

# Fluorescence Imaging of Mobility Shifts: An Expression Cloning Method for Identification of Cell Signaling Targets

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**SUMMARY:** There is a need for a simple global approach to identify signaling targets that are posttranslationally modified in response to physiologic or pathologic stimuli within living cells. Reported here is a simple method, fluorescence imaging of mobility shifts (FIMS), which relies on in-gel detection of cell-expressed green fluorescent protein fusion proteins undergoing electrophoretic mobility shifts. This detection method is applied to a small pool cDNA library screening protocol. The readout is essentially a differential display of posttranslational modifications. Unlike biochemical approaches to identifying signaling targets, the screen is performed in living cells using standard methods for transient transfection. This enables detection of intracellular targets modified in response to either molecularly defined stimuli, such as growth factors or drugs, or complex pathologic stimuli, such as oxidative stress or hypoglycemia. FIMS is rapid, sensitive, inexpensive, and nonradioactive and easily adapted to automated high throughput methods, including capillary electrophoresis. The technique is sufficiently sensitive to easily detect fluorescent proteins expressed in a single well in 384-well format. FIMS is applicable to traditional cDNA library screening, but the method will be especially attractive for screening preselected collections of autofluorescent fusion proteins. A bonus of the technique is that examination of transfected cells by fluorescence microscopy provides immediate information about intracellular localization and stimulus-induced translocation of putative targets. We illustrate the utility of the technique with pilot screens for apoptotic and mitogenic targets modified by staurosporine and serum stimulation, respectively. (*Lab Invest* 2002, 82:1631–1636).

A major challenge in functional genomics will be the identification of functional targets of intracellular signaling pathways. Large-scale efforts are now focused on the identification of networks of protein-protein interactions. However, the major currency of intracellular signaling is posttranslational modification of proteins, including such diverse chemistries as phosphorylation, dephosphorylation, ubiquitination, neddylation, sumoylation, and proteolytic cleavage. Previous approaches to identifying novel targets of cell signaling pathways have been largely biochemical, ie, not cell-based. For example, to identify novel targets of ERK/MAP kinase, the purified kinase was applied to candidate substrate proteins expressed from a cDNA library and putative targets identified by incorporation of radioactive phosphate (Fukunaga and Hunter, 1997). Another elegant approach, but again limited to screening for targets of a specific kinase, is the use of an unnatural nucleotide analog in conjunction with expression of a mutated kinase that is

enabled to use the analog for phosphorylation (Shah et al, 1997).

A more global approach is enabled by in vitro expression cloning (IVEC), developed by Kirschner and colleagues (Cryns et al, 1997; King et al, 1997; Stukenberg et al, 1997). In this biochemical approach, small pools of cDNAs are transcribed and translated in the test tube and then subjected to posttranslational modifications either by purified enzyme or crude cell extracts. Putative targets are identified by electrophoretic mobility shifts, a feature common to many if not most posttranslational modifications. IVEC is limited to situations in which the investigator has in hand either purified enzyme or a known crude source of the modifying enzyme under study, eg, a mitotic cell extract. IVEC and other in vitro methods are prone to false-positive results, in that some modifications occurring in the artificial environment of the test tube fail to be reproduced within the complex environment of a living cell. Conversely, false-negative results occur because many posttranslational modifications require the presence of intact intracellular organelles or macromolecular complexes, which are lacking in the test tube approach.

We sought to develop a method that combines the best aspects of cell culture-based expression cloning and IVEC. This approach would allow the cell biologist or experimental pathologist studying a complex cellular reaction to screen for targets of signaling pathways

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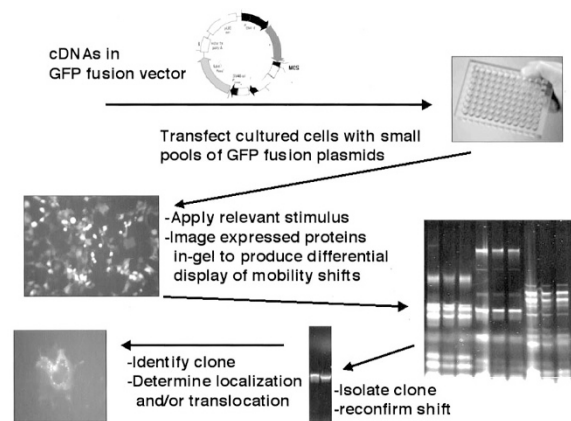
without a priori knowledge of the specific signaling enzyme(s) involved. We chose to use libraries or collections of green fluorescent protein (GFP) fusion proteins for several reasons. As demonstrated for the first time here, mammalian cell-expressed GFP fusions are easily detectable in-gel using commercially available laser scanners. In addition, as documented in hundreds of publications, GFP tagging enables rapid intracellular localization of the expressed protein without the development of costly antibody probes. Moreover, GFP fusions have been shown to act as bona fide kinase substrates both in vitro and in vivo (Mandell and Gocan, 2001; Sato et al, 2002; Tavare et al, 2001; Yang et al, 1999). Finally, GFP fusions are also ideal sensors for specific proteolytic events because the GFP moiety itself is highly resistant to proteolysis (Chiang et al, 2001). Thus, electrophoretic mobility shifts observed in expressed fusion proteins are likely to be a result of modifications of the putative target rather than of GFP itself.

Fluorescence imaging of mobility shifts (FIMS) is based on the expression of small pools of autofluorescent fusion proteins in cultured cells. Our method uses standard methods of mammalian cell expression cloning (Seed, 1995; Simonsen and Lodish, 1994). Posttranslational modifications are detected in-gel by laser-induced fluorescence as band mobility shifts, which indicate phosphorylation/dephosphorylation, ubiquitination or other peptide modification, or proteolytic cleavage. Once a target is identified and confirmed, existing biochemical and immunochemical approaches are used to determine the precise mode of posttranslational modification.

## Results

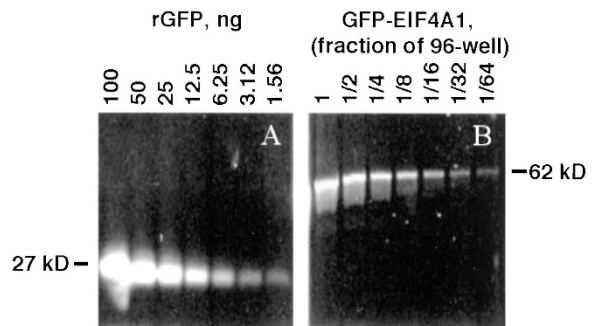
The FIMS strategy is illustrated schematically in Figure 1. cDNA libraries from a cell type or tissue of interest are prepared using a GFP vector via standard techniques. Alternatively, collections of identified genes prepared as GFP fusions are used for the screen. Using previously described techniques, small pools of plasmids (25–50 clones/pool) are prepared in 96-well format (Cryns et al, 1997; Stukenberg et al, 1997). Cultured mammalian cells are transfected with these small pools, in replicate wells, which are subjected to relevant stimuli. The crux of the technique is the in-gel visualization of expressed GFP fusion proteins, allowing rapid detection of mobility shifts, indicative of posttranslational modification. Finally, the plasmid encoding the band of interest is cloned by sib-selection, retransfected to confirm the stimulus-induced shift, and sequenced. A bonus of the technique is immediate visualization of the expressed protein's intracellular localization and possible stimulus-induced translocation.

We first tested the sensitivity of a commercially available fluorescence laser scanner (FluorImager; Molecular Dynamics, Sunnyvale, California) for in-gel detection of mammalian cell-expressed GFP fusion proteins (Fig. 2). As previously documented (FluorImager Technical Documentation, Molecular Dynamics),



**Figure 1.**

Schematic representation of FIMS. Genes from a random cDNA library or identified gene collection are subcloned into a mammalian green fluorescent protein (GFP) fusion expression vector. Small pools of plasmids are transfected in replicate in 96-well format. After expression, cells are subjected to relevant stimuli, lysed, and separated by either gel or capillary electrophoresis. A differential display of protein modifications is obtained by visualizing the expressed proteins by laser-induced fluorescence. Putative signaling targets undergoing mobility shifts are cloned by a sib-selection strategy, or for arrayed collections, simply by row/column position. An added bonus of the technique is immediate acquisition of information about protein localization and possible stimulus-induced translocation.



**Figure 2.**

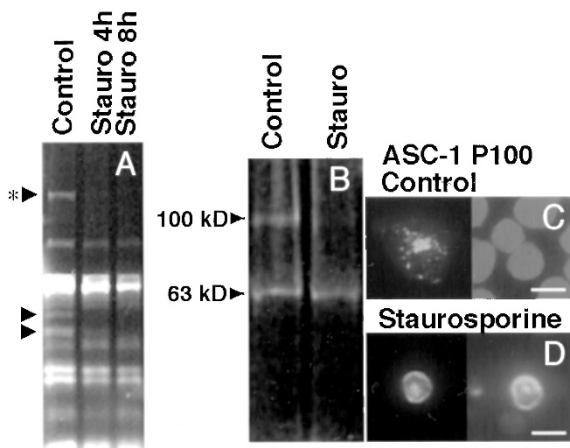
Sensitivity of in-gel detection of a mammalian cell-expressed GFP fusion. A, A serial dilution of recombinant EGFP (Clontech, Palo Alto, California) imaged in-gel by fluorescence laser scanning reveals detection to 1.56 ng. B, Serial dilutions of a whole cell lysate made from Cos-1 cells transiently expressing a GFP-EIF4A1 fusion demonstrate detection of fusion protein easily from 1/32, and faintly from 1/64, of a single 96-well, corresponding in intensity to approximately 1 ng of recombinant EGFP. *kD* = kilodalton.

purified bacterially expressed recombinant enhanced green fluorescent protein (EGFP) could be detected at low nanogram levels (Fig. 2A). We discovered that an EGFP fusion protein (EIF4A1-GFP) transiently expressed in mammalian cell cultures (Cos-1) could also be detected in-gel. In fact, the cell-expressed GFP fusion protein could be detected from as little as 1/64 of the lysate from a well of a 96-well plate (Fig. 2B).

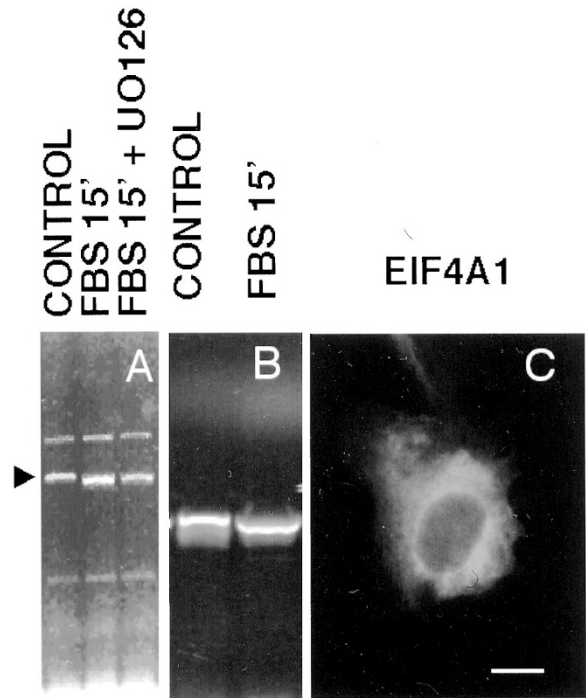
Given that we could easily detect a single EGFP fusion protein expressed from the equivalent of 1/32 of a 96-well plate, we reasoned that we could detect multiple fusion proteins simultaneously expressed from small pools of plasmids. We used a cDNA library from human HT1080 fibrosarcoma cells, in which cDNAs are inserted at the carboxy-terminus of a

modified EGFP vector (Fujii et al, 1999). Given the random nature of insertion, only 1/3 of clones are expected to be in-frame with GFP. We made small pools of 25 to 50 GFP fusion clones, expected to produce approximately 8 to 16 fusion proteins per pool. Cos-1 cells were transfected with pools in duplicate or triplicate, and successful expression was confirmed by fluorescence microscopy. Most pools contained roughly the expected number of autofluorescent protein bands (Figs. 1, 3A, and 4A). We then performed two pilot screens for apoptotic and mitogenic signaling targets.

We screened 48 small pools for putative apoptotic targets using staurosporine-induced death. Treatment of Cos cells with staurosporine is known to lead to caspase activation, reaching maximum activity at 4 hours, and eventual apoptotic cell death (Tyas et al, 2000). One pool contained three bands that reproducibly disappeared after 4 to 8 hours of staurosporine treatment (Fig. 3A). The clone encoding the largest band (Fig. 3A, *asterisk*) was isolated by sib-selection and its staurosporine-induced mobility shift reconfirmed (Fig. 3B). A single plasmid gave rise to two bands at approximately 100 and 60 kDa. The upper band disappeared after 4 hours of staurosporine target, leaving only the approximately 60-kDa band (Fig. 3A). The cloned cDNA was sequenced and found to be identical to the human ASC-1 P100 component (gi:12061187), a newly identified transcription coactivator complex component (Jung et al, 2002). Our clone encoded amino acids 154 to 757 fused in-frame



**Figure 3.** Pilot screen for apoptotic signaling targets. A, One pool contains three bands (*arrowheads*), which disappear after staurosporine (apoptotic) treatment. B, The plasmid encoding the largest band (*asterisk* in A) was isolated by sib-selection and expressed in Cos-1 cells, yielding two bands at ~100 and ~60 kDa. The *upper band* disappeared after 4 hours of staurosporine target, leaving only the ~60 kDa band. Sequencing revealed its identity to the human ASC-1 P100 component (gi:12061187), a transcription coactivator complex component. C, GFP autofluorescence in *left panel*, 4,6-diamidino-2-phenylindole (DAPI) stain in *right panel*. Fluorescence microscopy of control (untreated) cells revealed a punctate, predominantly cytoplasmic localization of GFP-ASC-1. D, GFP autofluorescence in *left panel*, DAPI stain in *right panel*. Apoptotic cells in staurosporine-treated cultures, identified by condensed nuclear DAPI staining, revealed a striking translocation of GFP-ASC-1 to the condensed nuclear DNA (D). This was also observed in spontaneously apoptotic cells in control cultures (not shown). Bar, 10  $\mu$ m.



**Figure 4.** Pilot screen for mitogenic signaling targets. A, One pool contains a putative serum-stimulated (mitogenic) target at ~65 kDa (*arrowhead*), which undergoes a serum-induced increased mobility shift after 15 minutes of stimulation. The shift is unaffected by pretreatment with the MEK inhibitor, UO126. B, The plasmid encoding the putative target was isolated by sib-selection and expressed in Cos-1 cells, yielding a single ~65-kDa band, which also shifted upon serum stimulation. C, The target, identified as EIF4A1, a known translation initiation factor component, shows a perinuclear and cytoplasmic localization, consistent with its known function. Bar, 10  $\mu$ m.

to the C-terminus of EGFP. Inspection of the predicted amino acid sequence revealed four previously undescribed consensus caspase cleavage sites: VELD (466–469), DEFD (543–546), DEYD (614–617), and DEED (663–666). If these sites are all cleaved after staurosporine treatment, the GFP-ASC-1 fusion protein is predicted to be reduced from 98 kDa to 64 kDa, consistent with the observed bands. The presence of some 60-kDa product in the untreated control lane is probably a result of the presence of a significant basal level of caspase activity and apoptosis in Cos-1 cells (Tyas et al, 2000). Fluorescence microscopy revealed a punctate, predominantly cytoplasmic localization of GFP-ASC-1 (Fig. 3C). Intranuclear speckles were observed in a subset of transfected cells. Apoptotic cells in staurosporine-treated cultures, identified by condensed nuclear 4,6-diamidino-2-phenylindole staining, revealed a striking translocation of GFP-ASC-1 to the condensed nuclear DNA (Fig. 3D). This was also observed in spontaneously apoptotic cells in control cultures (not shown).

To search for targets of mitogenic signaling, we screened 48 pools using brief (15 minutes) serum stimulation. An example of a putative approximately 65-kDa serum-regulated target is shown in Figure 4A. This band underwent an increased mobility shift that was insensitive to the MEK inhibitor, UO126, a potent and selective inhibitor of MEK→ERK-mediated mito-



genic signaling. Isolation of the plasmid encoding this band confirmed its serum-induced shift (Fig. 4B). The plasmid encoded the full-length eukaryotic translation initiation factor eIF4A, isoform 1 (gi:417180), fused in-frame to GFP. eIF4A1 is an RNA helicase of the DEAD-box family necessary for unwinding of mRNAs before translation (Gingras et al, 1999). Fluorescence microscopy of eIF4A1-GFP revealed its intracellular localization, previously undescribed (Fig. 4C). There was a diffuse, predominantly cytoplasmic localization, with some perinuclear concentration, compatible to the expected distribution of a translation factor. We did not observe any serum-induced translocation (data not shown). Posttranslational modification of mammalian eIF4A1 has not been reported, but other components of the eIF4 complex are phosphorylated (Pyronnet et al, 1999). We tested the possibility that the serum-induced mobility shift of eIF4A1 was a result of phosphorylation/dephosphorylation, using an alkaline phosphatase pretreatment protocol (Chen et al, 1999). Neither the mobility of the upper (serum-starved) nor the lower band (serum-stimulated) was altered by shrimp alkaline phosphatase pretreatment, arguing against phosphorylation as the serum-induced modification of eIF4A1 (data not shown).

## Discussion

The method described here provides a powerful yet simple approach for research laboratories, both large and small, to screen for signaling targets involved in a wide range of physiologic and pathologic cellular responses. All of the techniques used in FIMS are routine in laboratories equipped for molecular biology. The only special equipment needed is a laser scanner suitable for gel analysis, available as shared equipment in most academic institutions. Performing only small-scale pilot screens, we identified interesting targets for apoptotic and mitogenic signaling, which were not previously recognized as players in these responses. The method as described should be easily scaled up such that a near-saturating screen of a library could be completed in a few weeks of work.

Once targets undergoing mobility shifts are identified, the nature of the posttranslational modification can be determined by existing pharmacologic, biochemical, and immunochemical methods. For example, phosphorylation-induced shifts should be reversed by alkaline phosphatase pretreatment of lysates (Chen et al, 1999). For the case of proteolytic cleavages, there are several possible approaches. If the mobility shift or band disappearance is the result of cleavage by a specific protease, the change should be inhibitable by appropriate selective cell-permeant pharmacologic inhibitors, such as z-VAD-FMK for caspases, MDL-28170 for calpain, and lactacystin or MG132 for proteasome targets. Ubiquitination might be suspected if the target shows an early appearance of high-molecular weight smearing followed in time by degradation. To prove ubiquitination, the putative target is immunoprecipitated using commercially available anti-GFP antibody, separated on standard SDS-

PAGE, blotted, and then probed with an anti-ubiquitin antibody. A similar approach could be taken for sumoylation, neddylation, nitrosylation, and any other modification for which a specific antibody is available.

Great improvements in sensitivity and reliability of target detection will be achievable by integrating several available technologies with FIMS. Capillary electrophoresis with laser-induced fluorescence detection is well suited to sensitive detection of GFP fusion proteins (Korf et al, 1997). Use of a multicapillary electrophoresis apparatus like those used for large-scale DNA sequencing, in SDS-denaturing and/or isoelectric-focusing mode, will allow extremely rapid, sensitive, and reproducible detection of mobility shifts. Standard electrophoretic separation methods may miss a substantial fraction of subtle modifications, including one of the most important, addition of a single phosphate group. To increase the detection sensitivity for phosphorylation, isoelectric focusing, either in gel or capillary format, would provide a superior method for detection of phosphorylation.

An alternative to transfection-based methods is the use of collections of stably transfected mammalian cell lines or yeast strains, each of which harbors a single identified GFP fusion protein. This would eliminate the variation inherent in transient transfections and avoid the redundancy of library screening.

Large efforts are currently directed at obtaining complete collections of expressed eukaryotic and prokaryotic genes (Brizuela et al, 2001). Many of these gene collections are made in versatile cloning vectors using Cre/lox or other recombinational cloning technologies. This will allow rapid and efficient transfer of thousands of identified, full-length cDNAs into GFP fusion vectors. Screening of arrayed collections of GFP fusions will provide a great increase in efficiency compared with screening of traditional redundant cDNA libraries. In fact, it should be feasible to create a collection of all human genes (~30,000), made in a GFP vector, and arrayed in small pools (10 identified clones/pool). These approximately 3,000 pools could be easily manipulated in eight 384-well plates, with the cell transfections performed in the same high-throughput format. Entire coverage of all expressed human genes using FIMS would thus be feasible in a small laboratory. Application of FIMS to diverse cell culture models should yield many interesting signaling targets involved in physiologic and pathologic cell reactions.

## Materials and Methods

### *Construction and Screening of the GFP-cDNA Fusion Library*

Total RNA was isolated from HT1080 cells, a human fibrosarcoma cell line, with Trizol (Invitrogen, San Diego, California), and poly(A) RNA was prepared using the Oligotex mRNA purification kit (Qiagen, Studio City, California). The cDNAs were generated using the SuperScript Plasmid System (Invitrogen). This procedure yielded cDNAs that were directionally

cloned into the *SaI*I and *Not*I sites of a GFP expression plasmid, pCIneoHGFP (Fujii et al, 1999). The cDNA library was electroporated into DH10B cells (Invitrogen) that were plated onto 150-mm Petri dishes. The library was estimated to have greater than  $2 \times 10^4$  independent clones.

### **Preparation of Small cDNA Pools**

Colonies from the library were grown on 10-cm Luria-Bertani (LB)/ampicillin plates and pooled by manually scraping 25 to 50 colonies into 1 ml of LB/ampicillin. A 100- $\mu$ l aliquot from each of these pools was stored in a sealed 96-well plate containing LB/7% dimethyl sulfoxide; the remaining bacterial suspension was immediately pelleted (not further amplified), and plasmid DNA was purified using a 96-well low-endotoxin purification method (Neudecker and Grimm, 2000).

### **Transfection of Cos-1 Cells**

Cos-1 cells, grown in 96-well plates, were transfected with plasmid pools using 0.2  $\mu$ g of DNA and 0.6  $\mu$ l of Fugene 6 per well (as per manufacturer's instructions; Roche, Welwyn Garden City, United Kingdom). After 24 hours in complete DMEM/10% fetal bovine serum, transfected cells were serum-starved overnight and then subjected to serum stimulation for 15 minutes. Cells were rinsed once with PBS and harvested on ice for 30 minutes in RIPA buffer with added 1 mM sodium orthovanadate and protease and phosphatase inhibitors (Sigma protease inhibitor cocktail [P8340; Sigma, St. Louis, Missouri] containing a proprietary mixture of 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), bestatin, leupeptin, aprotinin, and Sigma phosphatase inhibitor cocktail [P2850] containing a proprietary mixture of microcystin LR, cantharidin, and (-)-p-bromotetramisole. After freeze-thawing, aliquots of the RIPA extracts were mixed with  $2 \times$  Laemmli sample buffer just before gel loading. Samples were not boiled or heated at any point in the protocol because this leads to loss of GFP fluorescence.

### **In-Gel Imaging of Expressed Proteins Using Fluorescence Laser Scanning**

Cell lysates were separated in 10% polyacrylamide gels by standard SDS-PAGE in minigel or standard format. A prestained protein ladder mixture (1  $\mu$ l; Benchmark, Invitrogen) was loaded for molecular weight estimation. The pink-stained marker protein (~64 kDa) proved to be a brightly autofluorescent landmark. After a rinse in distilled water, gels were scanned wet with a FluorImager (Molecular Dynamics) set for excitation at 488 nm and a photomultiplier setting of 800.

### **Sib-Selection Strategy Using Group Testing**

Once a band of interest was identified, an aliquot (0.05  $\mu$ l) of the corresponding cryopreserved bacte-

rial pool was replated. Ninety-six individual colonies were picked to inoculate a 96 deep-well plate, each well containing 1 ml of LB/ampicillin. For a pool size of 40 clones, screening 96 randomly chosen clones from the pool gives a 91.2% ( $1 - (39/40)^{96}$ ) chance of inclusion of the clone of interest. The plate was sealed and incubated overnight at 37° C with gentle shaking. The 96 clones were tested using a row/column group-testing procedure to minimize the number of plasmid preparations and transfections. Eight-row pools (consisting of 80  $\mu$ l from each well in a row) and 12-column pools (consisting of 80  $\mu$ l from each well in a column) were prepared with a multipipetter, from which a total of 20 plasmid preparations were made. These row and column pool preparations were used for another round of Cos-1 transfections, and lysates were screened for the band of interest. The well containing the positive clone can be identified by noting the intersection of a positive row and column pool, except in cases in which a single row or column contains multiple positives. Once isolated, the plasmid was further purified (GeneClean, Qbiogene) and subjected to double-stranded sequencing from both ends of the insert using either a T3 sequencing primer for 3' end sequencing or a pair of custom primers (forward primer 5'-GAGCTGTACAAGTCCGGAC-3' and reverse primer 5'-GTATCTTATCATGTCTGC-3', respectively). Sequence analysis and searches were performed using BLAST (<http://www.ncbi.nlm.nih.gov/>).

### **Fluorescence Microscopy**

Cos-1 cells expressing GFP fusions were fixed for 15 minutes in 4% paraformaldehyde followed by 5 minutes in -20° C methanol, to optimally preserve GFP fusion localization (Brock et al, 1999). After washing with PBS, cells were mounted in 70% glycerol/1% diazo-bicyclo-octane and photographed using an Olympus BX50 fluorescence microscope (Tokyo, Japan) with Kodak Elite 400 slide film (Rochester, New York).

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