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Functional Characterization of Phosphorylation Sites in Dynamin-Related Protein 1

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Abstract

Dynamin-related protein 1 is member of the dynamin-family of large GTPases. Similar to the endocytosis motor dynamin, Drp1 uses GTP hydrolysis to power constriction of the outer mitochondrial membrane and ultimately mitochondrial division. Dynamin phosphorylation in its unique C-terminal proline-rich domain interferes with binding of accessory proteins that induce membrane curvature and inhibits clathrin-mediated endocytosis. Evidence within the last few years indicates that Drp1 is also regulated by the phosphorylation/dephosphorylation cycle. Drp1 regulation is complex, in that both inhibitory and activating phosphorylations have been described that lead to, respectively, mitochondrial elongation and shortening. In this chapter, we describe methods for the identification and functional characterization of Drp1 phosphorylation sites. Among these methods is replacement of the endogenous protein by phosphorylation-site mutant Drp1 via combined shRNA and RNAi-resistant cDNA expression from the same plasmid. We also discuss primary astrocyte cultures as a model for regulation of cell death and mitochondrial morphology by Drp1 and present ImageJ macro source code for unbiased quantification of mitochondrial shape changes.

1. Introduction

Highly conserved from yeast to man, Drp1 belongs to the dynamin-family of large GTPases. Drp1 most likely functions as a mechanoenzyme that forms ring- or spiral-shaped superstructures to constrict and eventually sever mitochondria (Hoppins *et al.*, 2007). In addition to catalyzing mitochondrial division, Drp1 participates in cristae remodeling and cytochrome *c* mobilization during apoptosis (Suen *et al.*, 2008). In mammalian cells, Drp1 is recruited from the cytosol to the mitochondrial membrane by a multi-protein complex that includes the membrane-anchored adaptor protein Fis1. Fis1/Drp1 is also responsible for fission of peroxisomes, much smaller organelles delimited by a single membrane (Koch *et al.*, 2003, 2005). Even though Drp1 knockout mice have not been reported, it is likely that Drp1 is an essential enzyme in mammals, because a dominant-negative mutation in the fission GTPase causes severe birth defects in humans (Waterham *et al.*, 2007).

Drp1 is a ~80 kDa protein with a domain structure similar to the dynamins (Fig. 13.1). Shared with the dynamins are the N-terminal GTPase, the middle (MID) domain, and the GTPase effector domain (GED). Drp1 lacks the poly-proline domain that follows the GED in the dynamins. The poly-proline domain is the major regulatory region of the dynamins, containing PKC and CDK5 phosphorylation sites as well as docking sites for SH3-domain

containing proteins including amphiphysin (Smillie and Cousin, 2005; Takei *et al.*, 2005). Whereas a lipid-binding PH domain separates the MID and GED in the dynamins, a poorly conserved variable domain (VD) of unknown function takes its place in Drp1 (Fig. 13.1). Mammalian Drp1 is diversified by alternative splicing of three short (11–26 amino acid) exons, the most N-terminal of which separates subdomains A and B of the GTPase domain, whereas the other two alternative exons are in the VD. While the functional consequences of alternative splicing are unknown, all eight possible splice variants are represented as expressed sequence tags (ESTs). The existence of multiple Drp1 variants has confused the phosphorylation site nomenclature (Jahani-Asl and Slack, 2007), and care should be taken to refer to sequence numbers only in conjunction with the species origin and splice variant.

Posttranslational regulation of Drp1 is an active area of research, with ubiquitylation, sumoylation, and phosphorylation having been documented (Chang and Blackstone, 2007; Cribbs and Strack, 2007; Han *et al.*, 2008; Harder *et al.*, 2004; Nakamura *et al.*, 2006; Wasiak *et al.*, 2007). Phosphorylation at two sites has been shown to have functional consequence (Fig. 13.1). The first report came from Mihara's group, who reported that CDK1/cyclin B phosphorylates Drp1 at a Ser residue in the VD drives the breakdown of the mitochondrial reticulum during early mitosis (Taguchi *et al.*, 2007). Only 21 residues upstream of this activating phosphorylation site at the border of the GED is a Ser residue, phosphorylation of which by PKA inhibits the mitochondria-severing activity of Drp1 in HeLa and PC12 cells (Chang and Blackstone, 2007; Cribbs and Strack, 2007). This residue is dephosphorylated by the calcium/calmodulin-dependent phosphatase calcineurin (a.k.a. protein phosphatase 2B, PP2B), contributing to the calcium-dependent fragmentation that ensues following mitochondrial depolarization (Cereghetti *et al.*, 2008; Cribbs and Strack, 2007). However, the opposite result was also reported, in that calcium/calmodulin-dependent kinase I (CaMKI)-mediated phosphorylation of the same Drp1 site was found to participate in high potassium-induced mitochondrial scission in neurons (Han *et al.*, 2008). While different Drp1 splice variants were examined in these conflicting studies, our results so far suggest that alternative exons in the VD do not influence mitochondrial shape changes induced by Drp1 phosphorylation-site mutants (Fig. 13.5). Clearly, more work is required to understand the interplay between different phosphorylation events and the mechanism by which phosphorylation of Drp1 leads to mitochondrial shape changes and altered apoptotic sensitivity. This chapter describes some of the methods that our laboratory uses to characterize Drp1 phosphorylation sites.

2. Replacement of Endogenous with Phosphorylation-Site Mutant Drp1

A common way to infer the effect of site-specific phosphorylation on protein function is to replace the phospho-acceptor with an amino acid that cannot be phosphorylated, or with a negatively charged amino acid that mimics the phosphate group. To create non-phosphorylatable mutants, Ser/Thr residues are most often replaced by Ala, while Tyr is best replaced with Phe. Constitutive phosphorylation may be achieved by introduction of an Asp or Glu. We usually replace Ser with Asp and Thr with Glu to match for size. As there are no naturally occurring amino acids with a negatively charged aromatic side chain, phosphomimetic substitution of Tyr is difficult. Even for Ser and Thr, there is no guarantee that an acidic amino acid substitution will faithfully replicate the effects of phosphorylation,

since a phosphate group is considerably larger and carries twice the negative charge of a carboxylate. For instance, autophosphorylation of calcium/calmodulin-dependent protein kinase II (CaMKII) at Thr286 results in activation of the enzyme to ~50% of its maximal activity in the presence of calcium/calmodulin. However, replacing Thr286 with Asp only results in ~10% CaMKII autonomous activity, implying that this supposedly phosphomimetic substitution does not fully remove the auto-inhibitory region from the catalytic cleft of the kinase (Brickey *et al.*, 1994).

In the most easily interpretable scenario, the cellular activity of the wild type, phosphorylatable protein will fall between that of the Ala and that of the Asp/Glu substitution. If the Ala substitution alters activity, but the acidic substitution does not, then either Glu/Asp is not a faithful phosphomimetic or basal phosphorylation levels at the site in question are high. An essential structural role of the Ser/Thr is suggested in the case that both types of mutations are found to impair activity of the protein.

Overexpression of proteins can lead to artifactual results. Especially in the case of homo-oligomeric proteins like Drp1, the presence of the endogenous protein may obscure effects of mutating specific phosphorylation sites. For these reasons, we routinely combine shRNA-mediated knockdown with expression of the RNAi-resistant cDNAs to replace wild type with mutant Drp1 in the cell. To ensure co-expression of shRNA and cDNA, we express both from the same plasmid backbone.

2.1. Selection and characterization of Drp1-specific shRNAs

To select RNAi target sites, we use criteria derived from an empirical study of siRNA efficiency by Reynolds and colleagues at Dharmacon (Reynolds *et al.*, 2004). Criteria include low G/C content, low internal stability at the sense strand 3'-end, lack of inverted repeats, and base preferences at specific positions. This target selection algorithm has been implemented as an Excel macro and as a Web tool accessible at the Dharmacon Web site. We also use other Web tools (siRNA scales: http://gesteland.genetics.utah.edu/siRNA_scales/; DSIR: <http://cbio.ensmp.fr/dsir/>) that scored favorably in a recent evaluation of available siRNA selection algorithms (Matveeva *et al.*, 2007). A point to consider is that these tools may not optimally predict the efficiency of vector-encoded shRNAs, which generally do not reach cellular concentrations of chemically synthesized siRNAs.

We generate between 3 and 5 shRNA encoding plasmid for each gene target and find that generally one or two silences the expression of the cotransfected target's cDNA by 80% or better. Target sites are selected that fulfill three criteria: (1) an efficiency score in the upper 10% (score 6 or greater with the Reynolds *et al.* algorithm), (2) perfect conservation in human and rat, the origin of most of our cell lines and primary cell cultures, (3) no more than 15 consecutive matches with mRNA from any other vertebrate organism to minimize off-target effects. We use the hairpin design and H1 promoter of the original pSUPER vector described in Brummelkamp *et al.* (2002). The H1 promoter-shRNA cassette is amplified from pSUPER or related vectors by PCR using a forward primer that anneals 5' of the promoter sequence:

`cgcacatgtgaacgctgacgtcatcaaccg`

and a reverse primer that encodes the specific shRNA (in this example, Drp1-specific bases are in capital letters):

cgcacatgtccggattccaaaaGAGTGTAAGTTCAtctcttgaa

TGAATCAGTTACACTCTTCggggatctgtgtctcatacagaac

Both primers include a *PciI* site (underlined) for ligation; the reverse primer also contains a *BspEI* site, so the direction of insertion can be easily determined. We use a proofreading polymerase, such as Pfu Ultra (Stratagene) to minimize PCR errors and an initial annealing temperature of 60 °C. Depending on the stability of the hairpin in the reverse primer, PCR conditions may have to be optimized, for instance by varying the annealing temperature from 55 to 65 °C. The PCR product is gel purified (we use a Qiagen kit), digested with *PciI*, and ligated into the *PciI* site of the pEGFP series of vectors from Clontech/Takara. We found that insertion of the H1 promoter:shRNA cassette into the unique *PciI* site, which is located near the plasmid's origin of replication, does not interfere with cDNA expression from the CMV promoter or expression of the G418/kanamycin resistance gene.

To select the most efficient shRNA for subsequent functional studies, we initially test three to four candidate shRNA plasmids for silencing of cotransfected expression plasmids, transfecting COS or HEK293 cells with increasing shRNA to cDNA plasmid ratios (e.g., 0.2:1 to 3:1). If the target cDNA is available fused to a fluorescent protein, knockdown efficiency can be visually estimated by live cell imaging. More commonly though, we quantify knockdown of epitope-tagged cDNAs by western blotting and subsequent densitometry using the gel analysis macro set of ImageJ.

2.2. Site-directed mutagenesis

To replace endogenous with phosphorylation-site mutant fission/fusion proteins (e.g., Drp1), the cDNA has to be first rendered insensitive to the co-expressed shRNA. To this end, two to five silent (non-coding) base changes are incorporated into the shRNA target site using protocols adapted from the QuikChange mutagenesis kit. Briefly, double-stranded oligonucleotides with 19–25 bp flanking a central region with up to eight base changes are used for linear amplification of the template plasmid with Pfu Ultra. For screening purposes, we additionally incorporate a silent restriction site into the mutagenic oligonucleotide. To render Drp1 resistant to the shRNA described above, we used the following sense primer (shRNA target sequence underlined, base changes capitalized):

gccaaactggacattaacaataagaaATCAgtGactgattcaatccgtga tgagtatgc

The plasmid template is destroyed by digestion with *DpnI*, a methylation-specific restriction enzyme, and the remaining mutated DNA is transformed into competent *E.coli*, where the DNA is circularized by single-strand break repair. Through additional subcloning, the H1 promoter:shRNA and cDNA expression cassettes are incorporated into the same plasmid backbone to ensure co-expression. Mutagenesis of Ser/Thr phosphorylation sites is carried out identically, using the plasmid that expresses both shRNA and shRNA-resistant cDNA as a template.

2.3. Transient transfection and generation of stable cell lines

Vectors for replacing endogenous with phosphorylation-site mutant fission/fusion enzymes can be used for both transient and stable expression of shRNA and cDNA, with stable expression utilizing the neomycin (G418) resistance gene that is part of the pEGFP series of plasmids. We use transient transfection for analysis of mitochondrial morphology (see Section 6), subcellular localization of Drp1, and most biochemical assays, while stable cell lines are desirable for assays that require a homogenous cell population (e.g., 96-well plate growth and viability assays). We most commonly employ chemical transfection using Lipofectamine 2000 for adherent cells according to the manufacturer's instructions (BD Biosciences). Most cell lines we use routinely (COS, HEK293, PC12) are transfected with 2 μ g DNA and 4 μ l Lipofectamine 2000 per ml of OptiMEM growth media. For primary neuronal cultures, which do not tolerate high concentrations of cationic lipids as well, we decrease the Lipofectamine concentration to 0.8–1.5 μ l/ml and shorten the incubation period from 4–5 to 3 h or less. While cDNA expression may peak earlier, we mostly analyze cells around 72 h after transfection to ensure that the endogenous protein is maximally downregulated by the co-expressed shRNA.

To generate stable cell lines, cells are trypsinized 48–72 h after transfection and divided over several larger dishes (e.g., cells transfected in a 60 mM dish are re-seeded into 5–6 100 mM dishes), adding 500 μ g/ml G418 to the media to select for stable genomic integration of the transfected plasmid. After 2–3 weeks, individual colonies are isolated and expanded, lowering the G418 concentration to 100 μ g/ml for maintenance. We pick individual colonies under the dissecting microscope, using a p200 pipettor to scrape cells from the substrate while releasing the plunger to draw up the cell suspension. If the overexpressed protein carries a fluorescent protein tag (e.g., GFP-Drp1), positive clones can be identified by epifluorescence microscopy prior to isolation, which reduces the number of clones that have to be expanded for characterization. Clones are characterized for expression of the transfected cDNA and knockdown of the endogenous protein by immunoblotting (Fig. 13.2).

3. Analysis of Drp1 Phosphorylation

Metabolic labeling with radioactive orthophosphate ($^{32}\text{PO}_4^{2-}$) is a relatively straightforward method to assess the degree to which a protein is phosphorylated in intact cells under various stimulation conditions. Mass spectrometry has emerged as a powerful tool for the unbiased identification of phosphorylation sites. However, despite recent advances (Macek *et al.*, 2009), full sequence coverage and reliable quantification of phosphorylation is currently difficult to achieve by mass spectroscopy. For now, metabolic labeling with ^{32}P remains the method of choice for initially establishing a protein as phosphorylated, and for implicating specific kinases and phosphatases in its regulation. Once phosphorylation sites have been confirmed *in vitro* phosphorylation assays and by metabolic labeling of phosphorylation-site mutant proteins, production of phosphorylation-specific antibodies should be initiated to allow for quantitative analysis of site-specific phosphorylation in intact cells and organisms.

3.1. Metabolic labeling with ^{32}P and immunoprecipitation of Drp1

To assess ^{32}P incorporation into your protein of interest in intact cells, it is necessary to separate it from other ^{32}P -labeled proteins and nucleic acids. While this can be achieved by multiple methods, most commonly, proteins are immunoprecipitated with specific antibodies. For Drp1, we either immunoprecipitate the endogenous protein using a monoclonal antibody available from Transduction Laboratories, or we use hemagglutinin (HA) tag-directed antibodies to isolate HA-Drp1 (e.g., carrying mutations of phosphorylation sites) after transient transfection using Lipofectamine 2000 (see above).

Transfected or untransfected cells are grown to subconfluency in 60 mm dishes. To reduce isotope dilution with non-radioactive phosphate, cells are incubated for 30–60 min with phosphate-free RPMI (BD Biosciences) containing 1% dialyzed fetal bovine serum (labeling media) prior to labeling. The media are then replaced with the same media containing 0.5 mCi/ml ^{32}P -orthophosphate, and dishes are placed in a Tupperware or similar container before returning them to the incubator to minimize radioactive contamination. Cells are allowed to incorporate ^{32}P for 5–6 h in the incubator, including kinase and phosphatase activity modulators (e.g., 50 μM forskolin to stimulate PKA, 250 nM okadaic acid to inhibit PP2A) during the last 1–2 h of incubation. After a rinse in phosphate-free RPMI, cells are lysed in 400 μl buffer containing 1% Triton X-100, 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA and both protease (1 mM PMSF, 1 mM benzamidine, 1 $\mu\text{g}/\text{ml}$ leupeptin) and phosphatase inhibitors (1 mM β -glycerolphosphate, 1 mM Na_3VO_4 , 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 μM microcystin-LR). After precipitating insoluble material (10 min centrifugation in a table top centrifuge at maximum speed at 4 $^\circ\text{C}$), lysates are incubated with antibody and agarose beads (10 μl HA antibody–agarose conjugate for transfected Drp1 or 4 μl Drp1 antibody and 10 μl protein G-agarose for endogenous Drp1) for 3–4 h with end-over-end rotation at 4 $^\circ\text{C}$. Immunoprecipitates are recovered by centrifugation (2 min at 2000g) and washed four times in lysis buffer, transferring beads to a fresh tube after the first wash to minimize carry-over of proteins from the lysate. Proteins are eluted from the beads by boiling in SDS sample buffer and resolved on 10% polyacrylamide gels. After transferring proteins to nitrocellulose membranes, blots are first labeled for Drp1 by immunoblotting, and then exposed to PhosphorImager screens to visualize ^{32}P incorporation. Relative phosphorylation is quantified as the ratio of the ^{32}P signal to the Drp1 immunoblot signal in each lane. We use the Gel Analyzer plug-in from ImageJ for densitometry of blots and ^{32}P images.

3.2. Generation of phosphorylation-specific antibodies

Once sufficient evidence has been gathered that a residue is phosphorylated (e.g., by *in vitro* phosphorylation and metabolic labeling of wild type vs. phosphorylation-site mutant proteins), phosphorylation-specific antibodies are useful tools for tracking site-specific phosphorylation in intact cells and tissues. Antibodies are raised against a short peptide into which the phosphorylated residue is incorporated during peptide synthesis. Phosphopeptides are directionally coupled to maleimide-activated keyhole limpet hemocyanin (KLH, Pierce) via a cysteine residue added to either the N- or C-terminus of the peptide. Employing the services of an on-campus facility or a company, we then inject the peptide–KLH conjugated into mice or rabbits for mono- or polyclonal antibody production, respectively. Design considerations for the phosphopeptide include: (1) Short peptides (12

amino acids) are more likely to yield antibodies that include the phospho-Ser/Thr as part of their epitope. (2) The end of the peptide that is farthest from the phosphorylated residue should be sulfhydryl-coupled to the carrier protein so as to maximize accessibility of the phosphorylation site. (3) The unconjugated, exposed end of the peptide should be modified by acetylation (N-terminus) or amidation (C-terminus) to increase resemblance to the native protein. To generate antibodies against the PKA site in Drp1 (Ser656 in rat splice variant 1), we conjugated the following peptide via the C-terminal Cys to maleimide-activated KLH and raised mouse monoclonal antibodies at the University of Iowa Hybridoma Facility:

acetyl-Arg-Lys-Leu-[phospho-Ser]-Ala-Arg-Glu-Gln-Arg-Asp-Cys

Supernatants of hybridoma clones were screened by immunoblotting GST fusion proteins of the Drp1 GED domain (residues 643–755) with or without prior phosphorylation by PKA (see Section 4.2). Several clones were identified that recognize Drp1 only when phosphorylated at the PKA site.

We also generated polyclonal rabbit antibodies against the CDK1/cyclin B phosphorylation site in Drp1 that was initially identified by Mihara's group (Taguchi *et al.*, 2007) using the following peptide as the immunogen:

acetyl-Met-Pro-Ala-[phospho-Ser]-Pro-Gln-Lys-Gly-His-Ala-Cys

The rabbit's immune system is likely to produce not only the desired phospho-specific antibody, but also non-discriminating antibodies that recognize an epitope adjacent to the phosphorylated residue, or even antibodies specific for the non-phosphorylated peptide (presumably as a result of dephosphorylation in the animal). Therefore, polyclonal antisera generally have to be affinity purified to increase their specificity. Minimally, antibodies are subjected to a positive selection step by passing antisera over a column to which the phosphorylated peptide was covalently coupled (ultralink Iodoacetyl gel, Pierce), and then eluting immunoglobulins with 20 mM glycine-HCl, pH 2.0. If the eluted antibodies display significant reactivity toward the non-phosphorylated form of the protein, they should be depleted by adsorption to a second column containing the dephosphoform of the peptide (synthesized separately or enzymatically dephosphorylated by alkaline phosphatase). In the case of the phospho-CDK1/cyclin B site antibody, affinity purification against the phospho-peptide was sufficient to produce antibodies that discriminate between phospho- and non-phospho-Drp1.

4. Production and Assays of Recombinant Drp1

Recombinant proteins are indispensable for showing that a particular kinase or phosphatase directly targets a cellular substrate (as opposed to acting indirectly by modulating the activity of other kinases or phosphatases). When combined with phosphorylation-blocking or -mimicking mutations, they are also useful for demonstrating effects of phosphorylation on *in vitro* enzymatic activities or protein-protein interactions.

4.1. Expression and purification of recombinant Drp1

To express recombinant, full-length Drp1 in *E. coli*, cDNAs are cloned into the pCAL-n-EK vector (Stratagene) using PCR-based strategies. The pCAL series of vectors includes an N-terminal calmodulin binding peptide (CBP) tag, which is used for one-step purification of Drp1 on calmodulin-agarose in the presence of calcium. CBP-Drp1 is then eluted by chelating calcium with EGTA. Because calmodulin is a eukaryotic protein and because both binding and elution conditions are gentle, the CBP purification tag results in a relatively pure (~90%) Drp1 preparation compared to other affinity tags (e.g., His- or GST-Drp1).

BL21 (DE3) Star chemically competent *E. coli* (Invitrogen) are transformed with Drp1 expressing vectors, and 100 ml cultures of SOB media (20 g/l Tryptone, 5 g/l yeast extract, 100 mM NaCl, 25 mM KCl, 1 mM MgCl₂, 1 mM MgSO₄) containing ampicillin (100 µg/ml) are inoculated with individual colonies and grown over night at 37 °C. One liter cultures are inoculated with starter cultures to an OD₆₀₀ of 0.3 and grown to an OD₆₀₀ of 0.6–0.7 before 0.5 mM isopropyl-thiogalactoside (IPTG) is added to induce expression of CBP-Drp1. After 5–6 h continued growth at 37 °C, bacterial pellets are collected by centrifugation (30 min at 20,000g) and stored at –70 °C until further processing. Bacteria are lysed by 30 min shaking incubation at 4 °C in 25 ml of Lysis Buffer (1% Triton X-100, 1 mg/ml lysozyme, 25 mM HEPES, 25 mM PIPES pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine), followed by sonication with a probe-tip sonicator (1 min at maximum power) and clarification by centrifugation (25 min at 30,000g). After addition of 5 mM MgCl₂ and 2 mM CaCl₂ to the lysate, CBP-Drp1 is allowed to bind to 1 ml calmodulin-Sepharose for 2 h at 4 °C, followed by centrifugation (5,000g, 5 min), aspiration of unbound proteins, and washing of the beads with 50 ml Wash Buffer (25 mM HEPES pH 7.5, 50 mM KCl, 5 mM MgCl₂, 2 mM CaCl₂, 10 mM β-mercaptoethanol). Calmodulin-bound Drp1 is eluted by incubation of the beads with 15 ml Elution Buffer (25 mM HEPES pH 7.5, 50 mM KCl, 5 mM EGTA) for 1–2 h at 4 °C. Calmodulin eluates are concentrated with Amicon ultra filters (Millipore, 50,000 molecular weight cut-off) to ca. 200 µl, and supplemented to 50% glycerol for storage at –20 °C. Protein concentrations are determined using the Bradford assay (Pierce) or by Coomassie blue staining of SDS–PAGE gels with known amounts of BSA as standards.

To demonstrate site-specific phosphorylation of Drp1 *in vitro*, we express fragments of the protein as GST fusion proteins and use them in *in vitro* phosphorylation reactions with purified kinases. To this end, Drp1 domains are PCR-amplified and subcloned into pGEX-2T or -4T vectors. Bacterial growth and induction protocols are similar to those described above for full-length CBP-Drp1 expression, except that 100 ml cultures are grown over night (16–18 h) at 23 °C in the presence of 0.5 mM IPTG to induce GST-Drp1 expression. Bacterial pellets are lysed in 5 ml 1% Triton X-100, 1 mg/ml lysozyme, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM benzamidine, 0.1 mM PMSF (30 min, 4 °C, with shaking), and sonicated and centrifuged as above. Cleared supernatants are incubated with 1 ml of a 50% slurry of glutathione–agarose for 2 h at 4 °C. Beads are washed once with 10 ml 0.05% Triton X-100, 20 mM HEPES pH 7.5, 0.1 mM EDTA and are stored at –20 °C in ~0.5 ml of the same buffer containing 50% glycerol. GST fusion proteins are retained on glutathione–agarose beads, which facilitates removal of unincorporated ³²P after

in vitro phosphorylation. Protein concentrations are determined by SDS–PAGE with BSA standards and Coomassie blue staining.

4.2. *In vitro* phosphorylation

In vitro phosphorylations with PKA are carried out in a 200 μ l reaction volume containing 10–20 μ l GST-Drp1 (or GST only control) agarose slurry, 25 units of PKA catalytic subunit (2.5 μ l 10 U/ μ l stock of PKA_c from bovine heart, Sigma), 5–15 μ l [γ -³²P]ATP (5 mCi/ml, Amersham), 200 μ M unlabeled ATP, 50 mM Tris pH 7.5, 10 mM MgCl₂, 2 mM DTT, 1 mM EGTA, 0.01% Triton X-100. Reactions started by the addition of GST-Drp1 proceed for 15 min at 30 °C on an Eppendorf thermomixer (2 s 1000 rpm mixing every 1 min) and are stopped by the addition of 1 ml Tris-buffered saline containing 10 mM EDTA. After precipitation of the GST-Drp1 agarose (2 min at 3,000g) and removal of the supernatant, SDS sample buffer is added and proteins are separated by 10% polyacrylamide gels, stained with Coomassie blue, and exposed to PhosphorImager cassettes to quantify ³²P incorporation. Coomassie blue-stained proteins are imaged using the 700 nm laser line of the Odyssey imager (Licor). Relative phosphorylation levels of, e.g., wild type versus phosphorylation-site mutant GST-Drp1 fusion proteins are determined by digital densitometry utilizing the Gel Analyzer plug-in of ImageJ, dividing ³²P signals by protein signals in each lane.

4.3. GTPase activity assays

For assaying the GTPase activity of wild type and mutant Drp1, we use a rapid method based on the release of ³²P-labeled orthophosphate from [γ -³²P]GTP and adsorption of non-hydrolyzed [γ -³²P]GTP by charcoal (Brandt and Ross, 1986). Reactions are started by the addition of 50 μ l purified CBP-Drp1 (~2 μ g) to 150 μ l of 25 mM HEPES pH 7.5, 50 mM KCl, 10 mM MgCl₂, 25 μ M unlabeled GTP, 2 μ l [γ -³²P]GTP (10 mCi/ml, Amersham). Reactions proceed at 30 °C with intermittent mixing (2 s 1000 rpm every min) using an Eppendorf thermomixer, while 10 μ l aliquots are removed at 0, 5, 15, 30, 45, 60 min, etc. time points and added to 700 μ l of an activated charcoal suspension (10% w/v Charcoal Norit A in PBS, 50–200 mesh, Fisher). For determination of total [γ -³²P]GTP, 10 μ l reaction aliquots are added to 700 μ l PBS. After centrifugation of the charcoal-containing tubes (7 min at 20,000g), triplicate 50 μ l aliquots of the supernatants are added to 1.5 ml Biosafe II scintillation fluid and subjected to liquid scintillation counting. Percent hydrolysis is calculated by dividing the cpm in the charcoal supernatants by the cpm in the PBS-diluted aliquots.

5. Cell Death Assays

The mitochondrial fission/fusion equilibrium is an important determinant in cellular life and death decisions. In particular, inhibiting mitochondrial fission by RNA interference or expression of dominant-negative of Drp1 (GTPase-defective Lys38 mutant) slows both apoptotic and non-apoptotic cell death (Suen *et al.*, 2008). Our laboratory has shown that PC12 cell lines expressing Drp1 pseudo-phosphorylated at the PKA site (S656D, rat variant 1 numbering) are remarkably resistant to several apoptotic stimuli; conversely, non-phosphorylatable Drp1 (S656A) confers increased sensitivity to the same set of stimuli

(Cribbs and Strack, 2007). In the following, we describe two cell death assays currently used in the laboratory.

5.1. Viability assays in stable PC12 cell lines

Cell viability assays in a 96-well plate format allow for rapid testing of multiple toxic compounds at different concentrations. However, these assays require a homogenous cell population (i.e., cell lines expressing phosphorylation-site mutant Drp1 stably, see Section 2.3) and cannot distinguish between apoptotic and non-apoptotic cell death mechanisms. Cell lines expressing proteins of interest from an inducible promoter allow comparison to an uninduced cell population (e.g., Strack *et al.*, 2004). If inducible cell lines are unavailable, multiple clonal cell lines expressing the same mutant protein constitutively should be analyzed to avoid clonal selection artifacts.

Stable PC12 cell lines are seeded at 8000 cells/well in 96-well plates (100 μ l media/well). Four wells (half a column) are assayed for each condition; however, we frequently exclude the edge and corner wells from the final analysis, because liquid in these wells is prone to evaporation. After 2 days of growth (to ca. 80% confluency), media are replaced with fresh media containing the desired concentrations of drugs, which are prepared by serial dilution. Cell viability is determined by a colorimetric assay that is based on reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to formazan (CellTiter 96TM AQueous non-radioactive cell proliferation assay, Promega). While the MTS assay measures metabolic capacity as opposed to cell density, we find that results closely mirror those obtained by counting viable (Trypan-blue excluding) cells. Two days after drug addition, we replace the old media with fresh media containing MTS and phenazine-methosulfate (PMS) using an 8-channel pipettor. Residual MTS/PMS-containing media is pipetted into an empty 96-well plate to provide blank values. The 96-well plates are returned to the incubator for 2–4 h until sufficient brown formazan reaction product has formed, and the absorbance at 490 nm is determined using a plate reader. After background subtraction, cell viability is expressed relative to control wells incubated without drugs.

5.2. Apoptosis/necrosis assays in primary astrocytes

Astrocytes from neonatal rodent brain provide a convenient and physiologically relevant cell system to investigate the role of Drp1 in mitochondrial restructuring and cell death. Tumor derived or artificially immortalized, established cell lines such as HeLa or COS cells produce much of their ATP through glycolysis. In contrast, primary cells depend on mitochondrial ATP generation, making them a superior *in vitro* model for all but cancer-related studies of mitochondrial processes. Still, primary astrocytes share many of the advantages of established cell lines, in that they proliferate robustly for several weeks in serum-containing media on unmodified tissue culture plastic, and are easily transfectable by standard techniques. Perhaps most importantly, astrocytes are large and flat, which facilitates visualization of nuclear and mitochondrial morphology by epifluorescence microscopy (Figs. 13.4 and 13.5).

To prepare primary astrocyte cultures, 4–8 neonatal rat pups (0–3 days old) are euthanized by cervical transection. Forebrains excluding the cerebellum and hindbrain are dissected and the meninges are removed with fine forceps under sterile conditions. The tissue is minced with a scalpel or razor blade and resuspended in 5–10 volumes of Hank's buffered salt solution (HBSS) containing 0.25% trypsin and 1 mM EDTA. Following 15 min incubation at 37 °C with intermittent mixing to digest the extracellular matrix, the tissue is triturated with a fire-polished glass pipette and passed through a cell strainer (70–100 μ m mesh size). The cell suspension is diluted to $0.5\text{--}1.0 \times 10^5$ cells/ml in DMEM with 10% fetal bovine serum and plated onto uncoated 100 mm dishes. After 3–4 h, dishes are tapped and the media are replaced to remove poorly adherent cells (neurons and microglia). This procedure can be repeated as needed. We culture astrocytes for up to 3 weeks, passaging cells when they reach confluency. Astrocytes can be frozen, stored in liquid N₂, and thawed with plating efficiencies similar to established cell lines. Astrocytes can be transfected using a variety of methods; we use Lipofectamine 2000 at a concentration of 0.2% to achieve about 20–30% transfection efficiency.

Assaying cell death in cells that have been transiently transfected to replace endogenous with phosphorylation-site mutant Drp1 requires microscopy and is therefore more time-consuming than the MTS assay described above. On the other hand, transient transfection-based assays avoid the delays and pitfalls associated with generating stable cell lines and allow differentiation of cell death mechanisms. Because the classification into live, apoptotic, and necrotic cells can be somewhat subjective, the experimenter scoring cell death should be blinded to the identity of the transfected plasmids. For cell death assays in primary astrocytes, cells are seeded in poly-L-lysine-coated 4-chambered coverglasses (#1, Nunc) and transfected at day 4–12 *in vitro* with GFP-tagged Drp1. Allowing 3 days for knockdown of endogenous Drp1, cultures are subjected to various toxic compounds for 3–10 h. We have employed 1 μ M staurosporine to induce apoptosis and 2–5 U/ml glucose oxidase, which generates H₂O₂ from glucose in the medium, to induce necrosis. Five minutes prior to the end of the incubation period, the red fluorescent dye propidium iodide (5 μ g/ml) is added to the cultures to label the nuclei of cells that have lost membrane integrity, one of the hallmarks of necrotic cell death. After a quick wash with PBS, cells are fixed with 4% paraformaldehyde in PBS for 15 min at 37 °C in the presence of 1 μ g/ml Hoechst 33342, a blue-fluorescent DNA dye. After 4–5 additional washes in PBS, astrocytes are scored for nuclear morphology and dye uptake using the 63 $\times$ lens of an inverted, fluorescence microscope. Transfected (GFP-positive) cells that have undergone apoptotic death are characterized by propidium iodide negative nuclei with aberrant morphologies (condensed, fragmented Hoechst-stain). Early-stage necrotic astrocytes have propidium iodide-positive nuclei of normal morphology, while late-stage necrotic cell death is associated with both propidium iodide uptake and nuclear fragmentation/condensation.

6. Mitochondrial Shape Analysis with ImageJ

Drp1 activity appears to be regulated at multiple interrelated levels, e.g., the intrinsic GTPase activity of the enzyme, its distribution between cytosolic and mitochondrial pools, and its assembly into homo- and hetero-oligomeric complexes. Ultimately however, changes in Drp1 activity (through for instance (de)phosphorylation) translate into changes in the rate

of mitochondrial fission which in turn alters mitochondrial morphology. Quantifying mitochondrial fission rates by time-lapse imaging is prohibitively labor intensive, leaving steady-state morphology assays as the readout for cellular Drp1 activity. Live cell-based assays of mitochondrial interconnectivity have been developed that are based on fluorescence recovery after photobleaching (FRAP) of matrix-targeted EYFP, or the spread of a photo-activatable, matrix-targeted fluorescent protein (mito-PAGFP (Karbowski *et al.*, 2004)). Because these methods are time-consuming and can be confounded by changes in mitochondrial motility, our laboratory prefers unbiased image analysis of fluorescently labeled and fixed mitochondria as the principal Drp1 activity assay. For image enhancement and quantification, we use the free ImageJ, a Java application that runs on virtually every computer platform (<http://rsbweb.nih.gov/ij/>), as well as the ImageJ plug-ins and macros described below.

6.1. Visualization of mitochondria

For microscopic analysis, cells (e.g., primary astrocytes or HeLa cells) are cultured on 4-chambered coverglasses (#1, Nunc) and transfected using Lipofectamine 2000. To label mitochondria, either we incubate cells with MitoTracker Red (Invitrogen, 200 nM) for 15 min prior to fixation, we co-transfect with matrix-targeted fluorescent proteins (EYFP, EGFP, dsRed), or we immunofluorescently stain endogenous mitochondrial proteins. EYFP/EGFP signals can be enhanced by GFP-directed primary antibodies and green-fluorescent secondary antibodies (we use the GFP ab290 antibody from Abcam, with Alexa 488-conjugated, rabbit-directed secondary antibodies from Invitrogen). The monoclonal antibody against cytochrome oxidase subunit II (MTCO2, Labvision) gives in our hands by far the brightest and most specific mitochondrial staining, with other antibodies (e.g., HSP60 Ab2 from Neomarkers, COXIV from Cell Signaling) requiring antigen retrieval for optimal results. Unfortunately, the MTCO2 antibody is primate specific, labeling mitochondria from HeLa and COS cells, but not, e.g., rat astrocytes. After fixation, optional immunofluorescence labeling, and staining of nuclei with 1 μ g/ml Hoechst 33342 to assess cell health, multicolor digital images are captured on an inverted epifluorescence or confocal microscope, using a 63 $\times$ oil immersion lens.

6.2. Image enhancement

Depending on the quality of the mitochondria channel in terms of signal to noise ratio and separation of individual mitochondria, images may be subjected to a 2D deconvolution step, which is meant to compensate for optical imperfections of the microscope. To this end, we use the “Interactive Deconvolution” and “Diffraction Limit PSF” plug-ins written by Bob Dougherty for ImageJ (<http://www.optinav.com/imagej.html>). The latter plug-in computes a point spread function (PSF), which can be used by the deconvolution algorithm in lieu of an image of a fluorescent particle close to the optical resolution limit. Both plug-ins accept several parameters, which should be optimized for each cell type, mitochondria staining method, and microscope. Because deconvolution can take several minutes depending on image dimensions and hardware, we have written a macro that calls the deconvolution plug-in to batch-process a folder-full of images (available by request from the authors). Figure 13.3 shows images of HeLa cells stained with the MTCO2 antibody before and after deconvolution. Note the overall improvement in contrast, but also the appearance of

artificial discontinuities in what appears to the eye as interconnected mitochondrial networks. We find that deconvolution of images of marginal quality decreases the cell-to-cell variability in mitochondrial size metrics, thus amplifying experimental effects (of e.g., phosphorylation-site mutant Drp1 expression). Higher quality images, like those of astrocytes expressing mitochondria-targeted dsRed shown in Figs. 13.4 and 13.5, may actually suffer from deconvolution, so we subject them to only minor image processing before quantifying mitochondrial shape. A relatively “safe” image manipulation is the “subtract background” function built into ImageJ, which is invoked by the morphometry macro listed in Section 7. ImageJ features several other useful image enhancement functions, including kernel-based convolution and fast Fourier transform bandpass filters.

6.3. Morphometry

After optional image enhancement, mitochondrial shape metrics are reported by the morphometry macro, which at its core calls and processes the output of ImageJ’s “Analyze Particles” function. Since this function requires a two-intensity (binary) image, the mitochondria channel is first transformed by binary thresholding (Fig. 13.3). This step invariably degrades the image, making closely spaced mitochondria appear as fused and introducing gaps into clearly contiguous mitochondria. A careful comparison between original and thresholded images is the basis for evaluating different preprocessing options (e.g., deconvolution parameters), and images in which mitochondrial morphology cannot be approximated by the binary transformation should be excluded from the analysis. For each mitochondrial particle, the “Analyze Particles” function tabulates area, perimeter, and major/minor axis of a bounding ellipse (Fig. 13.3). The macro uses these values to compute form factor, area-weighted form factor (AWFF), and aspect ratio, and outputs them to the “Log” window as an average for all particles in the selected region of interest. The aspect ratio (major axis divided by minor axis) is a useful shape metric for simple rodlike mitochondria, but it does not faithfully represent the shape of kinked, branched, or highly interconnected mitochondria. The form factor takes into account perimeter and area and can therefore capture complex mitochondrial shapes. As the inverse of circularity, form factors range from 1 for a perfect circle to infinity as the ratio of particle perimeter to area increases. The AWFF metric is an intuitively sensible modification that affects the way form factors are averaged across all particles in the current selection. In multiplying form factor by area, mitochondria contribute to the population’s AWFF in proportion to their mass. The AWFFs fluctuate with greater amplitude than form factors or aspect ratios and tend to better approximate subjective assessments of mitochondrial morphology in cells with mitochondria of broad size distribution. A comparison of aspect ratio, form factor, and AWFF of mitochondria from astrocytes treated for various times with staurosporine is shown in Fig. 13.4.

The benchmark for evaluating mitochondrial shape changes (at least at the light microscopic level) remains the observer’s perception. Especially when establishing a new cell model or mitochondria staining protocol, it is therefore important to validate the image analysis algorithms described above by comparison to subjective scores. For these visual scores, coded and randomized images are assigned a value between 0 (completely fragmented,

circular mitochondria) and 4 (highly elongated and interconnected) based on comparison to a set of reference images (Dagda *et al.*, 2008).

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7. Appendix: Morphometry Macro

```
// This ImageJ macro reports three metrics that describe
// the average particle shape in the current image (selection)
// ImageJ is available at: rsbweb.nih.gov/ij/
macro "Morphometry [F7]" { // F7 is short-cut
requires("1.41");
title = getTitle();
if (isOpen("binary")) // close previous working image
{selectWindow("binary"); close(); selectWindow(title); }
run("Duplicate. . .", "title=binary");
run("Subtract Background. . .", "rolling=50");
run("Make Binary");
run("Restore Selection");
run("Set Measurements. . .", "decimal=5 area perimeter fit");
run("Analyze Particles. . .",
"size=9-Infinity circularity=0.00-1.00 show=Nothing
clear");
awff = ff = ar = sum_a = 0;
for (i = 0; i < nResults; i++) {
a = getResult('Area', i);
p = getResult('Perim.', i);
ar += getResult("Major", i)/getResult("Minor", i);
// ar = length/width
sum_a += a;
awff += b = (p * p)/(4 * 3.14159265358979);
// awff = ff * (a/sum_area)
```

```
ff += b/a;
// ff = p^2 / (4 * pi * a)
}
awff = awff / sum_a;
ff = ff / nResults;
ar = ar / nResults;
print(title+" -\t area-weighted form factor=\t"+awff+
"\t, form factor=\t"+ff+"\t, aspect ratio=\t"+ar);
}
```

Figure 13.1.

Drp1 domain diagram with phosphorylation sites. Drp1 consists of an N-terminal GTPase domain, followed by a middle (MID) and variable (VD) domain, as well as a C-terminal GTPase effector domain (GED). Alternative splicing of three exons generates eight splice variants and changes the numbering of the two highly conserved phosphorylation sites that have been characterized thus far. Kinases (↓) and phosphatases (⊥) that target the two phosphorylation sites are discussed in Section 1.

Figure 13.2.

Stable replacement of endogenous with GFP-tagged, mutant Drp1. PC12 cells were transfected with a vector that expresses a Drp1-targeting shRNA together with the RNAi-resistant, GFP-tagged cDNA. After selection in G418, clonal cell lines were immunoblotted for Drp1, GFP, and the catalytic subunit of PP2A (PP2A/C) as a loading control. Cell lines analyzed in the first two lanes have replaced endogenous with similar levels of GFP-tagged Drp1, whereas the line in lane three failed to silence endogenous Drp1 expression.

Figure 13.3.

Flowchart of mitochondrial morphometry with ImageJ. The raw image (HeLa cells fluorescently labeled with mitochondrial MTC02 antibody) on the left is first enhanced (deblurred) by 2D deconvolution and then converted to a binary (black and white) image. Individual particles in the binary image are analyzed for area, perimeter, as well as major and minor axis of a bounding rectangle using the "Analyze Particles" function of ImageJ. These measurements are used to calculate aspect ratio, form factor and area-weighted form factor (product of area and form factor).

Figure 13.4.

Comparison of three mitochondrial shape metrics. Rat primary astrocytes were transfected with mitochondrial dsRed, treated for 0–3 h with 1 μM staurosporine, fixed, and analyzed for mitochondrial morphology using the ImageJ macro listed in Section 7. Representative epifluorescence images are shown in (A) (with magnified areas in the insets), while three mitochondrial shape metrics are plotted in (B) (means $\pm$ SD of ~ 130 cells per time point). Staurosporine results in mitochondrial fragmentation that is significant by all metrics (values at the 2 and 3 h time point are not significantly different from one another, while pairwise comparisons between all other time points result in p values < 0.01).

Figure 13.5.

Effect of Drp1 phosphorylation on mitochondrial morphology is not influenced by alternative splicing. Rat primary astrocytes were transfected with mitochondrial dsRed and plasmids expressing both Drp1-targeting shRNA and GFP-Drp1 cDNAs. Phosphorylation-blocking (S→A) and -mimicking (S→D) substitutions of the PKA/CaMKI phosphorylation site (Ser656 in rat splice variant 111) were compared to wild type (WT) in the context of Drp1 splice variants with (111) and without (100) the two alternative exons in the variable domain (see Fig.13.1). Shown are representative epi-fluorescence images of astrocytes with dsRed-labeled mitochondria (A), as well as mitochondrial shape quantified as form factor (mean ± SD/SEM (lower/upper bars) of ~100 cells/condition). * $p < 0.005$ by Student's t -test.