THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 38, pp. 29231–29238, September 17, 2010

Parkin Enhances the Expression of Cyclin-dependent Kinase 6 and Negatively Regulates the Proliferation of Breast Cancer Cells*[S]

Received for publication, January 27, 2010, and in revised form, June 18, 2010 Published, JBC Papers in Press, July 14, 2010, DOI 10.1074/jbc.M110.108241

Shiam-Peng Tay[‡§1], Calvin W. S. Yeo[‡¶1], Chou Chai[‖], Pei-Jou Chua[**], Hui-Mei Tan[‡], Alex X. Y. Ang[‡‡], Daniel L. H. Yip[‡‡], Jian-Xiong Sung[§§], Puay Hoon Tan[¶¶], Boon-Huat Bay[**], Siew-Heng Wong[§], Carol Tang[‖‖], Jeanne M. M. Tan[‡‖2], and Kah-Leong Lim[‡¶‖3]

From the [‡]Neurodegeneration Research Laboratory and the [‖‖]Neuro-Oncology Research Laboratory, National Neuroscience Institute, Singapore 308433, the Departments of [**]Anatomy, [¶]Physiology, and [§]Microbiology, National University of Singapore, Singapore 117597, the [‡‡]Raffles Institution, Singapore 575954, the [§§]Ngee Ann Polytechnic, Singapore 599489, the [‖]Duke-NUS Graduate Medical School, Singapore 169857, and [¶¶]Singapore General Hospital, Singapore 169608

Although mutations in the parkin gene are frequently associated with familial Parkinsonism, emerging evidence suggests that parkin also plays a role in cancers as a putative tumor suppressor. Supporting this, we show here that parkin expression is dramatically reduced in several breast cancer-derived cell lines as well as in primary breast cancer tissues. Importantly, we found that ectopic parkin expression in parkin-deficient breast cancer cells mitigates their proliferation rate both *in vitro* and *in vivo*, as well as reduces the capacity of these cells to migrate. Cell cycle analysis revealed the arrestment of a significant percentage of parkin-expressing breast cancer cells at the G1-phase. However, we did not observe significant changes in the levels of the G1-associated cyclin D1 and E. On the other hand, the level of cyclin-dependent kinase 6 (CDK6) is dramatically and selectively elevated in parkin-expressing breast cancer cells, the extent of which correlates well with the expression of parkin. Interestingly, a recent study demonstrated that CDK6 restrains the proliferation of breast cancer cells. Taken together, our results support a negative role for parkin in tumorigenesis and provide a potential mechanism by which parkin exerts its suppressing effects on breast cancer cell proliferation.

Mutations in the parkin gene, located on chromosome 6q25.2-27, are a predominant cause of inherited parkinsonism (1). Accordingly, much of the interest in characterizing the function of the parkin gene has been directed toward understanding its role in neurodegeneration. However, aberrant parkin function has also been linked to several other disorders (2, 3), among which, to the development of several types of cancers (4). Comparatively, the role of parkin in these disorders is less well characterized.

Supporting a role for parkin in cancers, a previous study by Cesari *et al.* (4) involving physical mapping combined with loss of heterozygosity (LOH)[4] analysis identified the 6q-located, 1.4 Mb *parkin* as a gene that is frequently targeted by hemizygous deletion and inactivation in both malignant tumors and tumor-derived cell lines. Following this discovery, several other groups have reported parkin gene alterations and expression variability in a variety of tumor biopsies and tumor cell lines representing a wide range of cancers including breast and ovarian cancers (4–8). Frequently, diminished or absent parkin expression was observed in these cancers, suggesting that parkin may have tumor suppression properties. Consistent with this, microcell-mediated transfer of human chromosome 6 suppresses tumorigenicity in several cancer cell lines (9), and introduction of an intact chromosome 6 into MCF7 (a breast cancer cell line) restores its ability to senesce (10). Collectively, these studies support the existence of a tumor suppressor gene (TSG) on chromosome 6q and the potential candidacy of parkin as a TSG. However, whether and how the loss of parkin function contributes to the development of cancers are currently not well understood, although one line of parkin-deficient mice exhibits a tendency to develop hepatocellular carcinoma (11) and more recently, somatic mutations of parkin have been detected in human glioblastoma (12).

Here, we examined a variety of breast cancer cell lines and found that the mRNA and protein expression of parkin are dramatically reduced in these lines compared with normal breast cells. Similarly, parkin expression is compromised in primary breast cancer tissues relative to adjacent normal tissues. Importantly, by means of a wide spectrum of assays, we found that the restoration of parkin expression in MCF7 breast cancer cells, which is otherwise highly deficient in parkin expression, mitigates their proliferation rate both *in vitro* and *in vivo*, as well as reduces their capacity to migrate. The reduced proliferation rate of parkin-expressing MCF7 cells

* This work was supported by grants from Khoo's Discovery Award (to K.-L. L.) and Singapore Millennium Foundation (to C. Y. and J. T.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

[1] Both authors contributed equally to this work.

[2] To whom correspondence may be addressed: Neurodegeneration Research Laboratory, National Neuroscience Institute, 11 Jalan Tan Tock Seng, Singapore 308433. Tel.: 65-6357-7520; Fax: 65-6256-9178; E-mail: tmm18sg@yahoo.com.sg.

[3] To whom correspondence may be addressed: Neurodegeneration Research Laboratory, National Neuroscience Institute, 11 Jalan Tan Tock Seng, Singapore 308433. Tel.: 65-6357-7520; Fax: 65-6256-9178; E-mail: kahleong.lim@duke-nus.edu.sg.

[4] The abbreviations used are: LOH, loss of heterozygosity; TSG, tumor suppressor gene; IRS, immunoreactivity score; PCA, principal component analysis; CAP, cysteine-rich secretory proteins antigen 5 and pathogenesis-related proteins; CFS, common fragile site.

is likely due to their tendency to arrest at the G1-phase of the cell cycle, although this phenomenon appears not to be driven by changes in the levels of the G1-associated cyclin D1 and E. Instead, microarray analysis of parkin-expressing MCF7 cells revealed a significant increase in the expression level of cyclin-dependent kinase 6 (CDK6). Consistent with this, we observed that CDK6 protein expression increases in a dose-dependent manner with the levels of functional parkin. This phenomenon is however not observed when parkin is substituted with a mutant that is devoid of catalytic activity or with CHIP, a related member. Interestingly, CDK6 has previously been demonstrated to down-regulate the proliferation of breast cancer cells (13). Taken together, our results suggest a novel relationship between parkin and CDK6 and at the same time, provide a potential mechanism by which parkin exerts its tumor suppressing effects on breast cancer cells.

MATERIALS AND METHODS

Antibodies and Reagents—The following antibodies were used: monoclonal anti-parkin clone PRK8 (Covance), monoclonal anti-CDK6 (Abcam), monoclonal anti-Flag peroxidase (Sigma), monoclonal anti-β-actin (Sigma), FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.). All the cell lines used in this study were purchased from American Type Culture Collection.

Analysis of Parkin Expression and Cellular Localization—Total RNA was isolated from various breast cancer cells using the RNAeasy Mini kit (Qiagen). Subsequently, the isolated RNA was reverse transcribed using the Superscript First-Strand Synthesis System (Invitrogen). Real-Time PCR was carried out in a Light Cycler (Roche) using the FastStart DNA Master Plus Sybergreen I system (Roche) according to the manufacturer's protocol. A pair of parkin-specific primers (Forward: 5′-GGAAGTCCAGCAGGTAGATCA; Reverse: 5′-ACCCTGGGTCAAGGTGAG) were generated for this purpose. Concurrently, Real-Time PCR with a primer pair specific for GAPDH (Forward: 5′-GAAGGTGAAGGTCGGAGTCAACG; Reverse: 5′-TGCCATGGGTGGAATCATATTGG) was also included in the same run as an internal control. Western blot analysis and cellular localization studies of parkin were performed as previously described (14).

Primary Breast Cancer Tissue Analysis—Human breast cancer tissue qPCR array Panel I plate (OriGene Technologies, Inc, Rockville, MD) was removed from −20 °C storage and allowed to warm to room temperature. Master mix was prepared according to manufacturer's protocol. The stock solution contains 2× QuantiTect SYBR Green PCR master mix, parkin forward and reverse primers, and RNase-free water. 30 μl of PCR pre-mix was then aliquoted to each of the 48 wells containing cDNA, and the plate was covered with adhesive cover sheet. The plate was then centrifuged at 2000 rpm for 5 min and allowed to sit on ice for 15 min to allow the dried cDNA to dissolve. Real time PCR was done using Applied Biosystems 7500 Real-Time PCR Systems, and the amplification condition is as follows: activation at 95 °C for 15 min, followed by 40 cycles of denaturing at 94 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 60 s. The Ct value of the PCR products was obtained, and analysis was done. Automated immunohistochemistry was performed on 6 pairs of adjacent benign and malignant breast cancer tissues using the Leica Bond^{TM} system using parkin antibody (1:500 dilution). Each breast tissue section was evaluated for parkin immunoreactivity using a semi-quantitative scoring system based on the staining intensity. The immunoreactivity scores (IRS) were calculated as $\sum$(intensity$_n$ × percentage of positive stained cells with intensity$_n$).

Generation of Stable Cell Line and Cell Proliferation Assays—MCF7 cells stably expressing wild type or mutant parkin (or otherwise containing vector alone) were generated by means of a previously described procedure (15). All positive cell lines used for the experiments described hereafter were maintained in serum-containing DMEM supplemented with 200 μg/ml Geneticin (Invitrogen) to prevent extrusion of integrated constructs. A simple population growth assay was conducted by seeding cells to be counted in duplicates at a concentration of 2×10^4 cells in 6-well plates, and subsequently quantifying their number each day for a period of 5 days by means of a hemocytometer. BrdU-based proliferation assay (Roche) was carried out according to the manufacturer's instructions. For soft-agar colony formation, 0.3% agar containing 1×10^4 MCF7-vector or MCF7-parkin stable cells was overlaid onto pre-cast 0.5% bottom agar and allowed to solidify before incubating at 37 °C in a 5.0% CO_2 incubator for a period of 21 days. Colonies formed on the soft agar were visualized under light microscopy before and after MTT staining.

In Vivo NOD-SCID/J Model—All procedures involving animals were approved by and conformed to the guidelines of our Institutional Animal Care and Use Committee. 6-week-old NOD-SCID mice were allowed to acclimatize for 2 weeks before the start of the experiment. Each mouse was anesthetized before being injected subcutaneously into the right rear flank with 100 μl of phosphate-buffered saline (PBS) alone (as a control) or containing 3×10^6 MCF7-vector or MCF7-parkin stable cells. Tumor formation in injected mice was monitored daily. Where visible tumor was formed, its length (L) and width (W) were measured, and the tumor volume, *i.e.* $L \times W^2/2$, subsequently calculated.

Wound Scratch Migration and Matrigel Assay—Wound scratch assay was performed according to a method described by Suyama *et al.* (16). Briefly, 5×10^5 cells are plated in 35-mm dishes and allowed to grow to about 90% confluency. After which, a wound line was made by scratching the cell layer firmly with a pipette tip. Cells were then washed a few times with PBS to remove debris before recovery in fresh medium. The migration of cells into the wound area was monitored at regular intervals under a phase contrast microscope for a period of 84 h. Migration assay using the Matrigel transwell apparatus (BD Bioscience) was conducted according to the manufacturer's instructions.

Statistical Analysis—Statistical significance for all the quantitative data obtained was analyzed using Student's *t* test (*, $p < 0.05$; **, $p < 0.001$) unless otherwise stated.

RESULTS

Aberrant Parkin Expression in Several Breast Cancer Cell Lines—As an initial effort to characterize the role of parkin in breast cancer, we examined the expression level of parkin in a

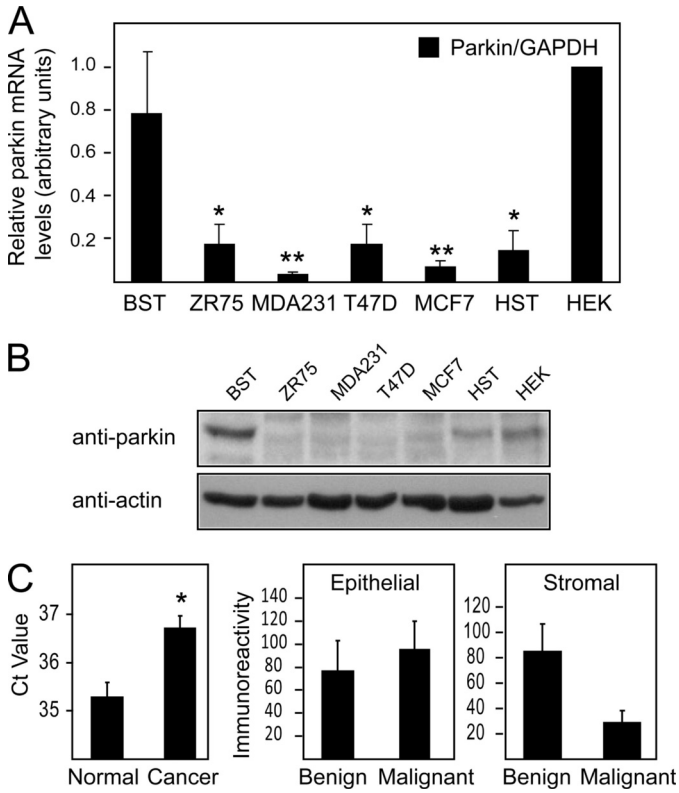

FIGURE 1. **Parkin expression is down-regulated in breast cancer cells.** *A*, bar graph depicting the real-time quantification of parkin mRNA levels in various breast cancer cell lines and a normal breast cell line Hs578BST (*BST*). The non tumor-derived HEK 293 cells were used as an additional control (*, $p < 0.05$, **, $p < 0.001$, Student's *t* test). *B*, representative anti-parkin immunoblot showing the relative expression of endogenous parkin protein in various lines. The blot was stripped and reprobed with anti-actin to reflect loading variations. *C*, graphs showing the Ct value of parkin expression in normal and cancer tissues (*left panel*), and parkin immunoreactivity scores derived from 6 pairs of adjacent benign and malignant breast cancer tissues. Statistical analysis was carried up using GraphPad Prism, Version 4.0 software (SanDiego, CA). Wilcoxon matched pair test was used to compare the difference between the adjacent benign and malignant breast cancer tissues (*, $p < 0.05$).

number of commercially available breast cancer cell lines including ZR75, MDA231, T47D, MCF7, and Hs578T (HST). Consistent with the observation by Cesari *et al.* (4), we found that parkin mRNA and protein levels are significantly reduced in all of these tumor-derived cell lines compared with normal breast cells Hs578BST (BST) or to the non-tumor derived HEK cell line (Fig. 1, *A* and *B*). On the other hand, neither DJ-1 nor PINK1, representing two other Parkinson disease (PD)-linked genes, exhibits a consistent pattern of down-regulation in this spectrum of breast cancer cells (supplemental Fig. S1*A*). To extend these findings, we also examined the expression of parkin in primary breast cancer samples. Quantitative PCR analysis of 5 identical sets of 48 tissues revealed that parkin expression is significantly lower in tumor compared with adjacent normal tissues (Mean Ct value: normal 35.28 ± 0.30 *versus* tumor 36.71 ± 0.26), which is in agreement with our earlier observations with cancer cell lines (Fig. 1*C*). Further, we also examined via immunohistochemical analysis whether parkin protein expression may be different between benign and malignant tissues. Although we recorded no difference in epithelial parkin expression between these tissues, the expression of stromal parkin in malignant tissues is apparently greatly reduced compared with their benign counterparts (Fig. 1*C* and supplemental S1*B*). Taken together, our results suggest that the function of parkin in breast cancer cells may be compromised via its down-regulation.

Ectopic Parkin Expression in Parkin-deficient MCF7 Breast Cancer Cells Mitigates Their Proliferation in Vitro and in Vivo—To examine the effects of restoring parkin expression in parkin-deficient breast cancer cells, we generated stable clones of MCF7 cells expressing FLAG-tagged parkin (MCF7-PK) or containing vector alone as a control (MCF7-Vector). The MCF7 cell line was selected for this study because it is a widely used *in vitro* model of breast cancer and also one that is highly deficient in parkin expression (Fig. 1, *A* and *B*). Three individual parkin-positive MCF7 clones (5, 7, and 11) were selected for our experiments to minimize clonal variation. Notably, all of these parkin-positive clones express parkin at a higher level than vector control or parental cells (Fig. 2*A*), but are otherwise similar morphologically (not shown). A simple population growth assay reveals that the proliferation rate of parkin-expressing MCF7 cells is significantly reduced compared with control cells (Fig. 2*B*), suggesting that ectopic parkin expression in MCF7 cells mitigates their growth. Supporting this, siRNA-mediated depletion of parkin expression in these stable clones reverses the inhibitory effects of parkin on their proliferation rate (Fig. 2*B*). Similarly, the incorporation of BrdU, a thymidine analogue, in parkin-expressing MCF7 cells also occurs significantly less frequently compared with control cells (Fig. 2*C* and supplemental Fig. S2*A*). Because cancer cells are anchorage-independent and have the ability to form colonies in soft agar, we also examined whether ectopic parkin expression in MCF7 cells compromise its ability to generate colonies in soft agar. We found that the number of soft agar colonies formed by parkin-expressing MCF7 cells is dramatically reduced and the size of these colonies also tend to be smaller compared with those generated by control MCF7 cells (Fig. 2*D*, not shown for MCF7-parkin 7 and 11). The ability of parkin to suppress the proliferation of MCF7 cells appears to be dependent on its catalytic activity, as MCF7 cells stably expressing a catalytically impaired parkin mutant (T415N) do not exhibit any alterations in their proliferation rate relative to control cells (supplemental Fig. S4*A*).

To extend our above findings, we examined the effects of parkin over expression on the ability of MCF7 cells to generate solid tumor *in vivo*. A flank tumor model ($n = 9$ for each group), where NOD-SCID mice were injected subcutaneously with parkin expressing or control MCF7 cells, or otherwise with PBS alone, was used for this purpose. Over a period of 4 weeks post-injection, we observed visible tumor formation that progressively increases in size in mice injected with control or parkin-expressing MCF7 cells, but not in those injected with vehicle alone (Fig. 2*E* and supplemental Fig. S2, not shown for PBS control). Consistent with our *in vitro* findings above, we found that parkin-positive clones tend to generate tumors of significantly smaller volume and mass *in vivo* compared with control cells (Fig. 2*E* and supplemental Fig. S2). Taken together, our results demonstrate that ectopic parkin expression in parkin-deficient MCF7 cells mitigate its proliferation both *in vitro* and

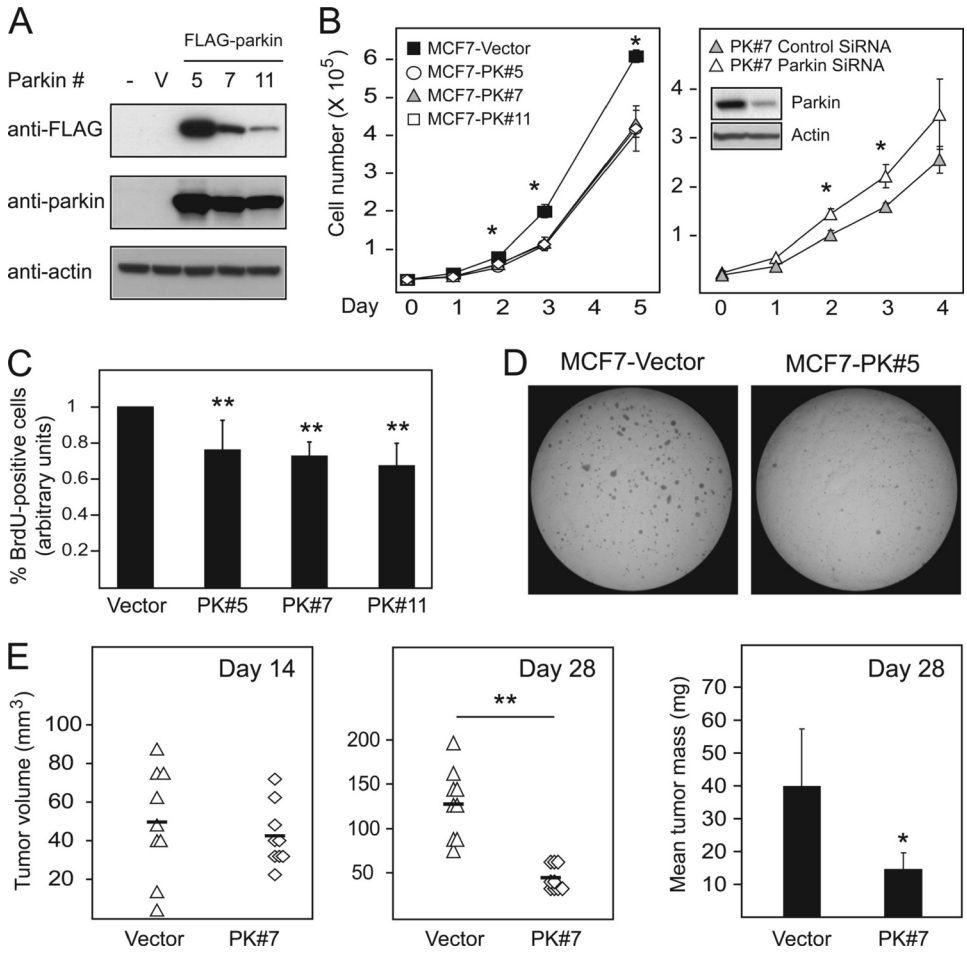

FIGURE 2. **Overexpression of parkin in MCF7 cells mitigates their proliferation rate.** *A*, anti-FLAG and anti-parkin immunoblots of total lysates prepared from MCF7 cells (−), vector control (*V*), and the parkin-expressing stable clones (5, 7, & 11). Equal loading of the different lysates was verified by anti-actin immunoblotting. *B*, graphical depiction of the percentage of cells undergoing cell proliferation, as measured by cell population number, in MCF7 vector control and parkin-expressing stable cell lines (*left*), or parkin-expressing cells in the presence of control or parkin siRNA (*right*). *Inset*, immunoblots showing the expression of parkin in cells transduced with control (*lane 1*) or parkin siRNA (*lane 2*) (*top panel*), and the respective actin levels (*bottom panel*). *C*, BrdU assay of MCF7 vector control and parkin-expressing stable cell lines. *D*, representative images showing apparent decreased ability of parkin-expressing MCF7 cells in forming colonies in soft agar compared with MCF7-vector control. *E*, graph showing the volume (*left* and *middle panels*) and mass (*right panel*) of tumor generated by different cell types in NOD-SCID mice ($n = 9$) at day 14 and day 28. The experiment was duplicated with essentially the same result (*, $p < 0.05$; **, $p < 0.001$, Student's *t* test).

in vivo, and strongly suggest a negative role for parkin in breast cancer cell proliferation.

Ectopic Parkin Expression in Parkin-deficient MCF7 Breast Cancer Cells Reduces Their Migration Rate—We next asked the question whether ectopic parkin expression in parkin-deficient MCF7 cells would influence their migration properties. To address this, we performed a simple wound scratch assay and monitored the re-colonization process for 84 h (3.5 days). From 36 h onwards, we found that MCF7 cells ectopically expressing parkin migrate significantly slower into the scratched area compared with control cells (Fig. 3, *A* and *B*). This trend persisted for all the parkin-expressing MCF7 clones until the end of our experiment (Fig. 3, *A* and *B*). Clearly, ectopic parkin expression in these cells markedly retarded their rate of migration. To verify our observations above, we repeated our migration assay using the standard matrigel invasion chamber (see "Materials and Methods"). Consistent with our above findings, we recorded a significantly reduced ability of parkin-expressing MCF7 cells to migrate to the underside of the matrigel-coated transwell compared with control cells (Fig. 3*C*, not shown for MCF7-parkin 7 and 11). Taken together, our results suggest that ectopic parkin expression influences the proliferation as well as migration properties of MCF7 breast cancer cells.

Parkin Promotes MCF7 Cell Cycle Arrest at G1 Phase—Conceivably, the reduced proliferation rate of parkin-expressing MCF7 cells may be due to alterations of its cell cycle program as a result of parkin overexpression. To examine whether parkin affects the cell cycle profile of MCF7 cells, we analyzed the cell cycle profile of control and parkin-expressing MCF7 cells by means of flow cytometry. Interestingly, compared with control cells, a significant number of parkin-expressing cells apparently accumulate at the G1 phase (Fig. 4). In agreement with this, the percentage of parkin-expressing cells at the S and G2/M phases is markedly reduced relative to MCF-vector controls (Fig. 4). Collectively, these results suggest that parkin reduces the proliferation rate of MCF7 breast cancer cells in part, by promoting their arrest at the G1 cell cycle phase.

Interestingly, cyclin E, a cell cycle regulator required for the transition from G1 to S phase, has previously been identified as a parkin substrate (17). Parkin apparently ubiquitinates cyclin E and promotes its turnover. Moreover, recent studies demonstrated defects in cyclin E proteolysis in colon and brain cancer cells harboring dysfunctional parkin (12, 18). We were therefore interested to examine whether cyclin E expression is affected in parkin-expressing MCF7 cells. Unexpectedly, we did not detect an apparent difference in the steady state level of cyclin E between parkin-expressing and control MCF7 cells (Fig. 5*A*). Like cyclin E, cyclin D1 is also involved in G1 to S transition. Hence, we also examined its levels in parkin-expressing MCF7 cells. Similar to cyclin E, the expression of cyclin D1 does not appear to be significantly affected in MCF7 cell stably expressing parkin compared with control cells (Fig. 5*B*). Aside from the above cyclin species, we also checked the expression of key mitogenic components that might differ between parkin-expressing and control MCF7 cells. A prime candidate central to mitogenic signaling is Erk (extracellular-regulated kinase), which exists in several isoforms. Another is Akt, whose hyperactivation is linked to several tumor types. Among the Erk iso-

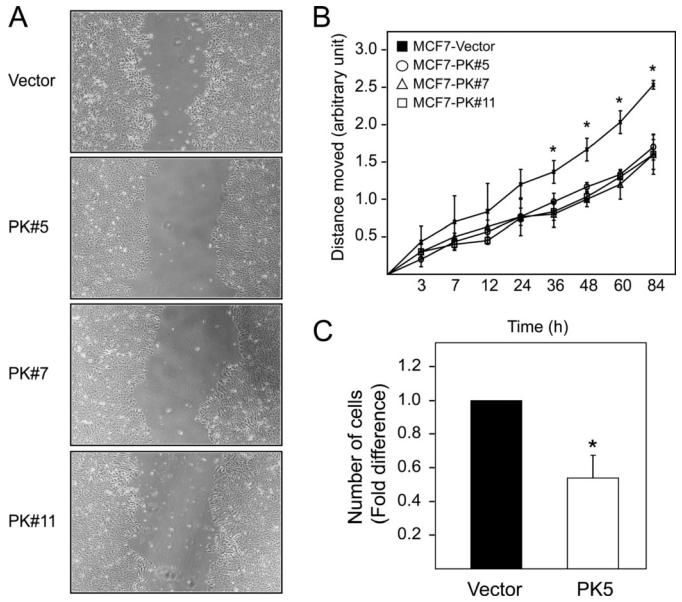

FIGURE 3. **Parkin-expressing MCF7 cells exhibits slower migration.** *A*, *phase-contrast images* showing the re-colonization of cells into the wound area at 60 h. *B*, graphical depiction of the distance moved by the various cell types (as indicated) into the wound area. (*, $p < 0.05$, Student's *t* test). *C*, *bar graph* showing the ratio between parkin-expressing and control cells that migrated to the underside of the matrigel-coated transwell after 24 h of incubation (*, $p < 0.05$, Student's *t* test).

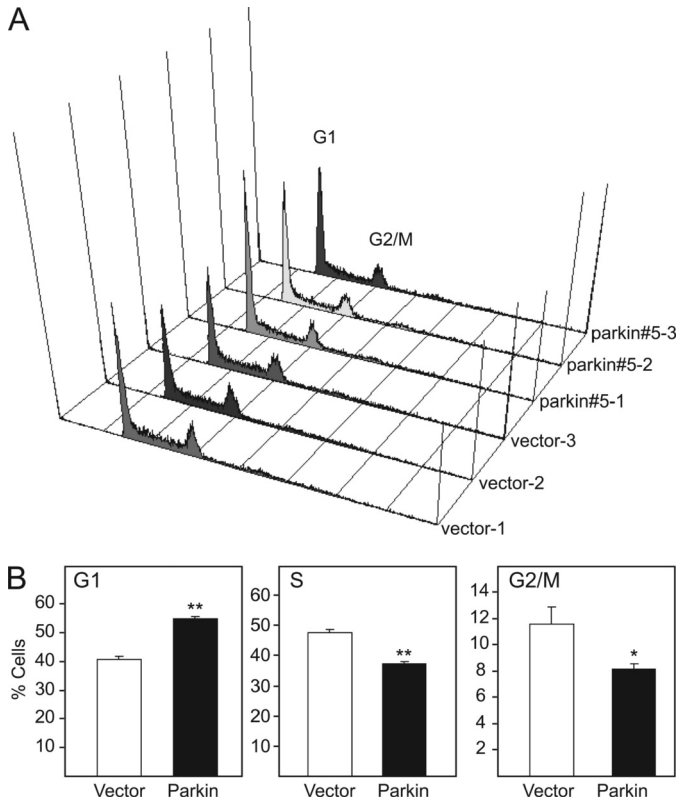

FIGURE 4. **Accumulation of parkin-expressing MCF7 cells at G1 phase.** *A*, graphical depiction of flow cytometry-based cell cycle analysis of control and parkin-expressing MCF7 cells. *B*, *bar graphs* showing the percentage of control (*vector*) or parkin-expressing MCF cells (*parkin*) in various phases of the cell cycle.

forms, Erk 1 and 2 are the main downstream effectors of mitogenic growth factor signaling. However, we failed to observe a clear trend in the level of activated, phosphorylated species of

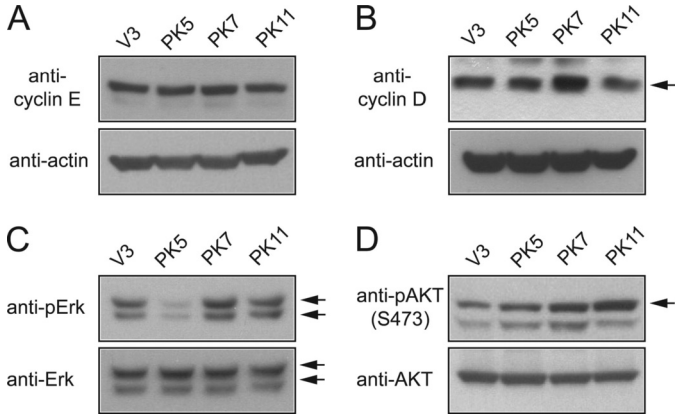

FIGURE 5. **Levels of cyclins and mitogenic components in control and parkin-expressing MCF7 cells.** *A*, anti-cyclin E; *B*, anti-cyclin D1; *C*, anti-phosphorylated Erk 1/2 and anti-Erk1/2; *D*, anti-phosphorylated Akt and anti-Akt immunoblots in vector control (*V3*) and parkin-expressing (PK5, -7 & -11) MCF7 cells. Equal loading of the different lysates was verified by anti-actin immunoblotting.

Erk 1 and 2 between parkin-expressing MCF7 cells and control cells (Fig. 5*C*). In contrast, the level of phosphorylated Akt appears to increase, rather than decrease in parkin-expressing MCF7 cells compared with control cells (Fig. 5*D*). It would thus appear that parkin-mediated suppression of MCF7 cells does not involve changes in the levels or activation of cyclin D1, E, Erk1/2, and Akt.

CDK6 Expression Is Significantly Enhanced in Parkin-expressing MCF7 Breast Cancer Cells—Although the precise mechanism by which parkin mediates the suppression of MCF7 breast cancer cell proliferation remains unclear, we reasoned that parkin likely participates in some cellular signaling events that trigger changes in the expression of specific genes, which in turn mediates its tumor suppression properties. We therefore subjected parkin-expressing and vector-control MCF7 cells to microarray analyses using Affymetrix chips. Principal component analysis (PCA) plots of the data obtained revealed that the gene expression changes in the two groups of cells are significant (not shown); demonstrating that parkin expression in MCF7 exerts profound effects on its transcriptional profile. In these analyses, the most significant alterations are a near 5-fold repression of the gene encoding the cysteine-rich secretory protein LCCL domain containing 1 (CRISPLD1) and a 3.4-fold up-regulation of the gene encoding cyclin-dependent kinase 6 (CDK6) in parkin-expressing cells compared with control MCF7 cells (supplemental Fig. S3). Whereas CDK6 is a cell cycle-related protein, less is known about the function of CRISPLD1 except that it belongs to the CAP (cysteine-rich secretory proteins antigen 5 and pathogenesis-related proteins) superfamily that has roles in cancer and immune response (19).

We next sought to address whether the increased CDK6 transcript levels in MCF7-parkin cells would translate to an increase in protein expression. Consistent with our microarray analyses result, anti-CDK6 immunoblotting of lysates prepared from MCF7-vector and MCF7-parkin cells revealed a significant elevation of the protein in parkin-expressing MCF7 cells, the expression of which correlates well with the level of parkin

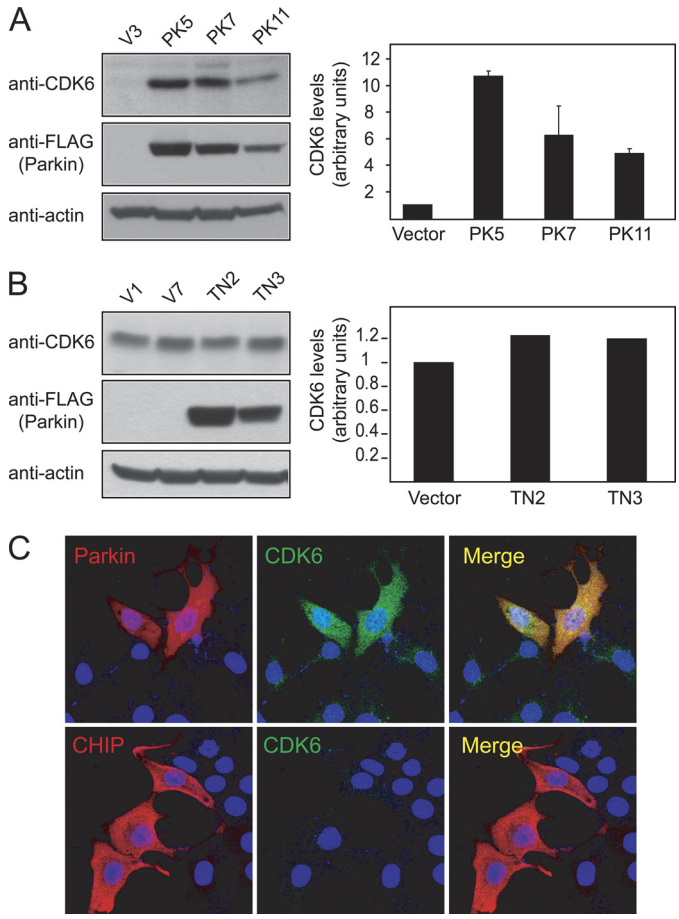

FIGURE 6. **CDK6 expression is enhanced in parkin-expressing MCF 7 cells.** *A*, *left*, immunoblots showing the levels of CDK6 and parkin in control (vector) or parkin-expressing MCF7 cells, as indicated. *Right*, *bar graph* showing the relative difference in CDK6 expression among the MCF7 clones examined as determined by densitometric quantification. *B*, as in *A* except that parkin is substituted with a catalytically-impaired T415N mutant. Two clonal populations of MCF7 cells stably expressing this mutant are indicated as *TN2* and *TN3*. *C*, representative confocal images showing CDK6 staining in MCF7 cells transiently transfected with parkin or CHIP, as indicated.

expression in the various stable clones (Fig. 6*A*). This relationship between parkin and CDK6 is clearly dependent on the catalytic activity of parkin, as substituting parkin with the catalytically-impaired T415N mutant failed to influence CDK6 expression (Fig. 6*B*). Similarly, substituting parkin with CHIP, a related E3 member, did not affect CDK6 expression (Fig. 6*C*). We also generated MCF7 cells stably expressing parkin R275Q and R275W, which are respectively associated with cancer and PD (12). Neither of these mutants alters CDK6 levels when expressed in the breast cancer cell line (supplemental Fig. S4*D*). Interestingly, the phenomenon appears unique to MCF7 cells, as CDK6 expression in a different cell line, *i.e.* SH-SY5Y neuroblastoma cells that ectopically expresses parkin is unaffected (supplemental Fig. S4*B*). Taken together, our results suggest a hitherto unknown cellular co-regulation of parkin and CDK6 expression in breast cancer cells. Given the recently described role of CDK6 as a negative regulator of breast carcinogenesis (13), our results also suggest a potential mechanism by which parkin exerts its suppression on the proliferation of breast cancer cells.

DISCUSSION

In this study, we have demonstrated that parkin expression is dramatically down-regulated in a variety of breast cancer cell lines as well as in primary breast cancer tissues. Further, we showed that restoration of parkin expression in a representative parkin-deficient line mitigates its proliferation rate both *in vitro* and *in vivo*, as well as reduces the capacity of these cells to migrate. Collectively, our results strongly support a role for parkin as a TSG.

Although a role for parkin in cancers may still come as a surprise to many, the 1.4 Mb parkin gene is located within the common fragile site (CFS), FRA6E, a mutational hotspot on chromosome 6 that is frequently deleted in several tumor types including breast, ovarian, kidney, and liver cancers (20). FRA6E, along with other CFSs such as FRA3B, FRA16D, and FRAXB, have been proposed to play a causal role in tumorigenesis and/or cancer progression (20, 21). Notably, the FRA3B-associated *FHIT* and the FRA16D-associated *WWOX* genes are commonly deleted or altered in various tumor types. Further, several lines of evidence suggest that functionally, FHIT and WWOX act as tumor suppressors (22–24). The residence on FRA6E that the enormous parkin gene takes thus immediately suggests its potential association with cancers. Consistent with this, Cesari *et al.* (4, 6–8) as well as a number of other groups have documented parkin gene alterations and concomitant reduction in parkin transcript abundance in a wide variety of cancers that includes breast, liver, non-small-cell lung, and ovarian carcinoma. Here, we extended these findings by showing that parkin expression down-regulation in several breast cancer cell lines is rather specific to the gene as both *DJ-1* and *PINK1* did not exhibit a consistent reduction in their expression in these cells.

Despite a consistent association between parkin expression alterations and cancers, there is currently a paucity of reports that demonstrates robustly the functional consequence of parkin expression restoration in parkin-deficient cancer cells, although a very recent study by Veeriah *et al.* (12) showed that restoration of parkin expression in parkin-deficient glioma cells clearly mitigates their growth. By means of a wide spectrum of proliferation assays that include population growth, anti-BrdU-staining, soft-agar colony formation as well as generation of solid tumors in NOD-SCID mice, we showed here that the restoration of parkin expression in MCF7 cells markedly reduces their proliferation rate both *in vitro* and *in vivo*, and significantly slows down their migration. Our results therefore provide further evidence supporting a tumor suppressor-like function of parkin. Mechanistically, exactly how parkin suppresses the growth of MCF7 cells remains to be elucidated. Because parkin functions as a ubiquitin protein ligase, and one of its identified substrates is cyclin E (17) (a G1 cyclin whose accumulation is associated with breast cancer development (25)), it is tempting to speculate that misregulation of cyclin E levels in parkin-deficient cells could promote tumorigenesis. However, we did not observe obvious difference in cyclin E levels between control and parkin-expressing MCF7 cells. Neither

did we observe a significant in the levels of cyclin D1 between these two cell types. Further, we failed to observe any reduction in growth factor signaling via Erk1/2 activation in parkin-expressing MCF7 cells. Surprisingly, the growth promoting PI3/Akt signaling pathway appears to be up-regulated in MCF7 cells stably expressing parkin. This is intriguing given our observation that parkin negatively regulates MCF7 cell proliferation, although a correlative relationship between Akt activation and parkin expression has been previously reported by others (18, 26). Conceivably, a network of other players might be involved in parkin-mediated suppression of breast cancer cell proliferation. In this regard, it is interesting to note that our microarray analysis have identified many interesting candidates whose expression is significantly altered in the presence of parkin expression. Among them is CDK6, whose expression increased by more than 3-fold in parkin-expressing relative to control MCF7 cells. Interestingly, Lucas *et al.* (13) have recently demonstrated that whereas normal human mammary epithelial cells contains high levels of CDK6 protein and activity, all the breast tumor-derived cell lines examined exhibit significantly reduced levels of CDK6, with several having little or no CDK6. Importantly, they showed that restoration of CDK6 in breast cancer cells restrained their proliferation (13). Similarly, NIH3T3 lines engineered to overexpress CDK6 all exhibit reduced rate of proliferation relative to parental control cells (27). It is thus attractive to speculate that enhanced CDK6 expression in parkin-expressing MCF7 cells participates to mitigate the proliferation rate of these cells. Notwithstanding this, it is important to point out that the reported anti-proliferative role of CDK6 is non-classic and currently controversial. However, this paradoxical activity of CDK6 is not without precedent. For example, Stat3, a widely accepted oncogene, was shown recently to play a tumor-suppressive role in PTEN-deficient human glioblastoma cells (28). It would therefore appear that the physiological manifestation of an oncogene (or tumor suppressor) is dependent on the cellular context. We have attempted to study the effects of CDK6 overexpression in MCF7 cells. However, unlike Lucas *et al.* (13), we have difficulties analyzing MCF7 cells expressing exogenous CDK6 as they detach from cell culture substratum and round up readily (supplemental Fig. S4*C*), suggesting that CDK6 expression above a certain threshold level potentially promotes apoptosis of MCF7 cells. Interestingly, our microarray analysis also revealed a significant down-regulation of a relatively novel protein known as CRISPLD1, a member of the CAP superfamily that apparently has roles in cancer and immune response (19). Although much less is known about the role of CRISPLD1 in breast cancer at this moment, future experiments should help elucidate the relationship between CRISPLD1 and its potential interaction with parkin in mediating the suppression of breast cancer cell proliferation. In essence, our results presented here strongly support a negative role for parkin in breast cancers, which likely involves a novel (albeit currently correlative) relationship between parkin and CDK6.

Acknowledgments—We thank Xiao-Hui Ng and Shijin Feng for technical support.

REFERENCES

1. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998) *Nature* **392,** 605–608
2. Mira, M. T., Alcaïs, A., Nguyen, V. T., Moraes, M. O., Di Flumeri, C., Vu, H. T., Mai, C. P., Nguyen, T. H., Nguyen, N. B., Pham, X. K., Sarno, E. N., Alter, A., Montpetit, A., Moraes, M. E., Moraes, J. R., Doré, C., Gallant, C. J., Lepage, P., Verner, A., Van De Vosse, E., Hudson, T. J., Abel, L., and Schurr, E. (2004) *Nature* **427,** 636–640
3. Mengesdorf, T., Jensen, P. H., Mies, G., Aufenberg, C., and Paschen, W. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 15042–15047
4. Cesari, R., Martin, E. S., Calin, G. A., Pentimalli, F., Bichi, R., McAdams, H., Trapasso, F., Drusco, A., Shimizu, M., Masciullo, V., D'Andrilli, G., Scambia, G., Picchio, M. C., Alder, H., Godwin, A. K., and Croce, C. M. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 5956–5961
5. Denison, S. R., Callahan, G., Becker, N. A., Phillips, L. A., and Smith, D. I. (2003) *Genes Chromosomes Cancer* **38,** 40–52
6. Denison, S. R., Wang, F., Becker, N. A., Schüle, B., Kock, N., Phillips, L. A., Klein, C., and Smith, D. I. (2003) *Oncogene* **22,** 8370–8378
7. Picchio, M. C., Martin, E. S., Cesari, R., Calin, G. A., Yendamuri, S., Kuroki, T., Pentimalli, F., Sarti, M., Yoder, K., Kaiser, L. R., Fishel, R., and Croce, C. M. (2004) *Clin. Cancer Res.* **10,** 2720–2724
8. Wang, F., Denison, S., Lai, J. P., Philips, L. A., Montoya, D., Kock, N., Schüle, B., Klein, C., Shridhar, V., Roberts, L. R., and Smith, D. I. (2004) *Genes Chromosomes Cancer* **40,** 85–96
9. Trent, J. M., Stanbridge, E. J., McBride, H. L., Meese, E. U., Casey, G., Araujo, D. E., Witkowski, C. M., and Nagle, R. B. (1990) *Science* **247,** 568–571
10. Negrini, M., Sabbioni, S., Possati, L., Rattan, S., Corallini, A., Barbanti-Brodano, G., and Croce, C. M. (1994) *Cancer Res.* **54,** 1331–1336
11. Fujiwara, M., Marusawa, H., Wang, H. Q., Iwai, A., Ikeuchi, K., Imai, Y., Kataoka, A., Nukina, N., Takahashi, R., and Chiba, T. (2008) *Oncogene* **27,** 6002–6011
12. Veeriah, S., Taylor, B. S., Meng, S., Fang, F., Yilmaz, E., Vivanco, I., Janakiraman, M., Schultz, N., Hanrahan, A. J., Pao, W., Ladanyi, M., Sander, C., Heguy, A., Holland, E. C., Paty, P. B., Mischel, P. S., Liau, L., Cloughesy, T. F., Mellinghoff, I. K., Solit, D. B., and Chan, T. A. (2010) *Nat. Genet.* **42,** 77–82
13. Lucas, J. J., Domenico, J., and Gelfand, E. W. (2004) *Mol. Cancer Res.* **2,** 105–114
14. Wang, C., Tan, J. M., Ho, M. W., Zaiden, N., Wong, S. H., Chew, C. L., Eng, P. W., Lim, T. M., Dawson, T. M., and Lim, K. L. (2005) *J. Neurochem.* **93,** 422–431
15. Wang, C., Ko, H. S., Thomas, B., Tsang, F., Chew, K. C., Tay, S. P., Ho, M. W., Lim, T. M., Soong, T. W., Pletnikova, O., Troncoso, J., Dawson, V. L., Dawson, T. M., and Lim, K. L. (2005) *Hum. Mol. Genet.* **14,** 3885–3897
16. Suyama, E., Kawasaki, H., Nakajima, M., and Taira, K. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 5616–5621
17. Staropoli, J. F., McDermott, C., Martinat, C., Schulman, B., Demireva, E., and Abeliovich, A. (2003) *Neuron* **37,** 735–749
18. Ikeuchi, K., Marusawa, H., Fujiwara, M., Matsumoto, Y., Endo, Y., Watanabe, T., Iwai, A., Sakai, Y., Takahashi, R., and Chiba, T. (2009) *Int. J. Cancer*, in press
19. Gibbs, G. M., Roelants, K., and O'Bryan, M. K. (2008) *Endocr. Rev.* **29,** 865–897
20. Smith, D. I., Huang, H., and Wang, L. (1998) *Int. J. Oncol.* **12,** 187–196
21. Smith, D. I., Zhu, Y., McAvoy, S., and Kuhn, R. (2006) *Cancer Lett.* **232,** 48–57
22. Bednarek, A. K., Keck-Waggoner, C. L., Daniel, R. L., Laflin, K. J., Bergsagel, P. L., Kiguchi, K., Brenner, A. J., and Aldaz, C. M. (2001) *Cancer Res.* **61,** 8068–8073
23. Druck, T., Berk, L., and Huebner, K. (1998) *Oncol. Res.* **10,** 341–345

24. Ishii, H., Dumon, K. R., Vecchione, A., Trapasso, F., Mimori, K., Alder, H., Mori, M., Sozzi, G., Baffa, R., Huebner, K., and Croce, C. M. (2001) *Cancer Res.* **61,** 1578–1584
25. Keyomarsi, K., Conte, D., Jr., Toyofuku, W., and Fox, M. P. (1995) *Oncogene* **11,** 941–950
26. Fallon, L., Bélanger, C. M., Corera, A. T., Kontogiannea, M., Regan-Klapisz, E., Moreau, F., Voortman, J., Haber, M., Rouleau, G., Thorarinsdottir, T., Brice, A., van Bergen, En Henegouwen, P. M., and Fon, E. A. (2006) *Nat. Cell Biol.* **8,** 834–842
27. Nagasawa, M., Gelfand, E. W., and Lucas, J. J. (2001) *Oncogene* **20,** 2889–2899
28. de la Iglesia, N., Konopka, G., Lim, K. L., Nutt, C. L., Bromberg, J. F., Frank, D. A., Mischel, P. S., Louis, D. N., and Bonni, A. (2008) *J. Neurosci.* **28,** 5870–5878