling Technology, 9272), anti-p44/42 MAP kinase (Cell Signaling Technology, 9102), anti-phospho-p70 ribosomal protein (Thr389) (Cell Signaling Technology, 9205), anti-phospho-S6 ribosomal protein (Ser235/236) (Cell Signaling Technology, 2211), anti-phospho-4E-BP1 (Thr37/46) (Cell Signaling Technology, 9459), anti-phospho-p44/p42 MAPK (Thy202/Tyr204) (Cell Signaling Technology, 9101), anti-Atg7 (Cell Signaling Technology, 2631), anti-phospho-Akt (Cell Signaling Technology, 4060), anti-actin (Millipore, clone C4), anti-LC3 (MBL, clone 4E12), anti-p62 (Progen Biotechnik, GP62-C) antibodies. Antibody signals were enhanced with chemifluorescent methods from GE HealthCare.

Immunofluorescent microscopy. Cells were embedded with 4% paraformaldehyde for 20 minutes. Following this, they were permeabilized with 0.1% Triton-X in 1x PBS. After incubation with 10% FBS and 1% bovine serum albumin in 1x PBS for 30 minutes, cells were immunostained with anti-LC3B (x500) (Sigma, L7543), anti-LAMP2 (x50) (Development Studies Hybridoma Bank, clone H4B4) overnight and incubated with anti-rabbit IgG tagged with AlexaFluor 488 or anti-mouse IgG tagged with AlexaFluor 546 for 1 hour. The cover slips were embedded with VectaShield, stained with DAPI and images were acquired on a Zeiss LSM510 META confocal microscope (63 x 1.4 NA) or a Leica TCS SP5 confocal microscope at room temperature using Zeiss LSM510 v.3.2 software or Leica LAS AF software. Adobe Photoshop 7.0 (Adobe Systems Inc.) was used for subsequent image processing. For colocalization assay in HeLa cells, an appropriate confocal image was taken with Leica LAS AF software. Then, these images were analyzed automatically with the ImageJ "Colocalization" Plugin (Settings: Each threshold: 25, Ratio: 75%) followed by "Analyze particles" (Settings: threshold 25; Pixel: 1) between endogenous LC3 positive and

LAMP2 vesicles. Experiments were done in triplicate at least twice.

Quantification of cells with GFP-LC3 vesicles. HeLa cells stable expressing GFP-LC3 were treated with various concentrations of caffeine for 24 or 48 hours and then fixed as described above. Analyses in triplicate were done for counting the proportion of GFP-positive cells with GFP-LC3 vesicles as previously described in reference 43.

Electron microscopy. SH-SY5Y cells treated with various concentrations of caffeine were prefixed in 2% glutaraldehyde in PBS at 4°C, treated with 1% OsO₄ for 3 hours at 4°C, dehydrated in a graded series of ethanol and flat embedded in epon. Ultra-thin sections were doubly stained with uranyl acetate and observed using a JEOL JEM-2000EX electron microscopy at 80 kV.

Statistical analysis. Densitometry analysis was performed using ImageJ 1.43 on immunoblots from three independent experiments. A t-test was performed with SYSTAT software (Hulinks).

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Note

Supplementary materials can be found at: www.landesbioscience.com/journals/autophagy/article/14074

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REVIEW

Molecular pathogenesis of Parkinson's disease: update

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ABSTRACT

Parkinson disease (PD) is a neurodegenerative disease characterised by progressive disturbances in motor, autonomic and psychiatric functions. Much has been learnt since the disease entity was established in 1817. Although there are well established treatments that can alleviate the symptoms of PD, a pressing need exists to improve our understanding of the pathogenesis to enable development of disease modifying treatments. Ten responsible genes for PD have been identified and recent progress in molecular research on the protein functions of the genes provides new insights into the pathogenesis of hereditary as well as sporadic PD. Also, genome wide association studies, a powerful approach to identify weak effects of common genetic variants in common diseases, have identified a number of new possible PD associated genes, including PD genes previously detected. However, there is still much to learn about the interactions of the gene products, and important insights may come from chemical and genetic screens. In this review, an overview is provided of the molecular pathogenesis and genetics of PD, focusing particularly on the functions of the PD related gene products with marked research progress.

INTRODUCTION

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease, named after James Parkinson's who provided a classic account of the condition in 1817. Affecting 1–2% of the population over the age of 65 years, the prevalence of PD increases by approximately 4% in those older than 85 years. Ten genes that contribute to the genetic aetiology of hereditary PD (hPD) were identified, mainly through positional cloning strategies in inherited PD patients and families (table 1).^{1–2} Several responsible genes for hPD have been identified, and based on functional studies in vitro and in vivo of gene products, some have been found to interact with each other in various cellular systems for homeostasis, such as synaptic homeostasis (α -synuclein), mitochondrial maintenance (PINK1, parkin, DJ-1, Omi/HtrA2), autophagy-lysosome pathway (α -synuclein, parkin, PINK1, Omi/HtrA2), axonal transport (LRRK2) and ubiquitin proteasome systems (α -synuclein, parkin, DJ-1, UCH-L1). Impairments in a number of cellular systems have been suggested to underlie hPD (figure 1). Also, more recent studies revealed that mutations in the same genes can be involved in familial PD and be risk factors for sporadic PD (sPD), suggesting that inherited and

sPD could have common pathological mechanisms.³ Therefore, understanding the function of the proteins encoded by hPD genes will hopefully further our understanding of the mechanisms leading to inherited and sPD.

In this review, we will summarise the latest research progress in the molecular mechanisms of hPD and genetic association studies of sPD.

HEREDITARY PD **α -Synuclein (PARK1 and PARK4)****Clinicogenetics**

SNCA was the first causal PD gene identified in a large Italian family.⁴ Mutations (A30P, E46K and A53T), duplications and triplications of the *SNCA* gene have been reported.² Clinical features of patients with the E46K mutation are similar to those of dementia with Lewy bodies, while A30P is not associated with severe dementia. Individuals with *SNCA* triplication developed an early onset form of PD with rapid progression and more extended neurodegeneration.⁵

Recent genome wide association studies (GWAS) have demonstrated a strong association between common single nucleotide polymorphism within the *SNCA* locus and PD in European and Japanese population, consistent with the finding that variation at the *SNCA* locus increases PD susceptibility.^{6–9} Although the *SNCA* single nucleotide polymorphism associated with sPD show a low OR (1.2–1.4), these findings are consistent with α -synuclein aggregation pathology.

Molecular biology

α -Synuclein is mainly expressed in the presynaptic terminal of the CNS. The protein binds with lipids and unfolds in the steady state. Although the exact function remains unclear, it regulates dopamine homeostasis in presynaptic vesicle cycling.⁵ The phenotype of α -synuclein knockout mice is unremarkable and only shows a mild decrease in dopamine levels in the striatum and a mild decrease in synaptic vesicles in the hippocampus. Compared with the wild-type α -synuclein, mutant forms easily aggregate in neuronal cells in vitro and in vivo.^{10–11} Transgenic mice with wild or mutant α -synuclein under various promoters have shown neuronal inclusions, mitochondrial abnormalities and neurodegeneration.^{12–14} Which type of α -synuclein species is the most toxic to cells remains unclear but some studies assert that mature aggregates are not themselves the toxic moiety but rather an attempt by the cell to clear small toxic oligomers.¹⁵ Hsp90 modulates the assembly of α -synuclein in an ATP

Table 1 Genetic and clinical characteristics of hereditary Parkinson's disease

Locus	Inheritance	Gene	Type of mutation	Clinical features
PARK1/PARK4	AD	SNCA	Missense, duplication, triplication	A30P: late onset, L-dopa responded parkinsonism; A53T: typical parkinsonism with rapid progression; E64K: DLB-like symptoms; duplication: typical parkinsonism; triplication: early onset parkinsonism with rapid progression
PARK2	AR	PRKN	Nonsense, frameshift, missense	Early onset, symmetric, slowly progressed parkinsonism with spasticity and sleep benefits
PARK3	AD	Unknown	—	—
PARK5	AD	UCH-L1	Missense	Similar to sporadic PD
PARK6	AR	PINK1	Nonsense, frameshift, missense	Early onset typical parkinsonism with psychiatric symptoms and L-dopa associated dyskinesia
PARK7	AR	DJ-1	Missense	Early onset parkinsonism with psychiatric symptoms, occasionally with scoliosis and blepharospasm
PARK8	AD	LRRK2	Missense	Middle to late onset typical parkinsonism with response to L-dopa
PARK9	AR	ATP13A2	Missense, deletion, insertion, duplication	Rapidly progressed parkinsonism with dementia and pyramidal features
PARK10	Sporadic	Unknown	—	—
PARK11	AD	Unknown	—	—
PARK12	Sporadic	Unknown	—	—
PARK13	AD	Omi/HtrA2	Missense	Typical parkinsonism
PARK14	AR	PLA2G6	Missense	Early onset parkinsonism with rapid progression, cognitive decline and brain atrophy (cerebellum and cerebrum)
PARK15	AR	FBX07	Missense, frameshift	Early onset parkinsonism with spasticity and response to L-dopa
PARK16	Sporadic	Unknown	—	—

AD, autosomal dominant; AR, autosomal recessive; DLB, dementia with Lewy bodies; PD, Parkinson's disease.

dependent manner by restricting conformational fluctuations of α -synuclein.¹⁶ Recent advances in research on the protein degradation system associated with PD revealed the importance of ubiquitin proteasome and the autophagy-lysosome pathway in disease pathogenesis.¹⁷ Wild-type α -synuclein is degraded by both chaperone mediated autophagy and macroautophagy, while A30P and A53T are degraded mainly by the latter.¹⁷⁻¹⁹ Furthermore, macroautophagy itself is blocked by α -synuclein via Rap1a dysregulation.²⁰

Several lines of evidence have shown that permeabilised α -synuclein from a neuron may be toxic to neurons and/or glia they are next to. Actually, grafted healthy neurons can gradually develop the same pathology as host neurons in PD brains.²¹ These findings have suggested that non-cell autonomous cell death as well as cell autonomous cell death may have an important role in disease pathogenesis.

Parkin (PARK2)

Clinicogenetics

The first genetic locus for autosomal recessive juvenile parkinsonism was mapped to chromosome 6, and the disease gene named parkin (*PRKN*) was identified in consanguineous families.²²⁻²⁴ Mutations in the *PRKN* gene are most common in autosomal recessive juvenile parkinsonism and many mutations have been reported.⁵ The clinical picture is similar to that of sPD except for earlier onset, dystonic features, brisk reflexes and sleep benefit. Pathologically, no Lewy bodies were seen in most cases.²⁵⁻²⁷ Whether or not heterozygous *PRKN* mutations may cause or increase the susceptibility to late onset typical PD remains controversial. [18F]Fluorodopa uptake by positron emission tomography was reduced in heterozygous carriers without symptoms.^{28 29} In addition, heterozygous carriers of *PRKN* mutations have been reported to have either minor motor signs or present with late onset parkinsonism, suggesting a link between heterozygous mutations and disease pathogenesis.^{27 30 31} On the other hand, screening for *PRKN* mutations in late onset PD and healthy controls revealed similar frequencies of genetic variants.^{32 33}

Molecular biology

Parkin is associated with the ubiquitin proteasome system as an E3 ubiquitin ligase.³⁴ The C terminal binds with ubiquitin E2 enzymes and recognises a substrate whereas the N terminal interacts with the 19S subunit of proteasome. A nonsense mutation lacking the rear RING finger motif had no E3 activity and sole IBR-RING2 retained E3 activity, and thus most parkin mutations do not lead to loss of kinase activity.³⁵ α -Synuclein and synphilin-1 were identified as parkin substrates and consist of Lewy bodies.^{36 37} Parkin mainly localises in the cytoplasm as well as in plasma membranes and partly in mitochondria. Under physiological or pathological conditions, parkin is involved in mitochondrial maintenance and recent evidence revealed that parkin with PINK1 physically associate and functionally cooperate to identify and label damaged mitochondria for selective degradation via autophagy (mitophagy).³⁸⁻⁴² Protein-protein interactions between parkin and other PD related genes are detailed in each gene section.

PINK1 (PARK6)

Clinicogenetics

PARK6 was first identified on chromosome 1p36.⁴³ The disease gene was identified as *PINK1* (PTEN induced kinase 1) containing eight exons.⁴⁴ The clinical characteristics are autosomal recessive, early onset, slow disease progression and L-dopa responsive parkinsonism. Most mutations were missense mutations, but whole gene deletions were also reported.^{45 46} Many putative pathogenic mutations were also observed in a heterozygous state in familial and sPD patients as well as in healthy controls. However, most of the studies have not checked the copy number variants, causing the mutation pathogenicity to remain controversial.² Lewy bodies, neuronal loss and astrocytic gliosis in the substantia nigra were detected in a patient with *PINK1* compound heterozygous mutations.⁴⁷

Molecular biology

PINK1 has eight exons encoding 581 amino acids, including a mitochondrial targeting sequence, transmembrane domain and

Movement disorders

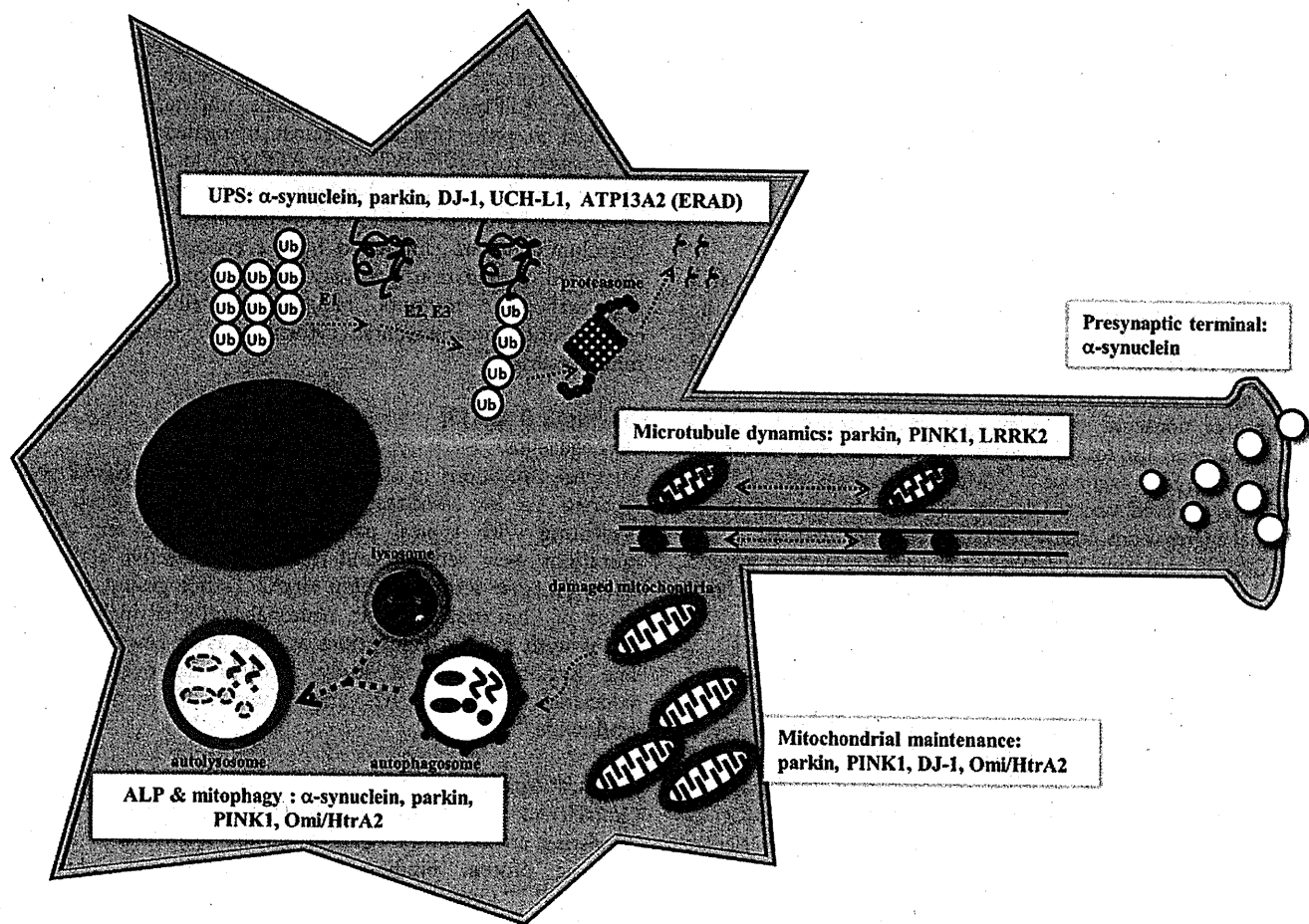


Figure 1 Schematic representation of the possible pathogenesis in hereditary Parkinson's disease. ALP, autophagy–lysosome pathway; ERAD, endoplasmic reticulum associated degradation; Ub, ubiquitin; UPS, ubiquitin proteasome system.

kinase domain.⁴⁸ The gene product is ubiquitously expressed in the brain and systemic organs. The protein mainly localises in mitochondria, especially in the outer membrane. PINK1 is a serine–threonine kinase and several pathological mutations in PINK1 have been reported to change their kinase activities.^{49–52} In addition, Rictor (a component of mTORC2),⁵³ tumour necrosis factor receptor associated protein 1 (TRAP1; a mitochondrial chaperone),⁵⁰ Omi (PARK13 gene product) and parkin (PARK2 gene product) were identified as substrates for PINK1.^{54 55}

PINK1 regulates mitochondrial dynamics and respiratory functions.^{58 53 56–58} Mitochondrial fission is accelerated by PINK1 overexpression accompanied by parkin.^{59 60} PINK1 ablation with siRNA in neurons reduces resistance against oxidative stress while its overexpression provides resistance.⁶¹ Using genetically modified *Drosophila* models, we see that PINK1 deficiency causes the same phenotype as parkin deficiency and the PINK1 deficiency phenotype is rescued by parkin complementation, suggesting that parkin is downstream of PINK1.^{62–64} Several lines of evidence have provided new aspects of the PINK1/parkin pathway associated with mitochondrial elimination via macroautophagy (mitophagy). When mitochondrial membrane potentials are lost, endogenous PINK1 is accumulated followed by parkin recruitment, and subsequently the depolarised mitochondria were eliminated by mitophagy.^{40 41 65 66} Mitochondrial targeting sequence, kinase activity of PINK1 and the linker domain of parkin are indispensable for the PINK1/parkin mediated mitophagy.

DJ-1 (PARK7)

Clinicogenetics

Clinical features of *PARK7* are characterised by early onset parkinsonism with scoliosis, blepharospasm and psychiatric symptoms, similar to those of *PARK2* and *PARK6*. The disease gene was identified as *DJ-1*, which has eight exons encoding 189 amino acids. Three missense mutations (L166P, M26I, E64D) in exons 1–5 of the gene have been identified in Italian, Dutch and Uruguayan families. *DJ-1* protein was detected around Lewy bodies, suggesting *DJ-1* is not in the main structure of Lewy bodies. However, the protein was detected in astrocytes and in a part of the cytoplasmic inclusions positive to tau in brains with corticobasal degeneration, progressive supranuclear palsy and multiple system atrophy.^{67–69}

Molecular biology

DJ-1 is almost ubiquitously expressed in organs, including the brain. Endogenous *DJ-1* is present in synaptic terminals, mitochondria and membranous organelles.^{70 71} *DJ-1* with the L166P mutation lost more stability compared with the wild-type and mutant *DJ-1* (M26I, E64D).⁷² In *DJ-1* knockout mice, no significant loss of dopaminergic neurons and decreased susceptibility to oxidative stress were noted.⁷³ *DJ-1* is a multifunctional redox sensitive protein regulating mitochondrial oxidative stress and increases expression levels of SOD1 in an Erk1/2-Elk1 pathway dependent manner,⁷⁴ and facilitates prosurvival factor Akt, leading to suppression of apoptosis.⁷⁵ Also, the protein