Autophagy-mediated clearance of huntingtin aggregates triggered by the insulin-signaling pathway

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Introduction

Polyglutamine (polyQ) disorders such as Huntington’s disease (HD) are caused by a dominantly heritable expansion mutation of a triplet repeat in the coding region of the gene. The expression of this mutant protein leads to the onset of a slow, progressive disorder that invariably leads to death. Thus far, neither an effective treatment nor viable targets for drug design are available.

A prevalent feature of HD and other polyQ diseases is the accumulation and aggregation of the mutant protein. These changes lead to the formation of cytoplasmic and nuclear inclusion bodies, the appearance of which indisputably signifies the inability of the cell to properly dispose of the mutant protein. Indeed, overexpression of expanded polyQ proteins has been shown to alter proteasome (Bence et al., 2001) and lysosome function (Qin et al., 2003). Over the past several years, animal models of HD (Yamamoto et al., 2000; Regulier et al., 2003; Harper et al., 2005) and spinocerebellar ataxia type 1 (Xia et al., 2004; Zu et al., 2004) revealed that cells have the capacity to clear these products if the continuous production of the mutant transgene is halted. Invariably, clearance of the protein is accompanied by reversal of the disease-like symptoms in the mice. In light of these findings, it is critical to determine the pathway responsible for alleviating this protein accumulation to define targets to fight these diseases.

To determine the pathway responsible for the clearance of mutant huntingtin (htt), we conducted a two-tiered functional genetic screen. We first used gene arrays to quickly assess the transcriptional changes induced by pathogenic polyQ lengths. Although these changes alone can be somewhat informative, it is difficult to determine the functional relevance of these changes. Thus, we next targeted transcripts of genes that were “increased” with chemically synthesized small interfering RNAs (siRNAs) to determine which of these proteins were required for mutant htt clearance. Those specific proteins revealed by the second screen then became the focus of further investigation.

Of the 56 up-regulated transcripts, 23 were required for mutant htt clearance. Interestingly, the pattern of genes revealed that activation of insulin receptor substrate 2 (IRS-2), a scaffolding protein that mediates the signaling cascades of growth factors such as insulin and insulin-like growth factor 1 (IGF-1; White, 2003), leads to a macroautophagy-mediated clearance of the accumulated polyQ proteins. Clearance is present despite
the activation of Akt, mammalian target of rapamycin (mTOR), and p70 S6 kinase. This is surprising because activation of mTOR is an inhibitor of the classic, starvation-induced macroautophagy (Meijer and Codogno, 2004). The significance of this is twofold: first, that macroautophagy in the presence of accumulated proteins can also occur in an mTOR-independent manner; and second, that this represents another important pathway through which factors such as insulin and IGF-1 may exert beneficial effects.

Results

Clearance of accumulated proteins in inducible cell lines

To determine if the clearance of mutant protein can be observed in stable cell lines, we designed a series of functional cell-based assays that were similar to the HD94 mouse model (Yamamoto et al., 2000). Cell lines inducibly express exon1 of htt (exon1 htt) carrying a polyQ expansion of 25, 65, or 103 residues. Inducibility is conferred using the tet-off system (Gossen et al., 1994). To monitor the state of the proteins, and to ensure that aggregation was mediated primarily by the polyQ repeat, the COOH termini were fused to monomeric enhanced CFP (mCFP; Zacharias et al., 2002). To ensure that our siRNA-based screen can be conducted as efficiently as possible, we first focused on HeLa-based cell lines (Fig. 1). siRNA transfection efficiency in these cells reaches >80% (Elbashir et al., 2001; Pelkmans et al., 2005; unpublished data). To confirm our findings, however, we also used a neuronal background with Neuro2a cell lines (N2a; Fig. 9), which have been previously used to characterize different cellular aspects of HD (Wang et al., 1999; Jana et al., 2000, 2001).

Next, we determined if the abolition of mutant exon1 htt expression would lead to protein clearance. Inhibition of polyQ expression with 100 ng/ml doxycycline (dox) led to clearance of both the soluble and aggregated protein (Fig. 1). Similar to primary cultures derived from the HD94 mice (Martin-Aparicio et al., 2001), within 6 d the inclusions cleared (Fig. 1 and see Fig. 9). Thus, both nonneuronal and neuronal cell lines are capable of clearing the mutant forms of exon1 htt. These findings indicate that the elimination of accumulated mutant exon1 htt is very slow. Furthermore, because the amount of time required for clearance is similar across cell types, including primary neurons, the process underlying the elimination of this protein may be a general cellular event.

Continuous expression of mutant exon1 htt leads to transcriptional changes

To identify genes that are altered because of expression of mutant exon1 htt, we tested our hypothesis that stable expression of 65Q or 103Q leads to transcriptional changes that reflect sequestration and elimination of inclusions. To examine

Figure 1. Stable cell lines with conditional expression of exon1 htt-polyQmCFP. (a) Representative images of stable cell lines with conditional expression of exon1 htt with 25QmCFP, 65QmCFP, and 103QmCFP. (b) Cell lines clear polyQmCFP inclusions within 6 d upon abolishing protein expression. Data represented as mean ± SEM, n = 6.

Figure 1.

Stable cell lines with conditional expression of exon1 htt-polyQmCFP. (a) Representative images of stable cell lines with conditional expression of exon1 htt with 25QmCFP, 65QmCFP, and 103QmCFP. (b) Cell lines clear polyQmCFP inclusions within 6 d upon abolishing protein expression. Data represented as mean ± SEM, n = 6.
these changes in an unbiased global manner, we determined the genetic profile of the cell lines using Affymetrix gene arrays. Comparisons between exon1 htt-65QmCFP (65Q) and 25QmCFP clones (25Q) revealed a total of 70 transcripts increased and 89 transcripts decreased. Comparisons between 103QmCFP (103Q) and 25Q revealed 132 increased and 96 decreased (Fig. 2a). Common across both pathogenic glutamine lengths was the 56 increased and 42 decreased (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200510065/DC1). Interestingly, increases were seen in only one proteasomal subunit (PSMD8), but in two lysosome-associated membrane proteins (LAMP1 and 2). The profile also revealed changes in vesicle trafficking, signaling proteins, metabolic proteins, and hypothetical proteins.

Functional consequence of siRNA-mediated knockdown of transcripts revealed from profiling

It is impossible to determine from profiling alone if the transcriptional changes have any functional relevance in our pathway. Therefore, we used RNA interference to functionally knockdown the transcripts that were increased in the genetic profile.
We reasoned that if up-regulated proteins were relevant to our pathway, then their loss would inhibit clearance and the polyQ protein would continue to accumulate despite abolishing expression.

Eight EST transcripts were excluded from the siRNA screen. For the remaining 48 transcripts, three to four siRNA sequences per gene were individually transfected into two different 65Q and 103Q cell lines. 48 h after transfection, cells were exposed to 100 ng/ml dox for another 48 h to shut down production of new protein and permit 50% of clearance to occur. Cells were fixed and analyzed for the number of inclusions per cell using InCell Analyzer (INCA) 3000 software and calculated for an accumulation index, as described in Materials and methods. An example is shown in Fig. 3.

Loss of function of 23 out of the 48 transcripts led to a complete or partial inhibition of clearance, of 9 led to cell death, and of 16 led to no change (Fig. 2). As predicted, genes involved in protein degradation were the most prevalent, including the lysosomal membrane proteins LAMP1 (Fig. 2 b, probes 3H5, 3H6, 3H7, and 3H8) and LAMP2 (Fig. 2 b, probes A7, A8, A9, and A10). LAMP2 has previously been shown as essential for lysosomal function in LAMP2 knockout mice. LAMP1, on the other hand, was not required; however, it was surmised that its function was redundant to LAMP2 (Andrejewski et al., 1999; Tanaka et al., 2000). The relatively acute loss of function offered by siRNA-mediated silencing may prevent the ability of such compensation to occur. Putative knockdown of the only proteasomal subunit that was altered in the gene arrays, PSMD8, led to cell death, and thus its role in clearance could not be elucidated.

IRS-2 is required for the elimination of mutant exon1htt aggregates

Unexpectedly, knockdown of IRS-2 led to an inhibition of aggregate clearance (Figs. 2 and 3). A scaffolding protein that transmits the phosphatidylinositol 3-kinase (PtdIns3K) signaling of growth factors like IGF-1 and cytokines, IRS-2 knockout mice have also revealed an important role in the brain (Schubert et al., 2003). Western blot analysis confirmed that siRNAs effectively down-regulates the protein (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200510065/DC1). Fig. 3 shows representative images taken from the INCA 3000 of one of the two 103QmCFP clones in the absence of dox, in the presence of dox, and in the presence of dox after transfection with siRNA number 8H06, which is one of the three siRNAs against IRS-2. Quantification using the INCA 3000 software of these images shows that, after transfection of 08H06, the clearance normally observed by abolishing transgene expression is inhibited (Fig. 3 b).

IRS-2 activation leads to enhanced exon1htt clearance

Because elimination of IRS-2 inhibited clearance, IRS-2 activation may stimulate clearance. Therefore, we tested a series of ligands known to activate IRS-2 in the following cells: insulin (Vassen et al., 1999), IGF-1 (Nakamura et al., 2000), and interleukin-4 (IL-4; Loh et al., 1992). All three cells demonstrate a dose-dependent clearance of accumulated polyQ proteins (Fig. 4 a). Unlike our normal clearance paradigm using dox, this clearance occurred despite maintaining continuous expression of
As shown in Fig. 4 b, silencing IRS-2 led to a complete inhibition of the enhanced clearance triggered by IGF-1. Western blot analysis also found that IGF-1–mediated clearance is inhibited by small interfering IRS2 (siIRS-2; Fig. S2). Silencing another IRS family member, IRS-1 (White, 2003), had no effect (Fig. 4 c and Fig. S1). Although this result is compelling, we cannot completely eliminate its role because up-regulation of IRS-2 may mask an effect. Similar dependence on IRS-2, but not -1, was observed with the enhanced protein degradation triggered by insulin and IL-4 (unpublished data).

**IRS-2–mediated clearance requires hVps34 and Beclin 1**

We next attempted to gain insight into the intracellular pathway by which IRS-2 activation triggers protein clearance. It has been previously shown that activation of IRS-2 can lead to the production of PtdIns[3,4,5]phosphate by turning on the class I PtdIns3K (Saltiel, 2001). However, it has also been shown that IRS-2 phosphorylation may lead to the production of PtdIns[3]phosphate (PI3P; Virbasius et al., 2001; Chaussade et al., 2003; Maffucci et al., 2003). These latter lipid products are predominantly formed by a class III PtdIns3K, called hVps34 (Schu et al., 1993; Backer, 2000).

We first determined whether the presence of PI3P leads to clearance. Synthetic dipalmitoyl-PI3P has been effectively delivered into the cell using liposomes (Franke et al., 1997; Petiot et al., 2000). Administration of these liposomes to the exon1hhtt-65Q or 103QmCFP cell lines for 3 d led to a significant decrease in the number of inclusions per cell (Fig. 4 c and not depicted). Coadministration of PI3P with IGF-1 or insulin did not significantly enhance this effect, suggesting that they lie in the same pathway (unpublished data). siRNA against hVps34 effectively eliminated the exon1hhtt clearance caused...
by IRS-2 activation (Fig. 4 c). Interestingly, similar to IRS-2, the loss of hVps34 also eliminated the clearance revealed by eliminating transgene expression with dox (unpublished data).

The requirement of hVps34, as well as LAMP1 and 2 (Fig. 2), suggests that macroautophagy is being triggered. Macroautophagy, which we will henceforth refer to as autophagy, is

Figure 6. **Exon1 htt-polyQmCFP htt inclusions can be found engulfed by an LC3-positive vesicle.** Z-sectioned image of an Exon1 htt-65QmCFP cell cotransfected with eYFP-LC3. Bar, 100 μM.
agy-mediated elimination of the accumulated protein, we exam-
in further ascertain that IRS-2 activation potentiates autoph-
activation leads to the activation of autophagy
sclerosis (ALS) and HD (Humbert et al., 2002; Kaspar et al.,
activation, we next knocked down the serine/threonine
silencing of Beclin1 abolished the clearance stimulated by IRS-
and the mammalian orthologue of Apg6, Beclin1 (Liang et al.,
3-methyladenine (3-MA) is caused by the inhibition of hVps34
function in exon1htt clearance and imply a role in autophagy.
Next, we examined if the inclusions colocalized with
autophagosomes or lysosomes. Administration of 65 nM of
red fluorescent dye that accumulates in acidic organelles, showed that mCFP-positive inclusions were in acid-
compartment (Fig. 5 b). Using the autophagosome marker
LC3 was also a significant interaction between siRNA
and treatment (P = 0.0022). Data represented as mean ± SEM. Asterisks indicate statistical significance, as indicated.

IRS-2 activation leads to the activation of autophagy
To further ascertain that IRS-2 activation potentiates autophagy-mediated elimination of the accumulated protein, we exam-
ined the effect of established inhibitors of lysosomal degradation and macroautophagy on clearance. As shown in Fig. 5 a, lyso-
osomal inhibitors inhibit the ability of the cell to eliminate the
inclusions. 3-MA and wortmannin also eliminated clearance. These findings further demonstrate the importance of lysosomal
function in exon1 htt clearance and imply a role in autophagy.

Class I PtdIns3K pathway is still active in the mutant exon1 htt cell lines
Growth factors like insulin are classic signaling molecules that inform the cell of the presence of nutrients. From yeast to mam-
malian cells, these factors generate PtdIns[3,4,5]phosphate. This leads to a signaling cascade that activates Akt, mTOR,
and the translation activator p70S6K (Jacinto and Hall, 2003). A known consequence of this pathway is an inhibition of macroautophagy, which is a catabolic process. This is contrary to our results, in which the same receptor activates degradation of mutant exon1 htt inclusions by autophagy. To ensure that our findings are not caused by a disturbance of normal signaling processes, we reexamined the effect of insulin and IGF-1 on both the proper phosphorylation of known downstream kinases and on autophagy caused by amino acid deprivation.

Whole cell lysates were collected from 65QmCFP- or 103QmCFP-expressing cells after exposure to IGF-1 or insulin for 30 min. In the presence of mutant exon 1, mTOR is phosphorylated and decreases after amino acid withdrawal (-aa; 4 h) or 20 nM rapamycin (1 h). IGF-1 and Ins inhibit the effect of amino acid withdrawal. [6] "[14C]valine-labeled long-lived protein degradation in response to 4 h amino acid withdrawal. ANOVA revealed a significant effect of treatment on the percentage of proteolysis [F(5,19) = 7.160; P = 0.0006]. 10 μM PI3P-containing liposomes also increased proteolysis in the presence of full serum, as previously shown (P = 0.0010). 100 nM Ins (P = 0.8763) and 10 nM IGF-1 (P = 0.3999) inhibited the enhanced proteolysis caused by amino acid withdrawal (P = 0.0016), as did 10 mM 3-MA (P = 0.3074). Data represented as mean ± SEM.

Figure 8. Macroautophagy caused by amino acid withdrawal is unchanged in mutant exon1 htt-polyQ cell lines. (a) In the presence of mutant exon1 htt expression, Akt and p70S6 kinase (S6K) are phosphorylated upon stimulation with IGF-1 and Ins for 30 min. In the presence of mutant exon 1, mTOR is phosphorylated and decreases after amino acid withdrawal (-aa; 4 h) or 20 nM rapamycin (1 h). IGF-1 and Ins inhibit the effect of amino acid withdrawal. (b) "[14C]valine-labeled long-lived protein degradation in response to 4 h amino acid withdrawal. ANOVA revealed a significant effect of treatment on the percentage of proteolysis [F(5,19) = 7.160; P = 0.0006]. 10 μM PI3P-containing liposomes also increased proteolysis in the presence of full serum, as previously shown (P = 0.0010). 100 nM Ins (P = 0.8763) and 10 nM IGF-1 (P = 0.3999) inhibited the enhanced proteolysis caused by amino acid withdrawal (P = 0.0016), as did 10 mM 3-MA (P = 0.3074). Data represented as mean ± SEM.
against mTOR were also used. Phosphorylated mTOR was readily detectable in the presence of mutant exon1htt expression, as well as in the presence or absence of mutant htt expression (Fig. 8 a and not depicted). Amino acid deprivation and rapamycin decreased mTOR phosphorylation in these cells, whereas insulin and IGF-1 inhibited the effect of amino acid withdrawal. Thus, the IGF-1 and insulin lead to the predictive phosphorylation of p70S6K and mTOR.

We next examined proteolysis of long-lived proteins. Proteolysis was measured by [14C]valine-labeled long-lived proteins. 103QmCFP cell lines were under dox suppression for 2 wk to ensure no transgene expression because the presence of these proteins led to high levels of baseline protein degradation, despite the presence of full serum. Insulin and IGF-1 significantly attenuated the amount of proteolysis, whereas PI3P induced proteolysis despite the presence of complete media. Furthermore, the administration of 3-MA also diminished degradation (Fig. 8 b). Thus, the autophagy- mediated clearance of inclusions by IGF-1 and Ins occurs despite proper signaling by mTOR.

IRS-2 activates autophagy in inducible neuronal mutant exon1htt cell lines IRS-2 is expressed in all insulin-responsive organs, including the brain. We examined if IRS-2 could activate macroautophagy in response to protein accumulation in neuronal cell lines (Fig. 9). Similar to the HeLa cells, elimination of novel polyQ protein production led to clearance over a period of 6 d. We stimulated IRS-2 using IGF-1 to determine if a similar mechanism was at play. IRS-2 activation using IGF-1 also led to an autophagy- mediated clearance of polyQ proteins in cells of a neuronal lineage. The clearance was accompanied by an increased colocalization of mCFP-positive inclusions in YFP-LC3 autophagosomes. Again, knockdown of Beclin1 inhibited the colocalization of inclusions in the autophagosomes.

Discussion

Using a unique two-tiered functional genetic screen, this study revealed an unexpected means by which autophagy-mediated clearance of accumulated mutant protein can be activated. We found that the activation of IRS-2 led to macroautophagy-induced clearance of the accumulated polyQ proteins (Fig. 10). The activation was dependent on class III PtdIns3K activation and occurred despite activation of Akt, mTOR, and p70S6K.

These findings highlight several points. The first is that activation of IRS-2 can lead to the clearance of accumulated mutant exon1 htt. IRS-2 is widely expressed and, together with IRS-1, mediates the signaling of insulin and IGF-1 in most tissue (Sun et al., 1992, 1995). White (2003) found that loss of IRS-2 function in knockout mice led to an accumulation of neurofibrillary tangles containing phosphorylated tau in the absence of changes in the kinase glycogen synthase kinase 3β. In light of our findings, it is possible that the loss of IRS-2 exposed a potential role for these proteins to mediate clearance of these complex proteins. It would be interesting to determine if protein accumulation is occurring in other tissues that are IRS-2 deficient.

Activators of IRS-2, such as insulin and IGF-1, have both been shown to strongly promote neuronal survival through stimulation of Akt. Consequently, its efficacy has been tested as such in other neurodegenerative diseases, such as ALS. For example, retroviral delivery of IGF-1 in a mouse model of
ALS led to amelioration of the phenotype, together with a diminishing of the accumulated mutant SOD1 (Kaspar et al., 2003). A placebo-controlled trial in American ALS patients found that the progression of functional impairment significantly slowed in the treated patients, with no adverse side effects (Lai et al., 1997). The outcome suggested an IGF-1 dose-dependent treatment effect. For HD, Humbert et al. (2002) have examined the neuroprotective effect of Akt stimulation by transiently transfecting mutant htt into primary neurons. Similar to the findings in ALS, they found that IGF-1 administration led to both a decrease in the number of aggregates formed and a decrease in cell death. The mechanism through which IGF-1 elicited both effects was believed to be directly downstream of Akt (Rangone et al., 2004, 2005). We were able to determine that the autophagic/cytolytic clearance can also occur independent of Akt because its knockdown did not eliminate clearance. Monomeric and aggregated proteins may be degraded differently. These findings indicate that the protection conferred by the insulin signaling pathway in diseases with protein accumulation may be twofold; the classical neuroprotective pathway triggered by Akt and the enhanced clearance stimulated by hVps34 activation. Because cytoplasmic inclusions are more readily degraded by autophagy, it is now crucial to determine to what extent these inclusions contribute to pathology.

Another point highlighted by this study is that macroautophagy is indeed capable of degrading large inclusions and is stimulated under conditions previously deemed inhibitory. Activation of IRS-2 in the HeLa cell lines was achieved by using insulin, IGF-1, or IL-4. Signaling through these receptors, however, is also known to inhibit mTOR-mediated autophagy through the activation of class I PtdIns3K and mTOR. Nonetheless, there is evidence that autophagy may occur despite mTOR activation. For example, transgenic expression of the autophagosomal marker LC3 demonstrates that in certain tissue autophagosomes constitutively form, even in the absence of starvation (Mizushima et al., 2004). Consistent with these findings, immortalized cells of certain lineages also have a higher basal activity of macroautophagy (Mizushima, 2004). More recently, Iwata et al. (2005a) found that proteasome inhibition was a potent activator of autophagy, although the role of mTOR in the response was not explored. In this study, our findings indicate that macroautophagy is the mechanism by which the aggregates are cleared, despite mTOR activation. This could be achieved if the autophagy regulators downstream of mTOR, such as hVps34, could be activated. Therefore, the activity observed in our results may represent a competition between the inhibitory effects of activating class I versus III kinases. Moreover, Scott et al. (2004) clearly demonstrated in the Drosophila melanogaster fat body that the regulation of autophagy may occur differently from what was previously believed. They found that constitutive activation of p70S6K was indeed required for autophagy, rather than inhibitory as previously described (Klionsky et al., 2005). The presence of expanded polyQ proteins may offset the balance of type II diabetes, and thus this line of research may also benefit other disorders. In any case, it is clear from these studies that more information regarding the importance of lysosomal degradation pathways such as autophagy in neuronal systems is absolutely crucial. Controlling these pathways that degrade mutant htt will allow us to finally begin to treat this terrible disease.

Materials and methods

Materials

N2a cells were purchased from American Type Culture Collection. Insulin, IGF-1, IL-4, and dox were purchased from Sigma-Aldrich. Antibodies were purchased from Upstate Biotechnology (anti–IRS-2, anti–phospho Akt, and anti–phospho S6 kinase), BD Biosciences (Akt, S6 kinase, and IRS-1), Cell Signaling Technology (anti–phospho-mTOR and anti–mTOR), and Roche (anti–GFP). htt-exon1 (CACGCAA) constructs were obtained from A. Kazantsev (Massachusetts General Hospital, Charlestown, MA). pYFP-LC3 was obtained from T. Yoshimori (National Institute of Genetics, Mishima, Shizuoka, Japan).

Creation of cell line

N2a were selected to be tTA-positive by transfection with P2A-tTA-IRE-RES-neo and selection with 800 μg/ml G418. PolQ2 cell lines were created by cotransfecting Hela and N2a with tetO-htt (25Q, 65Q, or 103Q) exon1-mCFP and P2A-hydro (CLONTECH Laboratories, Inc.) and then selected with hygromycin using 800 and 250 μg/ml, respectively. 100 ng/ml dox was also maintained in the culture media during selection to maintain suppression of transgene expression. Hela cells were maintained in DME with 10% FCS, whereas N2a cells were maintained in 50% DME/50% OptiMEM in 10% FCS.

RNA preparation and gene arrays

Cells were plated in a 100-mm dish, harvested using 100 μL TRIzol reagent (Invitrogen) and isolated per the manufacturer’s instructions.
RNA was resuspended and further purified using the RNAeasy kit (Qiagen). RNA was labeled and hybridized onto human U133A chips at the Genome Core Facility at Memorial Sloan Kettering Cancer Center (MSKCC). Array results were analyzed using GeneSpring 2.0 (Agilent Technologies) and an Affymetrix software package 5.0.

**Transfection**
siRNAs were designed using an algorithm designed by Jagla et al. (2005). siRNA were created at either Integrated DNA Technologies or the Functional Proteomics Project at MSKCC. Scramble siRNA sequences were purchased from Dharmacon. A final concentration of 10 or 20 nM of siRNAs was used for silencing. Cells were transfected using OligofectAMINE per the manufacturer’s instructions. 7.5 × 10^4 cells were plated in 96-well ViewPlates (Packard Instrument Co.) and transfected the next day. 48 h after transfection, cells were split across two wells and treated with dox. Cells were examined 48 h later. Transfection of plasmid DNA was accomplished using LipofectAMINE per the manufacturer’s instructions. Compounds were administered 48 h after transfection unless otherwise noted.

**Image analysis with the INCA 3000**
For the high throughput screen, images were collected on the INCA 3000 (GE Healthcare). Cells in 96-well ViewPlates were fixed for 10 min with 4% PFA. After scanning the plates, images were analyzed using the granularity analysis module on the accompanying software. In brief, the granularity analysis quantifies the number of inclusions (grains) within a cell, using a two-color strategy to identify individual cells and to analyze associated grains. After recognizing the objects, in this case the Hoechst-positive nuclei, the algorithm next identifies, using a specified size range (in pixels) and fluorescent intensity gradient, the grain in the proximity of the object. Both the Ngrains (number of qualifying grains per cell) and the fraction of fluorescent within the qualifying grains gave similar results. To calculate the accumulation index used in Fig. 2, values were first normalized as a percentage of control for scramble siRNA–transfected cells treated with 2 days of 100 ng/ml dox, ded used in Fig. 2, values were first normalized as a percentage of control (number of qualifying grains per cell) and the fraction of fluorescent within the qualifying grains gave similar results. To calculate the accumulation index used in Fig. 2, values were first normalized as a percentage of control for scramble siRNA–transfected cells treated with 2 days of 100 ng/ml dox, and set to 0 by subtracting 100%. This would permit comparison across all of the experiments conducted and a quick assessment of the direction of the change—an increase in accumulation index (I-I0) or an increase in clearance (I0/I). The kinase cascade was considered required when the absolute value of accumulation index was greater than two standard deviations of the Scramble siRNA + 2 d dox. Each siRNA was transfected on 8 wells per 96-well plate, and each experiment was repeated five to eight times. Cell viability was determined through several measures. First, we examined the number of cells per well scanned. If a drug or siRNA led to significantly fewer cells, they were initially considered toxic. If this toxicity consistently appeared across experiments, we next confirmed toxicity using an inclusion/exclusion assay of cell death, known as a LIVE/DEAD assay (Invitrogen).

**Other image analysis**
Cells were grown on glass (HeLa) or poly-L-lysine–coated (N2a) coverslips in 24-well plates. Cells were fixed for 10 min with 4% PFA. Nuclei were stained with Hoechst 33342 for 30 min, and membranes were stained using Alexa Fluor 633-labeled cholera toxin subunit B obtained from Invitrogen. Images were acquired using a confocal microscope (TCS SP2; Leica) at 63× magnification, along with the accompanying software package. Data acquisition was performed using National Institutes of Health Image 4.0. Analysis of variance (ANOVA) and post hoc analyses were conducted using Statview 5.0 (SAS Institute, Inc.).

**P13P liposomes**
Synthetic dipalmitoyl-P13P liposomes (Matreya) were dried together with phosphatidylserine at a 1:1 concentration under argon and vacuum and resuspended in 25 mM Hepes, 100 mM EDTA to a total lipid concentration of 800 μM. Liposomes were freeze-thawed, then manually extruded through two 50-nm polycarbonate membranes. P13P/phosphatidylserine liposomes were administered to cells at 20 μg P13P liposomes. PI3P liposomes were created at either Integrated DNA Technologies or the Functional Proteomics Project at MSKCC. Scramble siRNA sequences were purchased from Dharmacon. A final concentration of 10 or 20 nM of siRNAs was used for silencing. Cells were transfected using OligofectAMINE per the manufacturer’s instructions. 7.5 × 10^4 cells were plated in 96-well ViewPlates (Packard Instrument Co.) and transfected the next day. 48 h after transfection, cells were split across two wells and treated with dox. Cells were examined 48 h later. Transfection of plasmid DNA was accomplished using LipofectAMINE per the manufacturer’s instructions. Compounds were administered 48 h after transfection unless otherwise noted.

**References**


