Pescadillo, a Novel Cell Cycle Regulatory Protein Abnormally Expressed in Malignant Cells*

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Using a culture model of glial tumorigenesis, we identified a novel gene that was up-regulated in malignant mouse astrocytes following the loss of p53. The gene represents the murine homologue of pescadillo, an uncharacterized gene that is essential for embryonic development in zebrafish. Pescadillo is a strongly conserved gene containing unique structural motifs such as a BRCA1 C-terminal domain, clusters of acidic amino acids and consensus motifs for post-translational modification by SUMO-1. Pescadillo displayed a distinct spatial and temporal pattern of gene expression during brain development, being detected in neural progenitor cells and postmitotic neurons. Although it is not expressed in differentiated astrocytes in vitro, the pescadillo protein is dramatically elevated in malignant human astrocytomas. Yeast strains harboring temperature-sensitive mutations in the pescadillo gene were arrested in either G₁ or G₂ when grown in nonpermissive conditions, demonstrating that pescadillo is an essential gene in yeast and is required for cell cycle progression. Consistent with the latter finding, DNA synthesis was only observed in mammalian cells expressing the pescadillo protein. These results suggest that pescadillo plays a crucial role in cell proliferation and may be necessary for oncogenic transformation and tumor progression.

Astrocyte-derived neoplasms represent the most common type of primary central nervous system tumor. Recent studies have been directed toward defining the genes and gene products responsible for glial tumorigenesis and progression. Mutation or loss of the tumor suppressor gene p53 is thought to be an important event in the early neoplastic transformation of astrocytes (1, 2). Studies of human glial tumors have demonstrated that loss of the p53 gene is a frequent event in all grades of astrocytoma. A subpopulation of cells will then proceed to either mutate or delete the remaining copy of the p53 gene, leading to uncontrolled cellular proliferation. Of interest are studies suggesting that some high grade astrocytic malignancies result from clonal expansion of cells that have lost or mutated their p53 (3). The genes that may be adversely regulated following p53 alterations and that might contribute to enhanced growth and survival are just now being identified in different biological contexts but not yet in malignant astrocytes (4–6).

We have previously established, using cultured astrocytes from p53-deficient mice, an in vitro model of malignant transformation recapitulating glial tumorigenesis (7). Early passage p53⁻/⁻ astrocytes achieved higher saturation densities than p53⁺/⁺ and p53⁻/⁺ cells but did not exhibit evidence of neoplastic transformation. With continuedpassaging, p53⁻/⁻ astrocytes exhibit a multistep progression to a transformed phenotype displaying significant aneuploidy and acquiring the ability to form large, well vascularized tumors in nude mice. In marked contrast, p53⁻/⁺ astrocytes fail to show a transformed phenotype and senesce after 7–10 passages. Thus, loss of wild type p53 function promotes genomic instability, accelerated growth, and malignant transformation in astrocytes. Using this model system in conjunction with cDNA microarray technology, we sought to examine the relationship between the progressive phenotypic changes in astrocytes and alterations in gene expression.

Glass-based high density microarray hybridization is an efficient way to establish a detailed expression profile of up to 10,000 genes simultaneously from a single tissue or cell type and has been successfully used to analyze tumor- or tissue-specific patterns of gene expression (8, 9). We have utilized cDNA arrays to identify genes that are differentially expressed between malignant late passage p53⁻/⁻ astrocytes and nontransformed early passage p53⁺/⁺ and p53⁻/⁺ astrocytes. One expressed sequence tag that was up-regulated in malignant p53⁻/⁻ astrocytes represented the murine homologue of a novel zebrafish gene known as pescadillo (10).

The pescadillo gene was initially identified through an embryonic mutation in zebrafish, which resulted in animals bearing small eyes, impaired brain growth, and aberrant development of the liver and gut. Pescadillo is widely and highly expressed during the first 3 days of zebrafish development but

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was not observed in any adult tissues except for the ovary, suggesting that its expression is principally limited to developing tissues. In the present study, we present an analysis of the pescadillo protein and provide evidence that pescadillo is a nuclear cell cycle regulatory protein that is abnormally expressed in malignant astrocytomas and other transformed cell types.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—COS-7, HeLa, and SW480 colon carcinoma cells were obtained from the American Type Culture Collection. SNB-19 human glioblastoma cells (11) and p53+/+, p53−/−, and p53−/− astrocytes were prepared and maintained as described previously (7). Normal human mammary epithelial cells and MCF-7 human breast carcinoma cells were generously provided by Dr. Karen Swishelm (Department of Pathology, University of Washington). A primary culture of human glioblastoma cells was established from a primary glioblastoma resected at the University of Washington by previously described methods (12). The procedure for obtaining and culturing human tumor tissue received human subjects approval from the Institutional Review Board Committee of the University of Washington, and informed consent was obtained from the patient prior to surgery. All cells were routinely maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium, except for HeLa cells, which were maintained in Dulbecco’s modified Eagle’s medium, with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 37 °C in 5% CO2.

**Vector Construction and Cell Transfection**—Expression constructs encoding pescadillo fused to the enhanced green fluorescent protein (GFP) or to a Myc epitope were created using a full-length human pescadillo cDNA (derived from a Soares human fetal brain cDNA library obtained from Research Genetics) inserted into the vector pEGFP-C3 (CLONTECH) (13) or a Myc-tagged expression vector driven by the cytosine-aphenopyrimidine promoter (C2-Myc tag) (14), respectively. DNA transfections were performed using a calcium phosphate precipitation method (15) as described previously (16). Two days after transfection, cells were fixed with 4% paraformaldehyde and analyzed using fluorescence microscopy, or cells were processed for immunoprecipitation.

**Generation of an Anti-pescadillo Antibody**—A rabbit polyclonal antibody was generated against a peptide derived from the C-terminal 23 amino acids of the human pescadillo protein and used after affinity purification. Custom antibody production service was obtained from Quality Controlled Biochemicals (Hopkington, MA).

**Western Blotting and Immunoprecipitation**—Protein extracts were prepared from tumor and nonmalignant brain tissue samples as described previously (17). Cultured cells at confluency were lysed in an extraction buffer as described previously (18). Human glioblastoma and anaplastic astrocytoma surgical specimens, as well as brain tissue surrounding the primary tumors (marginal tissue) were generously provided by Dr. John Silber from the Department of Neurological Surgery’s Neuro-Oncology Research Laboratory obtained from Research Genetics) inserted into the vector pEGFP-C3 (CLONTECH) (13) or a Myc-tagged expression vector driven by the cytosine-aphenopyrimidine promoter (C2-Myc tag) (14), respectively. DNA transfections were performed using a calcium phosphate precipitation method (15) as described previously (16). Two days after transfection, cells were fixed with 4% paraformaldehyde and analyzed using fluorescence microscopy, or cells were processed for immunoprecipitation.

**Characterization of the Pescadillo Protein**—p53+/− astrocytes maintained in culture for 20 or more passages display the ability to form colonies in soft agar and form large, well-vascularized tissues. In the present study, we present an analysis of the pescadillo protein and provide evidence that pescadillo is a nuclear cell cycle regulatory protein that is abnormally expressed in malignant astrocytomas and other transformed cell types.

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larized tumors in nude mice, in contrast to p53<sup>+/−</sup> astrocytes and early passage p53<sup>−/−</sup> and p53<sup>+/−</sup> astrocytes (7). We attempted to characterize changes in gene expression that were associated with these markedly different behavioral phenotypes using glass-based high density cDNA arrays. We observed a reproducible series of genes whose expression pattern was altered as a result of continuous proliferation in the absence of p53. One cDNA in particular was expressed at higher levels in late passage p53<sup>−/−</sup> astrocytes relative to early passage p53<sup>−/−</sup> or p53<sup>+/−</sup> astrocytes and matched the sequence of an uncharacterized gene referred to as pescadillo (10). The pescadillo gene was selected for further study in malignant astrocytes because its expression was largely restricted to periods of embryonic development, and it was shown to be essential for nervous system development in zebrafish.

The human pescadillo gene is well conserved among a variety of species and shares 40 and 79% sequence homology with yeast and zebrafish pescadillo homologues, respectively (Fig. 1A). The human, zebrafish, and yeast pescadillo proteins range in size from 582 to 605 amino acids, and each contains several unique structural motifs including a BRCA1 C-terminal (BRCT) domain, two extensive acidic amino acid clusters (glutamic and aspartic acid) in the C terminus, numerous presump- tive tyrosine/serine/threonine phosphorylation sites, and several conserved consensus sequences for covalent attachment of SUMO-1 (small ubiquitin modification) (24) (Fig. 1A). All three pescadillo homologues contain putative nuclear localization signals, suggesting that pescadillo may function in the nucleus.

The subcellular distribution of the pescadillo protein was evaluated by immunostaining and by expressing a Myc or GFP fusion construct. Endogenous pescadillo detected by an affinity purified polyclonal antibody raised against a C-terminal peptide was typically observed as highly localized, punctate immunofluorescence in the nucleus with faint nucleoplasmic staining (Figs. 1B, panel A, and 4). This punctate immunofluorescence appeared to be associated with nucleoli when viewed under phase contrast optics, which was confirmed by double labeling with an anti-nucleolin antibody (Fig. 1B, panels B and C). The distribution of endogenous pescadillo protein was consistent with the pattern obtained after expressing tagged forms of pescadillo, GFP-pescadillo (Fig. 1B, panel D) and Myc-pescadillo (Fig. 1B, panel E). This punctate nuclear distribution was reproducibly observed in every cell type transfected with the GFP-pescadillo construct including human glioblastoma cells, HeLa cells, normal human diploid fibroblasts, and p53<sup>−/−</sup> mouse astrocytes (data not shown). These results confirm the specificity of the anti-pescadillo antibody as well as the normal distribution of the epitope-tagged forms of pescadillo. Interestingly, postmitotic primary cortical neurons in culture displayed a different distribution pattern than that observed in the proliferating cell types; the distribution was significantly more diffuse throughout the nucleus (data not shown). These results demonstrate that pescadillo is a nuclear protein whose compartmentalization within the nucleus may be regulated in a cell type-dependent manner.

The predicted molecular mass for the human pescadillo protein is 69.2 kDa. Immunoblots of extracts prepared from COS-7 cells transfected with human Myc-pescadillo and subjected to immunoprecipitation using an anti-Myc antibody displayed two bands corresponding to molecular masses of ~72 and 94 kDa (Fig. 2A). Both bands were absent in extracts prepared from cells transfected with the empty Myc-tagged vector. The 94-kDa protein (pes<sup>+</sup>) was a more prominent band and was also observed in total cell extracts not subjected to immunoprecipitation. The 72-kDa band corresponded to the predicted size of the full-length pescadillo protein plus the Myc epitopes contained in the expression construct. We hypothesized that the 94-kDa band represented a post-translationally modified form of pescadillo because it was only observed in Myc-pescadillo transfected cells. We directly tested this possibility in the following study.

The pescadillo protein sequence was evaluated for the presence of various post-translational modification motifs. We observed the presence of several potential consensus sequences ([I/L/K/K]XX[K/E/R]) for the covalent modification by the ubiquitin-like protein, SUMO-1 (24). The most highly conserved site was found at position 516-521 (LKLEDK). The size of the 94-kDa band was consistent with the covalent attachment of the SUMO-1 protein, which has been shown to increase the apparent molecular mass of proteins by ~20 kDa on SDS-PAGE (25). To test for the presence of the SUMO-1 modification, immunoblots were performed using extracts prepared from COS-7 cells transfected with Myc-pescadillo and subjected to immunoprecipitation using an anti-SUMO-1 antibody. The blot was subsequently probed with an anti-Myc antibody, and only the 94-kDa band was detected (Fig. 2B). Identical results were obtained when Myc-pescadillo was immunoprecipitated with the anti-Myc antibody, and the blot was probed with the anti-SUMO-1 antibody (data not shown). In contrast to the results obtained from pescadillo transfection studies, the unmodified band (70 kDa) represented the major endogenous form of pescadillo protein in a human glioblastoma cell line, COS-7 cells and in late passage p53<sup>−/−</sup> mouse astrocytes (Fig. 2C). A band migrating at about 92 kDa, consistent with the presence of SUMO-1, was detected in these cell lines if a film was overexposed (data not shown). These results confirm the presence and molecular mass of the pescadillo translation product and demonstrate that under some circumstances the pescadillo protein may be modified by the covalent attachment of SUMO-1.

**Pescadillo Expression Is Spatially and Temporally Regulated in the Brain**—The cellular distribution and the temporal pattern of pescadillo expression were evaluated in the mammalian brain to determine which cell types in the brain normally express pescadillo. Using *in situ* hybridization histochemistry, the pescadillo mRNA was found to be widely and highly expressed throughout the developing mouse brain and spinal cord at embryonic day 13, consistent with its expression in neural progenitor cells and developing neurons (Fig. 3A). No hybridization was detected with the sense probe or in tissue sections pretreated with RNase A (data not shown). At the day of birth (P0; Fig. 3, B and C), hybridization was present in the germinai zones and developing neuronal fields, with relatively intense hybridization detected in the developing hippocampus, piriform, and entorhinal cortices. No hybridization signal was present within developing white matter tracts, such as the corpus callosum (Fig. 3B). Throughout postnatal development, the general pattern of distribution did not appear to change (data not shown). In the adult, labeling was detected in many, if not all, neurons of the brain, but with some regional specificity. The most intense hybridization was present in allocortical regions such as the hippocampus (Fig. 3, E-G), especially in CA3 pyramidal neurons. Pescadillo mRNA was also present within the rostral portions of the germinai zone lining the lateral ventricles (Fig. 3D). This area is the site of continuing neurogenesis in the adult brain. Little or no hybridization signal was present within white matter in the adult (Fig. 3I), suggesting that normal glial cells do not express pescadillo.

**Pescadillo Protein Expression Is Elevated in Malignant Astrocytes**—The *in situ* hybridization studies described above demonstrate that pescadillo is not normally expressed by differentiated astrocytes *in vivo*. Therefore, to confirm that pes-
FIG. 1. Sequence conservation among pescadillo homologues and the subcellular distribution of the pescadillo protein. A, schematic depicting the structural conservation among human, zebrafish, and yeast pescadillo proteins. Identical amino acids are shaded dark gray, and similar amino acids are shaded light gray. This schematic illustrates the location of several important structural motifs including the BRCT domain, the two acidic domains, the presumptive nuclear localization signals, and the most conserved site for SUMOylation. The human pescadillo sequence was obtained from the GenBank™ data base and analyzed for structural and functional motifs using Pfam, a data base of multiple alignments of protein domains and conserved protein regions (33). The asterisks denote the location of the point mutations in the BRCT domain of the yph1–24 and yph1–45 mutant yeast strains described in Fig. 7. B, subcellular localization of pescadillo. Panels A–C, expression of endogenous pescadillo and nucleolin in HeLa cells. Pescadillo immunoreactivity was detected predominantly in the nucleolus as confirmed by colocalization with nucleolin, a nucleolar marker, with very faint nucleoplasmic staining seen in some cells. Essentially no staining above background was observed in the cytoplasm. Panel D, confocal microscopic image of a COS-7 cell transfected with a GFP-pescadillo fusion construct 2 days after transfection. Note that there is no cytoplasmic fluorescence observed. Panel E, malignant, late passage p53/f2 mouse astrocytes (passage 40) transfected with a Myc-tagged pescadillo construct and processed for Myc immunoreactivity 2 days later. Bar, 50 μm (except for D, where the bar indicates 18 μm).
cadillo is abnormally expressed in malignant astrocytes as suggested from our cDNA microarray results, we compared the expression of the pescadillo protein in normal and transformed mouse astrocytes in culture. Low levels of pescadillo immunoreactivity were detectable in primary cultures of p53-/- (Fig. 4, A and C) and p53-/- astrocytes (data not shown). As previously reported, p53-/- astrocytes and early passage p53-/- astrocytes display a normal pattern of contact-inhibited growth and fail to form tumors in nude mice (7). Pescadillo immunoreactivity in primary cultures of p53-/- astrocytes was localized to the nucleus and in the same punctate pattern that was observed following transfection with GFP-pescadillo. Also, pescadillo immunoreactivity remained constant in these cultures independent of cell density or length of time in culture (up to 3 weeks; data not shown). In marked contrast, pescadillo immunoreactivity was significantly elevated in malignant, late passage p53-/- mouse astrocytes (Fig. 4, B and D), and the subcellular distribution remained the same in malignant astrocytes. Significant up-regulation of pescadillo protein expression was also observed in MCF-7 human breast carcinoma cells (Fig. 4, F and H) compared with normal human mammary epithelial cells (Fig. 4, E and G). Additionally, significant levels of pescadillo immunoreactivity were detected in a variety of human cancer cell lines including glioblastoma, colon adenocarcinoma (Fig. 4), and cervical carcinoma cells (HeLa cells, Figs. 1 and 6).

To demonstrate that pescadillo expression was not elevated in established cell lines simply because of long term growth in culture, we evaluated pescadillo expression in a primary culture of human glioblastoma cells that was directly dissociated out of a surgically resected tumor specimen without being passaged. The primary glioblastoma culture expressed the same intense nuclear pescadillo immunoreactivity (Fig. 4J) that was seen in the established cell lines (Fig. 4, I, K, and L). This finding indicated that pescadillo expression may be elevated in malignant astrocytes in vivo. We evaluated this possibility by comparing the levels of pescadillo protein in matched tissue pairs resected from individual patients consisting of malignant brain tumor tissue and the brain tissue surrounding the tumor (margin). Pescadillo was also evaluated in nonmalignant brain tissue obtained from patients without neoplastic disease. Immunoblotting analyses demonstrated that the pescadillo protein was highly expressed in malignant human glial tumors including four glioblastomas and one anaplastic astrocytoma (Fig. 5). When pescadillo expression levels were normalized against actin expression levels, the malignant tumors exhibited up to a 12-fold increase in pescadillo expression relative to the matching margin tissue. Variations in pescadillo expression between tumor-margin pairs may reflect the fact that margin tissue is generally composed of normal glial cells and variable degrees of infiltrative tumor cells. These findings clearly demonstrate that the pescadillo protein is abnormally elevated in malignant human tumors of astrocytic origin.

Pescadillo Regulates Cell Cycle Progression—The BRCT (BRCA1 C terminus) motif has been identified in a superfamily of proteins involved in various aspects of DNA repair, recombination, and checkpoint control (26, 27). Having identified a BRCT domain in the pescadillo protein, we determined whether pescadillo was associated with cell cycle progression.

Preliminary observations indicated that pescadillo expression was suppressed when cells were maintained beyond confluence as shown for HeLa cells (Fig. 6, A and B), which was
that exceeded the original density (Fig. 6), the cells exhibited
of the cells had incorporated BrdU (Fig. 6) by 2.5 h after
re-expressed pescadillo immunoreactivity (Fig. 6).

When plated at a low density (Fig. 6) with concomitant robust
area re-expressed pescadillo (Fig. 6). Alternatively, when a
strip of cells was scraped away from the confluent HeLa cell
monolayer (Fig. 6J), cells that migrated out into the denuded
area re-expressed pescadillo (Fig. 6K) with concomitant robust
BrdU labeling (Fig. 6L). These results demonstrate that BrdU
labeling is only observed in cells expressing pescadillo immu-
noreactivity, suggesting that pescadillo may be required for
progression through the cell cycle.

Direct evidence that pescadillo is necessary for cell cycle
progression was obtained by analyzing the yeast pescadillo
homologue (YPH1). The human pescadillo protein shares sig-
nificant similarities with the yeast Saccharomyces cerevisiae
homologue, including the BRCT domain and putative SUMOy-
lation sites (Fig. 1A). In addition, the yeast homologue protein
localizes to the nucleus (data not shown), suggesting that hu-
man pescadillo may subserve the same function as its yeast
counterpart.

Deletion of the YPH1 open reading frame in a yeast diploid
strain and tetrad dissection demonstrated that YPH1 is an
essential gene. To gain more insight into the function of YPH1,
two temperature-sensitive mutant alleles of YPH1 were gener-
ated by PCR-based mutagenesis and integrated into the ge-
ome. At a permissive temperature (25 °C) the two yeast mu-
tant strains were able to grow on YPD plates (Fig. 7A). Cell
cycle progression of the two mutant strains was assessed by
monitoring their DNA content by FACS and cell morphology
by bright field microscopy. At 25 °C both wild type and mutant
yeast strains exhibited the same FACS profile, whereas at
37 °C yph1–24 (Fig. 7B) showed a G1 phase delay, and yph1–45
(Fig. 7B) showed a G2 phase delay. Cell morphology analysis
confirmed the FACS results obtained at the nonpermissive
temperature, showing that 70% of yph1–24 cells accumulated
in the G1 phase, whereas 43% of yph1–45 cells had a large bud
with the nucleus at the neck and a short spindle corresponding
to a delay in G2/M transition (Fig. 7C). Sequence analysis
indicated that both mutant strains contained a single point
mutation in the BRCT domain (position 435 for yph1–24, posi-
tion 360 for yph1–45) as well as two and one additional muta-
tion, respectively, outside of the BRCT domain. Collectively,
this study confirms that pescadillo is necessary for cell cycle
progression and further suggests that the BRCT domain is
essential for pescadillo activity.

DISCUSSION

Loss of the tumor suppressor gene p53 has been implicated
in the genesis of many human malignancies. We attempted to
define key transcriptional changes accompanying neoplastic
transformation in an in vitro mouse model of astrocyte tumo-
genesis (7). During this process we discovered that the murine
homologue of pescadillo was highly expressed in malignant
mouse astrocytes. The initial characterization of this novel
protein demonstrated: 1) that pescadillo expression in the
mouse brain is normally restricted to neural progenitor cells
and postmitotic neurons but is activated in malignant mouse
astrocytes in vitro following growth in the absence of p53; 2)
that pescadillo expression is significantly elevated in malig-
nant human astrocytomas in vivo; 3) that the loss of p53 is
necessary but not sufficient by itself to up-regulate pescadillo
expression in astrocytes; and 4) that pescadillo is a nuclear
protein necessary for cell cycle progression.

Pescadillo Protein Is Up-regulated in Malignant Cells—Our
results demonstrate that a novel regulator of embryonic de-
velopment in zebrafish, pescadillo, is aberrantly expressed in a
variety of malignant mammalian cells. Our results specifically
demonstrate that pescadillo protein expression is elevated in
malignant murine and malignant human astrocytes in vitro.
Moreover, the pescadillo protein is expressed at significantly
higher levels in malignant human brain tumors relative to the
adjacent tissue margins taken from the same patient and “nor-
mal” brain tissue obtained from patients without neoplastic
disease. The tissue surrounding a brain tumor is primarily

associated with reduced proliferation as measured by BrdU
labeling (Fig. 6C). To characterize the relationship between cell
density and pescadillo expression, HeLa cells grown to a very
high density (Fig. 6, A and B) were replated at different den-
sities. When plated at a low density (Fig. 6D), 100% of the cells
re-expressed pescadillo immunoreactivity (Fig. 6E), and ~20% of
the cells had incorporated BrdU (Fig. 6F) by 2.5 h after
plating. In contrast, when the cells were plated at a density
that exceeded the original density (Fig. 6G), the cells exhibited
a further reduction in pescadillo immunoreactivity (Fig. 6H)
and failed to incorporate BrdU (Fig. 6I). Alternatively, when a
strip of cells was scraped away from the confluent HeLa cell
monolayer (Fig. 6J), cells that migrated out into the denuded
area re-expressed pescadillo (Fig. 6K) with concomitant robust

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composed of normal cells with variable degrees of infiltrative tumor cells and inflammatory cells, suggesting that increased pescadillo levels in the tumor extracts reflect expression by malignant human mammary epithelial cells. Primary culture (7 days in culture) of p53−/− astrocytes (A and C) expressed a low level of pescadillo protein in contrast to late passage (passage 27), malignant p53−/− astrocytes (B and D), which showed significant up-regulation of pescadillo expression. Similarly, only weak immunoreactivity was seen in a normal human mammary epithelial cell line (E and G), whereas robust immunostaining was detected in the MCF-7 human breast carcinoma cell line (F and H). Thus, elevated pescadillo expression is associated with malignant transformation. I–L, pescadillo expression in established and malignant cell lines and in a primary culture of human glioblastoma. Note the strong nuclear staining in all types of cell lines. The insets in the upper right corner of F and H depict cells stained with normal IgG (0.5 μg/ml) in place of the pescadillo antibody. Bar, 50 μm.

**Fig. 4.** Pescadillo immunoreactivity is elevated in malignant cell lines. A–D, normal and malignant p53−/− astrocytes. E–H, normal and malignant human mammary epithelial cells. Primary culture (7 days in culture) of p53−/− astrocytes (A and C) expressed a low level of pescadillo protein in contrast to late passage (passage 27), malignant p53−/− astrocytes (B and D), which showed significant up-regulation of pescadillo expression. Similarly, only weak immunoreactivity was seen in a normal human mammary epithelial cell line (E and G), whereas robust immunostaining was detected in the MCF-7 human breast carcinoma cell line (F and H). Thus, elevated pescadillo expression is associated with malignant transformation. I–L, pescadillo expression in established and malignant cell lines and in a primary culture of human glioblastoma. Note the strong nuclear staining in all types of cell lines. The insets in the upper right corner of F and H depict cells stained with normal IgG (0.5 μg/ml) in place of the pescadillo antibody. Bar, 50 μm.

**Fig. 5.** Pescadillo protein is abnormally expressed in adult human glioblastomas. Pescadillo protein levels were compared between tumor tissues (T) and margin tissues (M) surrounding each tumor for glioblastoma multiforme and anaplastic astrocytoma, both of which are highly malignant, as well as nonmalignant temporal lobe cortex that was removed during the resection of a cavernous malformation (Normal temporal cortex). Each tumor-margin pair represents paired samples from the same patient. Protein extracts (20 μg of protein/lane) were resolved by SDS-PAGE on a 10% gel, and the blot was probed with the rabbit anti-pescadillo antibody, followed by probing with an anti-actin antibody to assess protein loading. For the four pairs of samples shown in the left blot, pescadillo expression was normalized against actin bands and compared between tumor and margin. The resulting fold increase in pescadillo expression was, from left to right, 1.3, 6.8, 6.2, and 12.2, respectively. The low but significant level of pescadillo expression in the normal cortex sample is presumed to represent neuronal pescadillo expression as temporal lobe contains numerous neurons that express pescadillo as shown by in situ hybridization in Fig. 3.

Pescadillo protein was also identified in a diverse array of malignant human cell lines, including glioblastoma, medulloblastoma, and colon carcinoma cell lines. Particularly noteworthy is the significant increase in pescadillo immunoreactivity that was detected in malignant human breast carcinoma cells relative to normal human mammary epithelial cells. Similarly, late passage malignant murine p53−/− astrocytes express much higher levels of pescadillo immunoreactivity than normal p53−/− or nontransformed early passage p53−/− astrocytes.

A. D. Jarell and R. S. Morrison, unpublished results.
Cells before manipulation | Replated at low density | Replated at high density | Scraped

HOECHST

A

B

PESCADILLO

C

D

E

F

G

H

I

J

K

L

BrdU

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z

FIG. 6. Pescadillo expression is associated with replicating cells. HeLa cells were grown to a post-confluent density (2 days after confluency), fixed and processed for nuclear staining (A) with Hoechst 33258 (2.5 μg/ml) and pescadillo immunoreactivity (B). A parallel culture was labeled with BrdU (10 μg/ml) for the next 8 h to assess proliferative activity at this high cell density (C). Confluent sister cultures, as shown in A, were trypsinized and replated either at a low density (1/8th of the original density) or at a higher density (1.3-fold denser than original), and cultured for 2.5 h in the presence of BrdU (20 μg/ml). The cultures were then fixed and processed for nuclear staining (D and G), pescadillo (E and H), and BrdU (F and I) immunoreactivity. In a separate experiment, a HeLa cell culture with a comparable post-confluent density as in A was scraped with a scalpel knife (seen in the phase-contrast image, J, as a horizontal line) and a strip of cells was removed (upper half in J) to create a denuded area in a post-confluent culture. The culture was allowed to recover for 2 days in the presence of BrdU (5 μg/ml), fixed, and processed for pescadillo (K) and BrdU (L) immunoreactivity. Nuclear pescadillo immunoreactivity in high density areas was primarily associated with cells that sit on top of the underlying cell layer. Conversely, there was increased cytoplasmic staining in the cells comprising the dense monolayer, suggesting that pescadillo may be translocated to the cytoplasm. Bar, 50 μm.

cytes. Although the p53 status of these various cell lines is not known, the results obtained with nontransformed early passage p53−/− astrocytes suggest that the loss of p53 alone is not sufficient to activate pescadillo expression.

The human pescadillo gene has been localized to chromosome 22q12.1 (28). Chromosome 22q deletions are common in human astrocytomas, but partial deletions may occur distal to the pescadillo locus involving a common region of deletion at 22q12.3q13.1 (29). Amplification of the human chromosome 22q12 locus has not been reported, suggesting that increased levels of the pescadillo protein in malignant gliomas are not the result of gene amplification. In addition, because the induction of pescadillo mRNA in malignant mouse astrocytes is small relative to increases in pescadillo protein,4 these changes may not directly involve pescadillo gene transcription but rather post-transcriptional mechanisms that influence pescadillo translation or protein stability. Irrespective of the mechanism, pescadillo induction in malignant cells is not restricted to cultured tumor cells as demonstrated by its elevated expression in malignant human glial tumors. These findings imply that pescadillo may subserve a function that is essential for maintaining the malignant phenotype.

Elucidating the Function of the Pescadillo Protein—The biochemical function performed by pescadillo has not been defined, but insights into its cellular role can be inferred from examining structural motifs contained within the pescadillo protein sequence. The most conspicuous identifiable domain in pescadillo is a protein motif originally identified in the breast1 and ovarian cancer gene BRCA1 (26, 27). A unifying theme that has evolved from studying the ~40 proteins that contain a BRCT domain is that many of these proteins participate in DNA damage-responsive checkpoints (26, 27).

Several conserved consensus sequences for the covalent attachment of SUMO-1 were also identified in pescadillo, and SUMO-1 modification was demonstrated in the present study. Although the consequences associated with SUMO-1 modification are largely unknown, there is evidence that SUMO-1 may be important for protein targeting and protein-protein interactions. For example, targeting of the RanGAP1 protein to the nuclear pore is dependent upon the SUMO-1 modification (25). The SUMO-1 moiety appears to interact with important regulatory proteins such as the death domain of the Fas/APO-1 and tumor necrosis factor receptors (30). Overexpression of SUMO-1 confers protection against both anti-Fas/APO-1 and tumor necrosis factor-induced cell death, suggesting that SUMO-1 binding suppresses the activity of these receptors (30). The SUMO-1 protein has also been shown to associate in a complex together with human RAD52, RAD51, p53, and a ubiquitin-conjugating enzyme, UBE2I (31). Such a complex is thought to be involved in DNA recombination and repair of double-strand breaks. These results illustrate that the SUMO-1 moiety and, by inference, proteins covalently modified by SUMO-1 can interact in protein complexes that regulate cellular processes essential for cell proliferation and survival. In support of this hypothesis, it has recently been shown that SUMO conjugation is essential for viability in S. cerevisiae and is required for entry into mitosis (32).

The convergence of function observed between proteins expressing a BRCT domain and proteins modified by SUMO-1 is

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4 J. Schuster, G. Foltz, P. S. Nelson, and R. S. Morrison, unpublished results.
consistent with the possibility that pescadillo is involved in the regulation of a cell cycle checkpoint. The contribution of pescadillo to the regulation of cell growth was further substantiated by the identification and characterization of temperature-sensitive pescadillo mutant yeast strains. Yeast expressing mutant pescadillo displayed growth arrest in the G1 or G2 phase of the cell cycle when shifted to a nonpermissive temperature. Interestingly, both mutant yeast strains contained a single point mutation in the BRCT domain, supporting the importance of this motif to pescadillo function. It is not presently clear why the two mutant strains arrest in different phases of the cell cycle. The yph1–24 mutant strain, which arrests in G1, contains an additional mutation at amino acid 337 (E337G) close to the start site of the BRCT domain. This additional mutation near the BRCT domain may further modify pescadillo binding affinity or specificity, thus altering its activity. The results obtained with the yeast mutants demonstrate that pescadillo is essential for cell cycle progression, providing definitive support for the correlation between pescadillo expression and S phase progression observed in HeLa cells. The absence of such a critical cell cycle gene during embryogenesis would account for the tissue abnormalities observed in zebrafish lacking a functional pescadillo gene (10).

The results of the present study demonstrate that pescadillo, a novel gene expressed principally in developing tissues, is inappropriately expressed in adult human malignant brain tumors. Pescadillo is also up-regulated in several different carcinoma cell lines, suggesting that abnormalities in pescadillo expression may be a common feature of malignancy. Interestingly, overexpressing pescadillo in nontransformed cell lines, such as NIH-3T3 cells, promoted cell death instead of inducing colony forming ability or serum-independent growth, suggesting that pescadillo is not directly oncogetic in nontransformed cells but rather may be necessary for integrating the complex array of signals that promote progression through the cell cycle. In contrast to nontransformed cells, pescadillo overexpression did not affect the viability or growth of malignant cell lines, which constitutively express high levels of the pescadillo protein. Thus, the precise function of pescadillo in cell cycle progression appears complex and requires further study. Nevertheless, elucidation of pescadillo function may afford unique insights into the process of neoplastic transformation and could provide a new target for suppressing malignancy.

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Fig. 7. The yeast pescadillo homologue is essential for growth and regulates cell cycle checkpoints. A, the wild type and the two YPH1 temperature-sensitive mutant strains (yph1–24; yph1–45) were streaked on YPD plates, placed at 25 °C or 37 °C for 3 days, and photographed. B and C, yeast strains expressing temperature-sensitive mutant pescadillo homologues exhibit cell cycle arrest at the nonpermissive temperature. Yph1–24 and yph1–45 cells growing in logarithmic phase at 25 °C were split and incubated at either 25 or 37 °C for 3 h and then subjected to DNA content analysis by fluorescence-activated cell sorting (B). Cells that were split and incubated at 37 °C were further analyzed for growth arrest characteristics based on morphological hallmarks as revealed by nuclear and tubulin staining (C).
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Pescadillo, a Novel Cell Cycle Regulatory Protein Abnormally Expressed in Malignant Cells

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