ABI3, a component of the WAVE2 complex, is potentially regulated by PI3K/AKT pathway

SUPPLEMENTARY MATERIALS

Plasmids constructions

The full length cDNA of ABI3 (accession number NM_016428.2) was synthesized from a RNA isolated from a FTA sample using using Super-Script III Reverse Transcriptase kit with an oligo(dT)$_{12-18}$ primer and 10 units of RNase inhibitor (Invitrogen, Life Technologies, Grand Island, NY), according to the manufacturers' recommendations. To insert EcoRI and BamHI restriction sites at the 5' and 3'-termini of the cDNA products, a sense primer containing an EcoRI (underlined) and an antisense primer with a BamHI restriction site (underlined) were used to generate the ABI3 cDNA (Supplementary Table 5). An aliquot of the cDNA was used as template in a 50-μL PCR reaction containing 0.8 mM dNTPs, 2 mM MgSO$_4$, 60 mM Tris–SO$_4$ (pH 8.9), 18 mM (NH$_4$)$_2$SO$_4$, 1 U Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and 10 pmol of each sense and antisense primers. The amplified product was purified and digested with restriction endonucleases EcoRI and BamHI (New England Biolabs, Hitchin, UK). The PCR fragment was cloned into pHCMV2 expression vector containing a HA-tag upstream the multiple cloning site using EcoRI/BamHI restriction sites generating the plasmid HA-ABI3. Ligation was performed using a 1:30 molar ratio of vector to insert and T4 DNA Ligase (New England Biolabs). The construct was fully sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit.

To generate the HA-GFP construct (control vector), both pCMV-FLAG-GFP [1] and HA-phCMV2 expression vectors were used. Precisely, the pCMV-FLAG-GFP and HA-phCMV2 expression vectors were digested with EcoRI restriction enzyme. The GFP cassette was gel-purified and inserted at the 3' end of the HA coding sequence, using EcoRI cloning site of vector. Insertion of the GFP cassette was confirmed by EcoRI digestion.

Generation of stable cell clones

Two follicular thyroid carcinoma cell lines were used. The WRO (UCLA RO-82W-1) was kindly donated by Alfredo Fusco (Facoltà di Medicina e Chirurgia, University Federico II, Naples, Italy). FTC133 was purchased from the European Collection of Authenticated Cell Cultures (ECACC; cat# 94060902). WRO cells were cultured in DMEM and FTC133 in DMEM:F12 (vo/vol) supplemented with 10% (vol/vol) fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco, Life Technologies). Cells were maintained at 37°C in a 5% CO2 humidified atmosphere. Cells were stably transfected with 10 μg of each construct encoding ABI3 gene (HA-ABI3) and controls (HA-phCMV2 and HA-GFP-phCMV2) by electroporation using a Gene Pulser II Electroporation System (Bio-Rad, Hercules, CA), as described [2]. Pools were selected in complete medium supplemented with Geneticin 800 μg/mL (WRO) and 700 μg/mL (FTC133). The stable expression of the different constructs was confirmed by Western Blot analysis of the target protein. At least two independent experiments were performed.

Preparation of protein lysates and western blot analysis

Cellular protein lysates were collected under cell exponential growth. Where indicated, cells have been also treated with LY294002 (Cell Signaling Technology). Cells were harvested on ice after washing with cold PBS and were lysed with Triton lysis buffer (1% Triton, 20 mM Tris-HCl pH 7.5, 130 mM NaCl, 2.5 mM sodium phosphate, 10 mM NaF and 1mM NaVO$_4$) supplemented with a Protease Inhibitor Cocktail Tablets (complete, Roche, New York, NY). The lysates were clarified by centrifugation at 8,000 g for 10 min at 4°C. Total protein concentration was determined using the BCA method (Thermo Scientific, San Jose, CA). For immunoblot analysis proteins (25 μg) was resolved on 8-12% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Subsequently, the membranes were blocked with 5% non-fat dried milk in 1X TBS 0.1% Tween-20 and...
incubated at 4°C for at least 16 hours with the indicate antibodies (Supplementary Table 2). Membranes were incubated with Pierce ECL Western Blotting Substrate (Thermo Scientific, San Jose, CA) or Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Darmstadt, GE) when we needed to detect lower levels of proteins. The chemiluminescent signals were detected by ImageQuant LAS4000 Analyzer (GE Healthcare, Chicago, IL). At least two experiments were performed. The band intensities were quantified using the ImageQuant TL software (GE Healthcare) and corrected for background differences. After background correction, the data were normalized to the expression of β-actin or α-tubulin (loading control) and expressed as fold-change.

Proteome profiler antibody array

Human Phospho-Kinase Array Kit and Human Apoptosis Array Kit (cat. # RY003), which detects relative phosphorylation levels of 46 intracellular serine/threonine/tyrosine kinases, and Human Apoptosis Array Kit (cat# ARY009) which detects levels of 35 apoptosis-related proteins, were purchased from R&D Systems (Minneapolis, MN). WRO cells were stably transfected with either ABI3 or control (empty vector), as described above. Cells were lysed with Lysis Buffer 6 (R&D Systems). Cell lysates were clarified by microcentrifugation at 14,000 rpm for 5 min, and the supernatants were subjected to protein assay. Membranes were incubated with 300 μg (ARY003) or 400 μg (ARY009) of cellular extract for 16 hours at 4°C, on a rocking platform. The membranes were washed twice with wash buffer (R&D Systems) and then were incubated with Chemiluminescent reagent (R&D Systems). Array images were analyzed using ImageQuant LAS4000 Analyzer (GE Healthcare, Chicago, IL) and quantified using ImageQuant TL software (GE Healthcare), as described above. Each spot was detracted by the averaged background level from negative control spots. The expression values were calculated as mean of the duplicated spots, representing each protein, normalized by the density levels of the positive control spots present on the membrane. For positive control present in the membrane. The fold change was calculated as the expression observed in the experimental membrane (WRO expressing ABI3) relative to the control membrane (WRO expressing empty vector). The fold changes were determined following two criteria: 1) a protein expression ratio of the control expressing cells to the ABI3 expressing cells ≤0.8 was considered down-regulation and 2) a protein expression ratio of the ABI3 expressing cells to the control cells ≥1.2 was considered up-regulation. The list of antibodies and their position on the arrays can be found at http://www.rndsystems.com/pdf/ARY003.pdf and http://www.rndsystems.com/pdf/ARY009.pdf.

Functional enrichment analysis

In order to visualize enriched pathways, the list of proteins that were found differentially expressed in WRO cells expressing ABI3, compared to the control, were upload at Enrichr analysis tool available online (http://amp.pharm.mssm.edu/Enrichr/).

HA-tag immunoprecipitation (HA-IP) and mass spectrometry analysis

WRO cells expressing the HA-tagged proteins (ABI3 and GFP) were washed harvested by scraping on using cold PBS and were lysed using cold lyses buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM NaF, 1 mM NaVO₄, 0.4% n-Octyl-β-D-glucoside) supplemented with 2X Protease Inhibitor Cocktail Tablets (Complete, Roche). The lysates were centrifuged at 8,000 g for 10 min at 4°C. The supernatants were collected and were incubated at at 4°C for overnight with an EZView Red Anti-HA agarose Affinity Gel (Sigma Aldrich, St. Louis, MO) on a rocking platform. The recovered gel was washed three times with wash buffer (50 mM Tris-HCl pH=8.0, 100 mM NaCl) and the bound proteins were eluted by competition with an HA peptide (300 ng/μL) (Sigma Aldrich) on a rocking platform at 4°C for 2 h. The resultant immunoprecipitation eluates were directly analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), as described below.

Protein digestion and LC-MS/MS analysis

Proteins from the HA-ABI3 and HA-GFP pull-downs were denatured in 6 M urea, 2 M thiourea, 10 mM HEPES, reduced with 1 mM DTT and alkylated with 5.5 mM iodoacetamide. The samples were diluted 1:5 with 50 mM ammonium bicarbonate and digested with trypsin (Promega) using a 1:50 mass ratio. The samples were acidified with 0.5% trifluoroacetic acid and desalted on C18 spin columns. Four micrograms of peptides were analyzed in triplicate by LC-MS/MS in a Thermo Scientific Easy-nLC 1000 system coupled to a LTQ Orbitrap XL ETD (mass spectrometry facility RPT02H PDTIS - Carlos Chagas Institute - FIOCRUZ Parana). Peptide separation was carried out on a 15 cm microcapillary column (75 μm I.D.; fused silica packed with reversed-phase C18, 3 μm resin). Chromatography runs were performed in a flow rate of 250 nL/min from 5 to 40% MeCN, 0.1% formic acid in a 120 min gradient. The mass spectrometer operated in a data-dependent mode to automatically switch between MS and MS/MS (MS²) acquisition. Survey full-scan MS spectra (at 350 – 1,650 m/z range) were acquired in the Orbitrap analyzer with resolution of 60,000 at m/z 400 (after accumulation to a target value of 500,000 in the C-trap). The ten most
intense ions were sequentially isolated and fragmented in the linear ion trap using collision-induced dissociation at a target value of 30,000. The “lock mass” option was set to 445,120025 m/z, enabling in all full scans an improvement of precursor ions mass accuracy [3].

Proteomic data analysis

Peaklist picking, protein identification, quantification, and validation were obtained using the MaxQuant platform (version 1.5.2.8) [4], which includes the algorithm Andromeda [5] for database searching. Default parameters of the software were used for all analysis steps, unless stated otherwise. Proteins were searched against a “decoy database” prepared by reversing the sequence of each entry of the used database and appending them to the forward sequences. The search was conducted against a human protein sequence database (containing 70,071 protein sequences, updated in August 09, 2015) and the green fluorescent protein sequence both from the UNIPROT database. This database was complemented with frequently observed contaminants (porcine trypsin, Achromobacter lyicus lysyl endopeptidase, and human keratins) and their reversed sequences. Search parameters specified a MS tolerance of 4.5 ppm, a MS/MS tolerance of 0.5 Da, and full trypsin specificity, allowing for up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification. Oxidation of methionine, and N-terminal acetylation (protein) were allowed as variable modifications. For validation of the identifications, a minimum of seven amino acids for peptide was required. In addition, a false discovery rate (FDR) threshold of 0.01 was applied at both peptide and protein levels. The option match between runs was enabled to increase protein identification. Protein quantification was performed using a label-free (LFQ) approach, where peptides eluting from each LC run are detected as three-dimensional features-retention time versus signal intensity (extracted ion chromatogram, XIC) versus mass/charge-aligned and compared across runs, as previously describe [6].

Protein G-sepharose immunoprecipitation with WAVE2 antibody (WAVE 2-IP)

WRO cells expressing the HA-tagged proteins (ABI3 and GFP) were harvested on ice after washing with cold PBS and were lysed in the lyses buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM NaF, 1mM NaVO₄, 0.4% n-Octyl-β-D-glucoside) supplemented with 2X Protease Inhibitor Cocktail Tablets (Complete, Roche). To reduce nonspecific binding to the Protein G-sepharose beads, the cells lysates were pre-cleared by incubation with rec-Protein G-Sepharose 4B (Invitrogen) for 30 min at 4°C. Subsequently, the supernatants were incubated at 4°C overnight with 1:50 WAVE2 antibody (Supplementary Table 4) on a rocking platform. As negative control, cells lysates were incubated without WAVE2 antibody. After antibody incubation, Protein G- sepharose beads were coupled to the extract for 3h at 4°C in a rocking platform and washed three times with lyses buffer. Complexes were eluted from the beads with 0.1 M glycine-HCl pH 2.2 and neutralized with 1 M Tris-HCl pH=8.0. Eluted proteins were stored at -80°C and Western blot analysis was performed as described above.

Alkaline phosphatase treatment

Under normal conditions ABI3 runs as doublet (a lower band of 52kDa an upper band of 54kDa) in both WRO and FTC1333 cells (Figure 1A and 1C). To determine whether the gel shift of ABI3 (upper band) is due to its phosphorylation, cells lysates were treated with Calf Intestinal Alkaline Phosphatase (CIP, New England Biolabs). WRO and FTC1333 cells expressing ABI3 were lysed in an EDTA-free lyses buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 0.1mM NaVO₄, 1% Triton, 10 μg/mL Aprotinin, 10 μg/mL Leupeptin and 10 μg/mL Pepstatin). Next, cell lysates were diluted 1:3 and incubated with CIP (1U/μg of protein) at 37°C for 1h. Cell lysates untreated with CIP were used as negative control.

Thyroid samples

The series consists of 23 thyroid samples (7 normal thyroid tissues and 16 follicular thyroid adenoma) obtained from patients who underwent thyroid surgery at Hospital São Paulo (Universidade Federal de São Paulo). The study was conducted under the approval of the Review Boards and Research Ethical Committee of the Universidade Federal de São Paulo (Process 1278/10).

RNA isolation, cDNA synthesis and quantitative RT-PCR (qPCR)

Total RNA was isolated from thyroid samples and Cell lines using TRIzol reagent (Invitrogen, Life Technologies, Grand Island, NY) according to the manufacturer’s recommendation. Complete homogenization was obtained by passing the lysate through a 23-gauge needle. RNA was quantified using a NanoDrop2000c spectrophotometer. Total RNA (1μg) was treated with DNase (Ambion, Life Technologies), reverse transcribed into cDNA using Super-Script III Reverse Transcriptase kit with an oligo(dT)₁₂₋₁₈ primer and 10 units of RNase inhibitor (Invitrogen), according to the manufacturers’ recommendations. An aliquot (1μL) of cDNA was used in a 12 μL PCR reaction containing SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) and 3,2 pmol of each specific primer for the
target genes (ABI3, WAVE2 and CYFIP1) or reference gene (RPS8). The PCR reactions were performed in triplicate, and the Ct was obtained using Applied Biosystem software and averaged (SD < 1.0). The relative expression (RE) was calculated according to the comparative ΔΔCt method. Normal thyroid, vehicle-treated control cells or empty vector transfected cells were used as the control group for ΔΔCt calculation. Primers and conditions for PCR are summarized in Supplementary Table 5.

Analysis of candidate phosphorylation sites in ABI3

A search for putative phosphorylation sites of human ABI3 was performed using ABI3 data registered in the public databases such as PhosphoSite Plus (http://www.phosphosite.org/), UniProt (http://uniprot.org/) and Human Protein Reference Database (HPRD, http://hprd.org/). Residue numbers were defined according to human isoform 1 (NP_057512.1). To further evaluate whether the residues identified as potentially phosphorylated could be phosphorylated by the protein kinases found modulated in the proteome antibody array analysis, we submitted the amino-acid sequence of ABI3 to Motif Scan analysis at Scansite 3 (http://scansite3.mit.edu/). This combined analysis revealed S342 as a likely candidate site for phosphorylation.

ABI3 site-directed mutagenesis

To determine if the phosphorylation at serine 342 (S342), we mutated the serine amino acid codon to alanine (S342A). The phCMV2-ABI3 vector was submitted to site-directed mutagenesis with primers for S342A conversion (Supplementary Table 5) using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. The S342A mutation was confirmed by sequencing. The phCMV2-ABI3-S342A vector was stable transfected into WRO and FTC133 cells and the expressed mutant protein was evaluated by western blot analysis.

LY4029002 treatment

The LY294002 (Cell Signaling Technology) PI3-kinase specific inhibitor was employed to test the ABI3 dependence on AKT pathway. About 3.5 x 10^5 WRO and FTC133 cells expressing ABI3 were seeded in 60-mm plates. Cells were starved overnight and pre-treated with 20 μM LY294002 for 2h. As negative control cells were incubated with vehicle (DMSO). Then, FBS was added and cells were collected after 5h and used in western-blot analysis as previously described using. Antibodies against pAKT(S473) and AKTpan were used to determine the efficiency of LY294002 treatment and ABI3 antibody for ABI3 phosphorylation analysis.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v5.01 Software (GraphPad Software). Comparisons between groups were performed using Student t test or Mann-Whitney test when data did not exhibit a normal distribution verified by Shapiro-Wilk test. Results were expressed as mean±SD and P<0.05 was considered statistically significant.
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Supplementary Figure 1: S342 site is highly conserved in other species such as *Macaca mulatta*, *Canis lupus familiaris*, *Bos taurus*, *Mus musculus* and *Rattus norvegicus*. 
### Supplementary Table 1: List of phosphokinases and apoptosis-related proteins modulated by ABI3

| Antibody array | Spot | Protein      | Phosphorylation site | Fold-change | Percentage of modulation | P-value |
|----------------|------|--------------|----------------------|-------------|--------------------------|---------|
| ARY003         | A3, A4 | p38α         | T180/Y182            | 0.58        | -42 %                    | 0.0402  |
| ARY003         | A7, A8 | JNK pan      | T183/Y185, T221/ Y223 | 0.39        | -61 %                    | 0.0275  |
| ARY003         | A9, A10 | GSK-3α/β     | S21/S9               | 0.17        | -83 %                    | 0.0155  |
| ARY003         | B5, B6 | MSK1/2       | S376/S360            | 0.67        | -33 %                    | 0.0255  |
| ARY003         | B7, B8 | AMPKα1       | T174                 | 0.46        | -55 %                    | 0.0235  |
| ARY003         | C7, C8 | AMPKα2       | T172                 | 0.73        | -27 %                    | 0.0290  |
| ARY003         | B9, B10 | Akt          | S473                 | 0.60        | -40 %                    | 0.0058  |
| ARY003         | B11, B12 | Akt         | T308                 | 0.73        | -27 %                    | 0.0028  |
| ARY003         | C1, C2 | TOR          | S2448                | 0.64        | -36 %                    | 0.0324  |
| ARY003         | C5, C6 | HSP27        | S78/S82              | 0.47        | -53 %                    | 0.0105  |
| ARY003         | C9, C10 | β-Catenin/ CTNNB1 | -                    | 1.24        | +24 %                    | 0.0313  |
| ARY003         | C11, C12 | p70 S6 Kinase | T389                 | 0.00        | -100 %                   | 0.0163  |
| ARY003         | D1, D2 | Src          | Y419                 | 0.66        | -34 %                    | 0.0302  |
| ARY003         | D3, D4 | Lyn          | Y397                 | 0.61        | -39 %                    | 0.0182  |
| ARY003         | D5, D6 | Lck          | Y394                 | 0.46        | -54 %                    | 0.0403  |
| ARY003         | D7, D8 | STAT2        | Y689                 | 0.72        | -28 %                    | 0.0115  |
| ARY003         | E9, E10 | STAT5b      | Y699                 | 0.57        | -43 %                    | 0.0215  |
| ARY003         | D17, D18 | PLCγ-1     | Y783                 | 0.74        | -26 %                    | 0.0349  |
| ARY003         | E1, E2 | Fyn          | Y420                 | 0.74        | -26 %                    | 0.0225  |
| ARY003         | E3, E4 | Yes          | Y426                 | 0.57        | -43 %                    | 0.0023  |
| ARY003         | E5, E6 | Fgr          | Y412                 | 0.37        | -63 %                    | 0.0479  |
| ARY003         | E15, E16 | c-Jun      | S63                  | 0.74        | -26 %                    | 0.0434  |
| ARY003         | E17, E18 | Pyk2       | Y402                 | 0.74        | -26 %                    | 0.0396  |
| ARY003         | F1, F2 | Hck          | Y411                 | 0.66        | -34 %                    | 0.0159  |
| ARY003         | F3, F4 | Chk-2        | T68                  | 0.66        | -34 %                    | 0.0262  |
| ARY003         | F5, F6 | FAK          | Y397                 | 0.70        | -30 %                    | 0.0294  |
| ARY009         | B23, B24 | Cytochrome C | -                    | 0.75        | -25 %                    | 0.0229  |
| ARY009         | D21, D22 | XIAP       | -                    | 1.55        | +55 %                    | 0.0190  |
Supplementary Table 2: List of proteins identified in the IP of HA-ABI3. The list includes the proteins that were identified in at least two replicates and that showed higher peak intensity relative to the ip with HA-EGFP. Peak intensity was calculated using the label-free quantitation method (lfq) of the maxquant platform.

See Supplementary File 1
### Supplementary Table 3: Site-specific phosphorylation of ABI3

| Position | Peptide sequence | UniProt (domain) | PhosphoSite plus | HPRD | Motif Scan - scansite 3 (score) | Conservation | Reference |
|----------|-----------------|-----------------|-----------------|------|-------------------------------|--------------|-----------|
| T134     | PENLPPPltPYCRRL | Abi_HHR         | H               | -    | Cdc2 (0.4317), Cdk5 (0.4303) and Erk1 (0.4811) | High/ Medium | -         |
|          |                 |                 |                 |      | 14-3-3 (0.4218), AKT (0.4741), AMPK (0.6313), CAMKK2 (0.5339), PKCa/β/γ (0.4444), PKCδ (0.4950) and PKA (0.5515) |              |           |
| T165     | TQLSRGtLsRKSik | Abi_HHR         | H/M             | -    | 14-3-3 (0.5966), PKCa/β/γ (0.4541), PKCδ (0.4407), PKCe (0.4208) and PKA (0.5007) | High         | -         |
| S170     | TGtLSRKsIKAPTP  | Abi_HHR         | H               | -    | -                             | High         | -         |
|          |                 |                 |                 |      | Aurora A (0.5746), Aurora B (0.5598) and GSK3 (0.6035) |              |           |
| S203     | VVPDGLSAAASSAs | -               | M               | -    | Aurora A (0.5746), Aurora B (0.5598) and GSK3 (0.6035) | High         | -         |
| S210     | SAAASsLASAGsA  | -               | H               | -    | PKCδ (0.4522) | High         | -         |
| S213     | SSASsLASAGsAEGV| -               | M               | -    | PDK1 (0.9491) | High         | [7, 8]    |
| S216     | SsLASAGsAEGVGGA | -               | H/M             | Phosphorylation | PKCδ (0.4728) | High         | [7-9]     |
| T258     | EVFQRPPtLEELSP | Pro-rich e      | H               | -    | 14-3-3 (0.6000), AMPK (0.7138), ATM (0.4690), DNA PK (0.6222), PKCe (0.5097) and PKA (0.5491) | Low         | -         |
| T338     | EGTVICtRRysDGW | SH3 f           | H               | -    | 14-3-3 (0.5574), AKT (0.6697), Aurora A (0.2924), Aurora B (0.3321) and PKA (0.4732) | High         | [7, 8, 10]|
| Y341     | VICtRRysDGWCEG | SH3             | H/M             | -    | -                             | High         | [7]       |
| S342     | IVCtRRysDGWCEG | SH3             | H/M             | Phosphorylation | - | High         | [7, 8, 10]|
| S365     | PGNVYVEPsC______ | SH3             | H               | -    | -                             | Medium       | -         |
Supplementary Table 4: Antibodies specifications

| Antibody     | Dilution | Blocking                  | Cat. number                                      |
|--------------|----------|---------------------------|--------------------------------------------------|
| HA tag       | 1:1000   | 5% w/v nonfat dry milk    | # 2367 (Cell Signaling Technology)               |
| ABI3         | 1:500    | 5% w/v nonfat dry milk    | HPA017345 (Sigma Aldrich)                        |
| pAKT(S473)   | 1:1000   | 5% w/v BSA                | # 4070 (Cell Signaling Technology)               |
| pAKT(T308)   | 1:1000   | 5% w/v BSA                | # 2965 (Cell Signaling Technology)               |
| AKTpan       | 1:2000   | 5% w/v BSA                | # 4691 (Cell Signaling Technology)               |
| pGSK3β(S9)   | 1:1000   | 5% w/v BSA                | # 5558 (Cell Signaling Technology)               |
| CYFIP1       | 1:1000   | 5% w/v nonfat dry milk    | ab108220 (Abcam)                                 |
| WAVE2        | 1:1000   | 5% w/v BSA                | # 3659 (Cell Signaling Technology)               |
| β-actin      | 1:2000   | 5% w/v BSA                | # 4967 (Cell Signaling Technology)               |
| α-tubulin    | 1:10.000 | 5% w/v nonfat dry milk    | T9026 (Sigma Aldrich)                            |
| Anti-Mouse   | 1:10.000 | 5% w/v nonfat dry milk    | SC2031 (Santa Cruz Biotechnology)                |
| Anti-Rabbit  | 1:10.000 | 5% w/v nonfat dry milk    | P0448 (Dako)                                     |

BSA = bovine serum albumin.
| Gene    | Sequence (5’-3’)                      | Assay   | Annealing temp (°C) | Product size (pb) |
|---------|---------------------------------------|---------|---------------------|------------------|
| ABI3    | Forward: TAAGCACGCTGGGCCAGAT          | Expression | 60                  | 166              |
|         | Reverse: CAGCCAAAGTTGAGGGGTCT         |          |                     |                  |
| WAVE2   | Forward: CCCACGTAAAATCAAGACACG        | Expression | 60                  | 129              |
|         | Reverse: AATGCTGCCATTCTGGTACAC       |          |                     |                  |
| CYFIP1  | Forward: CCATCAGGAAGACCGTGCGT        | Expression | 60                  | 114              |
|         | Reverse: CGCGGCGTGGTACTTTTAT         |          |                     |                  |
| RPS8    | Forward: AACAAGAAATAATCCGTGCC        | Expression | 60                  | 125              |
|         | Reverse: GTACGAACCAGCTCGTTATTA       |          |                     |                  |
| ABI3    | Forward: CCGGAATTCGTATGGCGAGCTACAGCAGCT | Cloning | 60                  | 1250             |
|         | Reverse: CCGGGATCCAGGACAGAACACAGCTCACAGC |          |                     |                  |
| ABI3S342A | Forward: CACTCGCCGCTACGCTGATGGCTGGTGTCG | Mutagenesis | 55                  | NA               |
|         | Reverse: CGCACCAGCCATCAGCTAGCGGCGAGTG |          |                     |                  |

NA = not applicable.