p63 Expression Profiles in Human Normal and Tumor Tissues[1]

Charles J. Di Como,[2] Marshall J. Urist,[2] Irina Babayan, Marija Drobnjak, Cyrus V. Hedvat, Julie Teruya-Feldstein, Kamal Pohar, Axel Hoos, and Carlos Cordon-Cardo[3]

Division of Molecular Pathology [C. J. D., M. J. U., I. B., M. D., C. C-C.], Departments of Pathology [J. T-F.], Urology [K. P.], and Surgery [A. H.], and Laboratory of Molecular Aspects of Hematopoiesis [C. V. H.], Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

Purpose: **The *p63* gene, located on chromosome 3q27-28, is a member of the *p53* gene family. The product encoded by the *p63* gene has been reported to be essential for normal development.**

Experimental Design: **In this study, we examined the expression pattern of p63 in human normal and tumor tissues by immunohistochemistry using a monoclonal antibody (clone 4A4) that recognizes all p63 splice variants, and by reverse transcription-PCR using isoform-specific primers.**

Results: **We found that p63 expression was restricted to the nucleus, with a nucleoplasmic pattern. We also observed that the expression was restricted to epithelial cells of stratified epithelia, such as skin, esophagus, exocervix, tonsil, and bladder, and to certain subpopulations of basal cells in glandular structures of prostate and breast, as well as in bronchi. Consistent with the phenotype observed in normal tissues, we found that p63 is expressed predominantly in basal cell and squamous cell carcinomas, as well as transitional cell carcinomas, but not in adenocarcinomas, including those of breast and prostate. Interestingly, thymomas expressed high levels of p63. Moreover, a subset of non-Hodgkin's lymphoma was also found to express p63. Using isoform-specific reverse transcription-PCR, we found that thymomas express all isoforms of p63, whereas the non-Hodgkin's lymphoma tended to express the transactivation-competent isoforms. We did not detect p63 expression in a variety of endocrine tumors, germ cell neoplasms, or melanomas. Additionally, soft tissue sarcomas were also found to have undetectable p63 levels.**

Conclusions: **Our data support a role for p63 in squamous and transitional cell carcinomas, as well as certain lymphomas and thymomas.**

INTRODUCTION

The recent discovery of two *p53*-related genes, *p63* and *p73*, has revealed an additional level of complexity to the study of p53 function (Ref. 1 and references therein). Both genes encode multiple proteins arising from alternative promoter usage and splicing, with transactivation, DNA-binding, and tetramerization domains. Whereas some isoforms of p63 (TAp63) and p73 (TAp73) are capable of transactivating p53 target genes and inducing apoptosis, other isoforms (ΔNp63 and ΔNp73) act in a dominant-negative fashion to counteract the transactivation-competent isoforms of not only p63 and p73, but p53 as well.

Data from p63-deficient mice have made it apparent that p63 plays a key role in regulating epithelial proliferation and differentiation programs (2, 3). It has been shown that absence of p63 leads to defective epidermal differentiation, as well as agenesis of mammary glands, lacrimal glands, and the prostate (2, 3). Among the various isoforms, normal human keratinocytes express mainly the truncated dominant-negative p63 isoforms, rather than isoforms with the NH_2-terminal transactivation domain (4). It has been reported that these ΔNp63-encoding transcripts are down-regulated during the irreversible growth arrest and differentiation of human keratinocytes. Similarly, ΔNp63α is the predominant isoform expressed in the basal cells of the normal human prostate (5).

SCCs,[4] but not adenocarcinomas, frequently show genomic amplification at 3q (6, 7). *p63* maps to 3q27-28, which may suggest that increased copy number contributes to its dysregulation. To address this issue, a number of studies have examined the expression levels of p63 in SCCs derived from lung, head and neck, and skin (4, 8, 9). One group has found that SCCs of the lung and head and neck display increased ΔNp63 mRNA levels with increased *p63* gene copy number (9). Others have found that SCC of the head and neck expresses both the transactivating (TA) and the dominant-negative (ΔN) p63 isoforms (4, 10, 11). In addition, p63 appears to play a role in cervical cancer (12, 13).

Data on p63 expression are limited mainly to Northern blots, thus lacking microanatomical details. Thus, we undertook the present study with the goal of expanding and consolidating the expression profile of p63 in human normal and tumor

Received 8/6/01; revised 10/2/01; accepted 11/6/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Special Fellowship 3956-01 from The Leukemia and Lymphoma Society (to C. J. D.), The Norman and Rosita Winston Foundation (to C. V. H.), and Grant AFUD MD0010 from the American Urological Association (to K. P.). This work also was supported in part by NIH Grants CA-87497, CA-47179, and DK-47650 (to C. C-C.).

[2] Both authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Division of Molecular Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021. Phone: (212) 639-7746; Fax: (212) 794-3186; E-mail: cordon-c@mskcc.org.

[4] The abbreviations used are: SCC, squamous cell carcinoma; IHC, immunohistochemistry; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FL, follicular lymphoma; DLCL, diffuse large B-cell lymphoma; EEC, ectrodactyly, ectodermal dysplasia, and facial cleft; NHL, non-Hodgkin's lymphoma.

tissues, using IHC. This study was aided by the use of tissue microarrays, which allow high-throughput screening of a large number and variety of neoplasms. In cases where we identified novel tumor types expressing p63, we used isoform-specific primers to determine the variant distribution.

MATERIALS AND METHODS

Antibodies. The mouse antihuman monoclonal antibody 4A4, raised against amino acids 1–205 mapping at the NH_2 terminus of ΔNp63, was used (sc-8431; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The mouse antihuman monoclonal antibody AE1/AE3, a pan-specific cocktail of antibodies for human cytokeratins, was used (M3515; DAKO, Corp., Carpinteria, CA).

Tissues. A broad spectrum of normal, benign, and malignant human tissues were studied for their expression of p63: 28 normal tissues from different organ systems (Table 1); and 68 benign and malignant tissues from 28 different organ systems (Table 2). To better understand the changes in p63 expression patterns observed during tumorigenesis, matched pairs of normal and tumor tissues of the same tissue type were analyzed.

Tissue Processing, Microarray Construction, and IHC. Tumor and normal tissues were embedded in paraffin, and 5-μm sections stained with H&E were obtained to identify viable, representative areas of the specimen. From the defined areas, core biopsies were taken with a precision instrument (Beecher Instruments, Silver Spring, MD) as described previously (14). Tissue cores with a diameter of 0.6 mm were punched from each specimen and arrayed in triplicate on a recipient paraffin block (15); 5-μm sections of these tissue array blocks were cut and placed on charged poly-lysine-coated slides. These sections were used for immunohistochemical analysis (16). Tissues and cell lines known to express p63 were used as positive controls.

Sections from tissue arrays were deparaffinized, rehydrated in graded alcohols, and processed using the avidin-biotin immunoperoxidase method. Briefly, sections were submitted to antigen retrieval by microwave oven treatment for 15 min in 10 mM citrate buffer (pH 6.0). Slides were subsequently incubated in 10% normal horse serum for 30 min, followed by overnight incubation at 4°C with appropriately diluted primary antibody. The mouse antihuman monoclonal antibody 4A4 (200 μg/ml) was used at a 1:200 dilution to a final concentration of 1 μg/ml. Samples were then incubated with biotinylated antimouse immunoglobulins at 1:500 dilution for 30 min (Vector Laboratories, Inc., Burlingame, CA), followed by avidin-biotin peroxidase complexes (1:25; Vector Laboratories) for 30 min. Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain.

Immunoreactivities were classified as continuum data [undetectable levels (0%) to homogeneous staining (100%)] for the marker. Slides were reviewed by several investigators (C. C-C., C. J. D., M. J. U., and M. D.), and results were scored by estimating the percentage of tumor cells showing characteristic nuclear staining (−, undetectable; ±, occasional; +, moderate; ++, strong).

RNA Isolation and RT-PCR. Total RNA was extracted from tissue samples by TRIZOL reagent (Life Technologies, Inc., Grand Island, NY) according to manufacturer's specifications. Total RNA (1 μg) was then amplified using p63 isoform-specific primers with the Superscript One-Step RT-PCR Kit with Platinum *Taq* (Life Technologies) according to the manufacturer's protocol (50-μl reaction volume). All reverse transcription reactions were carried out for 30 min at 50°C and then 3 min at 94°C, followed by isoform-specific PCR conditions for each primer set: (*a*) TAp63 (nucleotides 3–269 of *TAp63*γ), 40 cycles at 94°C for 30 s, 57°C for 40 s, and 72°C for 30 s, using SKO32 (sense; 5′-GTCCCAGAGCACACAGACAA-3′) and SKO33 (antisense; 5′-GAGGAGCCGTTCTGAATCTG-3′) primers; (*b*) ΔNp63 (nucleotides 10–207 of Δ*Np63*α), 40 cycles at 94°C for 30 s, 57°C for 40 s, and 72°C for 30 s, using SKO26 (sense; 5′-CTGGAAAACAATGCCCAGAC-3′) and SKO27 (antisense; 5′-GGGTGATGGAGAGAGAGCAT-3′) primers; (*c*) p63α-tail (nucleotides 1380–1568 of Δ*Np63*α), 2 cycles at 94°C for 30 s, 57°C for 40 s, and 72°C for 30 s, followed by 38 cycles at 94°C for 30 s, 55°C for 40 s, and 72°C for 30 s, using SKO28 (sense; 5′-GAGGTTGGGCTGTTCATCAT-3′) and SKO29 (antisense; 5′-AGGAGATGAGAAGGGGAGGA-3′) primers; (*d*) p63β-tail (nucleotides 1345–1550 of *TAp63*β), 2

Table 1 Distribution of p63 in normal adult tissues by IHC

Organ site	Score[a]	Positive component
Central nervous system		
Cortex	(−)	
Digestive tract		
Esophagus	(++)	Squamous epithelium
Stomach	(−)	
Small Intestine	(−)	
Colon	(−)	
Glands associated with digestive tract		
Liver	(−)	
Pancreas	(−)	
Endocrine system		
Adrenal	(−)	
Thyroid	(−)	
Lymphoid system		
Lymph node	(+/−)	Cells in germinal center
Spleen	(−)	
Tonsil	(++)	Squamous epithelium
Muscle tissue		
Skeletal striated muscle	(−)	
Reproductive system (female)		
Breast	(++)	Basal epithelium
Exocervix	(++)	Squamous epithelium
Endometrium	(−)	
Ovary	(−)	
Placenta	(+)	Cytotrophoblast
Umbilical cord	(−)	
Reproductive system (male)		
Prostate	(++)	Basal epithelium
Testis	(+/−)	Cells in seminiferous tubules
Respiratory system		
Larynx	(++)	Mucosa & glandular epithelium
Lung	(++)	Bronchiolar epithelium
Skin		
Epidermis	(++)	
Hair follicles	(++)	
Sweat glands	(++)	
Urinary system		
Bladder	(++)	Transitional epithelium
Kidney	(+/−)	Epithelial cells of Bowman's capsule

[a] −, undetectable; +/−, occasional; +, moderate; ++, strong.

Table 2 Distribution of p63 in human tumors by IHC ($n = 583$)

Neoplasms	n^a	Positive^b	Neoplasms	n^a	Positive^b
Carcinoma			Endocrine		
Lung primary	9	−	Thyroid		
Lung metastasis	3	−	Follicular	4	−
Colon primary	8	−	Papillary	4	−
Colon metastasis	1	−	Anaplastic	4	−
Stomach	9	−	Medullary	4	−
Pancreatic	4	−	Hurthle	4	−
Breast primary	12	−	Parathyroid		
Breast metastasis	1	−	Carcinoma (3); adenoma (1)	4	−
Ovary primary	15	1	Adrenal		
Ovary metastasis	2	−	Cortical adenoma (4) and carcinoma (4)	8	−
Kidney clear cell	4	−	Pancreas islet cell tumor	4	−
Endometrial	3	1	Pituitary adenoma	4	−
Prostate	74	−	Soft tissue sarcoma		
Bladder transitional cell carcinoma	4	3	Fibrosarcoma	41	−
Lung (SCC)	4	2	Malignant fibrous histiocytoma	25	−
Head and neck (SCC)	4	4	Desmoid	24	−
Cervix (SCC)	4	3	Leiomyosarcoma	7	−
Skin basal cell carcinoma	4	4	Rhabdomyosarcoma	31	2
Salivary gland carcinoma	4	2	Angiosarcoma	2	−
Benign mixed tumor, parotid	4	4	Liposarcoma	10	−
Hepatocellular carcinoma	3	−	Giant cell tumor	3	−
Mesothelioma	4	−	Neural crest derivatives		
Germ cell tumors			Pheochromocytoma	3	−
Seminoma testis	4	−	Melanoma	15	−
Embryonal carcinoma testis	4	−	Lymphoid		
Yolk sac tumor, testis	4	1	Thymoma	13	13
Teratoma			Non-Hodgkin's B-cell lymphoma		
Mature teratoma, testis	4	3	Anaplastic large cell	2	1
Immature teratoma, testis	4	3	Chronic lymphocytic/small lymphocytic	13	3
Leydig cell tumor	4	−	Diffuse large cell	27	9
Choriocarcinoma			Follicular (grade 1) small cell	12	−
Gestational/nongestational	8	−	Follicular (grade 2) mixed small & large cell	13	4
Implantation site trophoblastic tumor	2	−	Follicular (grade 3) large cell	11	4
Dysgerminoma ovary	4	−	Mantle cell	5	−
Sertoli-Leydig cell tumor	4	−	Marginal zone	4	1
Immature teratoma, ovary	4	2	Hodgkin's lymphoma		
			Nodular lymphocytic predominant	6	1
Malignant peripheral nerve sheath	18	4	Mixed Cellularity	7	−
			Nodular Sclerosis	21	−

[a] *n*, Number examined.
[b] Number staining positive for p63: −, undetectable.

cycles at 94°C for 30 s, 57°C for 40 s, and 72°C for 30 s, followed by 38 cycles at 94°C for 30 s, 55°C for 40 s, and 72°C for 30 s, using SKO30 (sense; 5′-AACGCCCTCACTCCTA-CAAC-3′) and SKO31 (antisense; 5′-CAGACTTGCCAGATC-CTGA-3′) primers; (*e*) p63γ-tail (nucleotides 1057–1270 of *TAp63*γ), 2 cycles at 94°C for 30 s, 57°C for 40 s, and 72°C for 30 s; 2 cycles at 94°C for 30 s, 55°C for 40 s, and 72°C for 30 s, and 36 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 30 s, using SKO22 (sense; 5′-ACGAAGATCCCCAGATGATG-3′) and SKO23 (antisense; 5′-GCTCCACAAGCTCATTC-CTG-3′) primers. The *GAPDH* gene was chosen as an endogenous expression RT-PCR standard and was amplified using SKO36 (sense; 5′-GAAGGTGAAGGTCGGAGT-3′) and SKO37 (antisense; 5′-GAAGATGGTGATGGGATTTC-3′) primers. Isoform-specific RT-PCR (including *GAPDH* and a one primer-only control) was performed in triplicate. Twenty-five microliters of RT-PCR products were resolved in 1.8% agarose gels.

RESULTS

In contrast to wild-type p53, where expression is rarely detectable by immunohistochemical methods but is present in all cell types (17), we found a relatively restricted tissue-specific distribution of p63 in normal tissues when we used an anti-p63 monoclonal antibody that detects all p63 isoforms. Table 1 summarizes the patterns of p63 expression in normal tissues, whereas Table 2 summarizes the p63 phenotypes observed in a large collection of primary and metastatic tumors.

Expression of p63 in Stratified and Transitional Epithelia. Stratified squamous epithelia include both keratinized and nonkeratinized mucosae. These epithelial compartments are arranged as discrete cell layers, with a basal layer that includes germinative cells and consecutive layers of differentiating and terminally mature cells (18). We found that all of the stratified epithelia studied, which included skin, tonsil, esophagus, and exocervix, displayed intense p63 nuclear immunoreactivity in

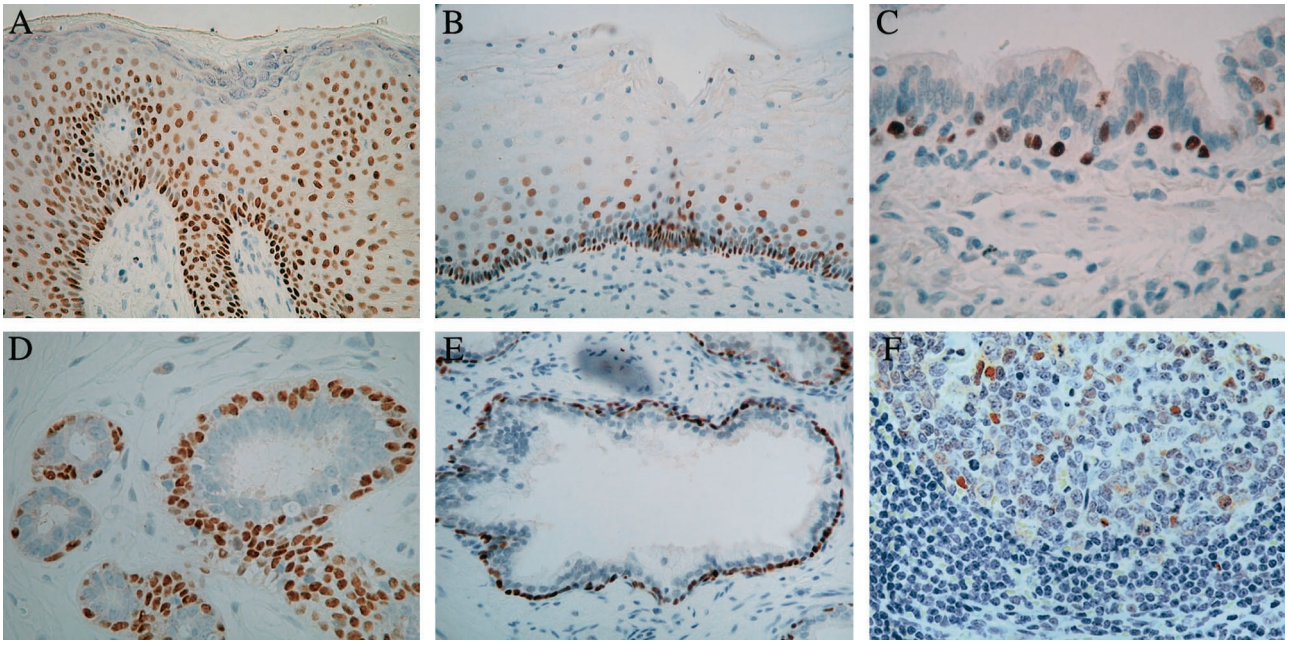

Fig. 1 Representative photomicrographs of immunophenotypes of p63 in normal human tissues, obtained using the anti-p63 4A4 monoclonal antibody. Stratified epithelia, such as the epidermis of the skin (*A*) and the exocervical mucosa (*B*), displayed intense p63 nuclear immunoreactivity in the basal layer with a gradual diminution of nuclear intensity in the more terminally differentiated cell layers. Note that the most apical cell layer(s) lack p63 staining (see *A* and *B*). Epithelial cells of specific tissues and organs, such as bronchium (*C*), breast (*D*), and prostate (*E*), displayed a moderate to intense p63 nuclear staining in basal cells, whereas the luminal cells lining the glandular spaces were unreactive. Occasional cells in germinal centers of lymph nodes (*F*) displayed a weak to moderate p63 nuclear immunostaining. Original magnifications: ×200 for *A*, *B*, and *E*; ×400 for *C*, *D*, and *F*.

the basal layer, with a gradual diminution of staining in the more terminally differentiated cell layers. Moreover, we observed that cells localized to the most superficial layers had undetectable p63 levels (Fig. 1). This pattern of staining had been previously observed for both murine and human tissues (3, 4, 8, 19).

The transitional epithelium is found in the urinary tract, where it lines the calyces of the kidney, the ureters, the bladder, and the urethra. We observed that all cell layers stained intensely, with the exception of the umbrella cells, which had undetectable p63 levels (data not shown).

Expression of p63 in Simple Epithelia. We observed that epithelial cells in specific organs, such as the acini and ducts of the breast and prostate, displayed moderate to intense p63 nuclear staining in basal cells, whereas the luminal cells lining the glandular lumen were unreactive (Fig. 1). Similar to this pattern, we found that various skin adnexa, including sebaceous and sweat glands, also showed intense p63 expression in their basal cell compartments (data not shown). Finally, we also observed an analogous pattern of staining in basal cells of the bronchial tree (Fig. 1).

We found moderate p63 nuclear expression in occasional cells of the Bowman's capsule of the glomerular tufts in the kidney (data not shown). However, epithelial cells of the proximal convoluted tubules and collecting ducts were unreactive. Similarly, we found that epithelial cells of the lower digestive tract and other organs, such as the liver and pancreas, had undetectable p63 levels (data not shown; see Table 1). However, a subpopulation of p63-positive epithelial cells was identified in the thymus (see below).

Trophoblastic epithelial cells of the placenta are categorized into inner layer (Langerhans cells or cytotrophoblasts) and outer layer (syncytial trophoblasts) cells. We observed that only the cytotrophoblasts expressed moderate to high levels of p63, whereas the syncytial trophoblasts had undetectable p63 levels (data not shown).

Expression of p63 in Other Normal Tissues. We observed p63 expression in certain cells in a few organs. Of interest, we found that occasional cells in the germinal center of lymph nodes displayed a weak to moderate p63 nuclear immunostaining (Fig. 1). We also identified occasional cells in the seminiferous tubules, which displayed low to moderate p63 levels (data not shown).

p63 was undetectable in mesenchymal elements, including endothelial cells, smooth muscle cells, and fibroblasts. Similarly, we observed that neuroendocrine cells, including those of the thyroid and adrenal gland, had undetectable p63 expression. Analysis of the cortical tissue revealed that both neuronal and glial cells were unreactive for p63, in agreement with previous studies showing a lack of p63 expression in human brain by Northern blot analysis (20, 21).

Expression of p63 in Squamous and Transitional Carcinomas. We found a good correlation between the staining patterns seen in normal tissues and the immunoreactivity encountered in corresponding neoplastic samples (see Table 2). In general, tumors derived from stratified epithelia showed strong p63 nuclear reactivities (Fig. 2). Similarly, transitional cell carcinomas of the bladder displayed p63 positivity (Fig. 2). Additionally, we observed p63 nuclear reactivity within regions of squamous cell differentiation in other tumors, such as ovarian endometrioid tumors and teratomas of both testes and ovaries. All adenocarcino-

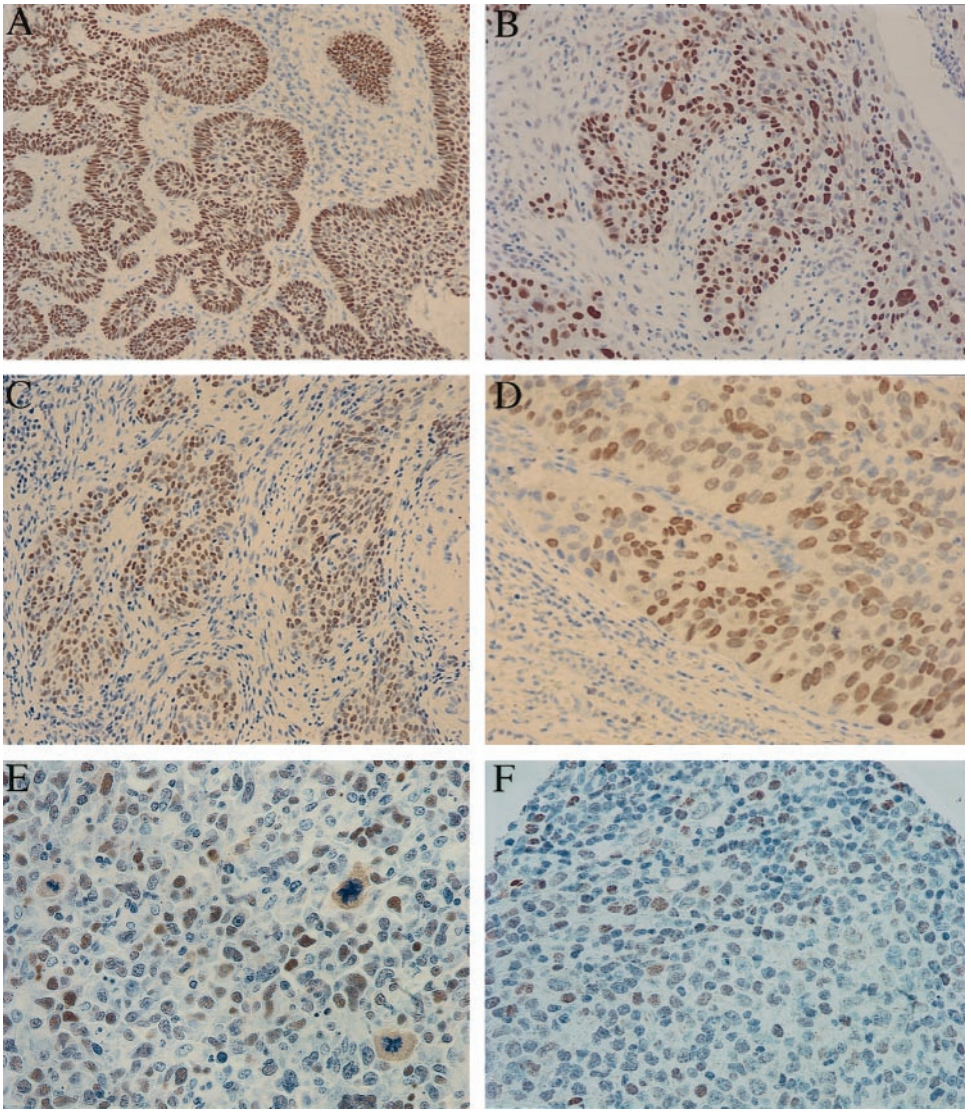

Fig. 2 Representative photomicrographs of immunophenotypes of p63 in different carcinomas and lymphomas, obtained using the anti-p63 4A4 monoclonal antibody. Tumors derived from stratified epithelia, such as basal cell carcinoma of the skin (*A*), SCC of the tongue (*B*), and SCC of the cervix (*C*), showed strong p63 nuclear reactivity. We also observed intense p63 nuclear immunoreactivities in transitional cell carcinoma of the bladder (*D*). NHLs, such as diffuse large B-cell lymphoma (*E*) and FL (*F*), displayed intense nuclear staining. Original magnifications: ×200 for *A*, *B*, and *C*; ×400 for *D*, *E*, and *F*.

mas analyzed, including those derived from breast and prostate, as well as mesotheliomas and hepatocellular carcinomas, had undetectable p63 levels (Table 2 and data not shown).

p63 Is Expressed in a Subset of B-Cell Lymphomas. We examined a number of non-Hodgkin's B-cell lymphomas, including those classified as chronic lymphocytic leukemia/small lymphocytic lymphoma, FL, DLCL, anaplastic large-cell lymphoma, mantle cell lymphoma, and marginal zone lymphoma (Table 2 and data not shown). Specifically, we observed diffuse to intense staining in DLCL and FL (grade 2 and 3; Fig. 2 and data not shown). Furthermore, in FL, the strongest staining was observed in grade 3 cases in the larger neoplastic cells. In chronic lymphocytic leukemia, the three positive cases showed focal, weak p63 reactivity in what appeared to be infiltrating normal lymphocytes rather than the neoplastic component (data not shown). We found no association between p53 and p63 immunoreactivity (data not shown).

To determine which p63 isoforms were present in lymphoid tissues, we performed RT-PCR using isoform-specific primers on total RNA from normal lymph node and six lymphomas (see below). With the exception of the ΔNp63 isoforms, all other TAp63 products were expressed in the normal lymph node (Fig. 3). The subset of six lymphomas analyzed, including three DLCLs and three FLs, showed a similar pattern of p63 expression (Fig. 3). Interestingly, we were able to detect the ΔNp63 isoform(s) in two of the three FL cases (Fig. 3), but not in the DLCL samples.

In addition, we also examined 34 Hodgkin's lymphomas representing several common subtypes, including nodular lymphocytic predominant, mixed cellularity, and nodular sclerosis. The majority of Hodgkin's lymphomas had undetectable levels of p63 expression; however, we observed occasional p63 nuclear-positive lymphocytes. Moreover, one of the nodular lymphocytic predominant samples revealed weak p63 nuclear staining of lymphocytic and histiocytic cells (Table 2 and data not shown).

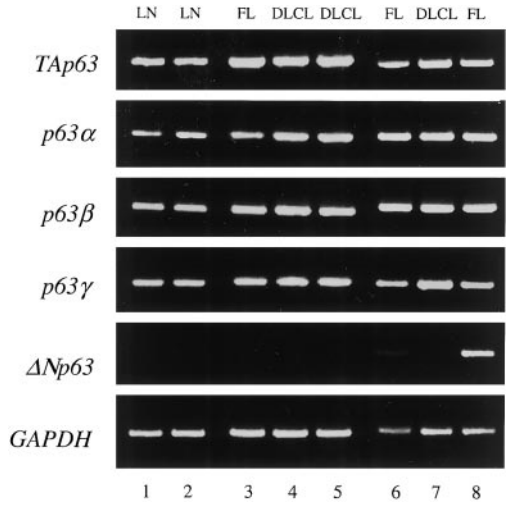

Fig. 3 Expression of p63 in normal lymph node and NHL. RT-PCR analysis for p63 using isoform-specific primers. *Lanes 1* and *2*, normal lymph node (*LN*); *Lanes 3–8*, DLCL and FL. *GAPDH* was used as an endogenous standard. A representative result from three experiments is shown. RT-PCR analysis with one primer was negative for all samples (data not shown).

p63 Is Expressed in Normal Thymus and Thymomas. We observed strong p63 nuclear staining in a cell population of the normal thymus that, after staining with anticytokeratin antibodies, was identified as the reticular epithelium (Fig. 4; Ref. 22). To determine which p63 isoforms are present in the thymus, we performed RT-PCR using isoform-specific primers and found that all p63 isoforms were expressed (Fig. 5).

We also analyzed a group of 13 thymomas covering the full spectrum of the disease, including noninvasive (spindle cell, lymphocyte-rich, and mixed), invasive, and thymic carcinomas. All of these cases showed strong p63 nuclear immunostaining in the neoplastic epithelial component (Fig. 4). For two of these cases, we were able to obtain frozen tissue to further investigate isoform distribution. As with the normal thymus, we found that all p63 isoforms were expressed (Fig. 5).

Analyses of p63 Expression in Other Tumor Types. We also studied a large number of adenocarcinomas, endocrine tumors, melanomas, sarcomas, and germ cell neoplasms (Table 2). In general, these lesions had undetectable levels of p63. However, 2 of the 31 rhabdomyosarcomas and 4 of the 18 malignant peripheral nerve sheath tumors analyzed showed p63 staining in occasional cells (data not shown).

DISCUSSION

This study confirms and expands on earlier results showing that p63 expression follows a restricted pattern in normal tissues. We observed intense p63 nuclear localization in the basal layer of stratified squamous epithelia, with a gradual diminution of nuclear intensity in the more terminally differentiated cell layers, whereas the most apical cells had undetectable p63 levels. We also observed p63 immunoreactivity in basal cells of certain glandular epithelia, such as breast and prostate, but not in the luminal cells. The identification of p63 in basal cell layers of stratified and transitional epithelia is significant because some of these cells act as progenitors of the suprabasal cells, which undergo differentiation and cell death (19). Furthermore, squamous cells appear to down-regulate the p63 α and β isoforms on differentiation (23). Data from *TP63* knockout mice point to a pivotal function for p63 in epithelial development and morphogenesis (2, 3). Hence, they displayed defective epidermal differentiation as well as agenesis of mammary glands, lacrimal glands, and the prostate.

It has been reported that p63 overexpression can mimic p53 activities by binding DNA, activating transcription, and inducing apoptosis (19, 20, 23, 24). Whereas p53 is ubiquitously expressed, although in low to undetectable levels by IHC, p63 is tissue and cell type restricted (4, 5, 8, 19). It is now evident that p53 has evolved to protect cells from genotoxic insults and unprogrammed proliferation, whereas p63 may have evolved to perform tissue-specific functions distinct from those of p53 (Ref. 1 and references therein).

Regarding patterns of expression of p63 in carcinomas, we observe that only those lesions derived from p63-positive squamous and transitional epithelial compartments also displayed p63 immunoreactivities. Squamous cell carcinomas of different organs, including head and neck, cervix, and lung, as well as basal cell carcinomas of the skin showed strong nuclear p63 expression. A similar pattern of immunostaining has been reported by several groups (4, 9, 19). In addition, we also observed that transitional cell carcinomas displayed intense nuclear staining. However, tumors derived from epithelia with p63 restricted to a basal cell population, such as breast and prostate, were p63 unreactive. It should be noted, however, that p63 knockout mice are defective in the development of all organs that express p63. Thus, it appears that, in addition to its role in normal development, maintenance of p63 expression is important in the neoplastic transformation of squamous and transitional epithelium.

Mesenchymal elements, including fibroblasts and smooth muscle cells, had undetectable p63. However, we found that skeletal muscle had TAp63 transcripts by RT-PCR (data not shown); nevertheless, we were not able to detect the protein by IHC in several different skeletal muscle samples. Interestingly, we found p63 expression only in a subset of rhabdomyosarcomas, whereas all other soft tissue sarcomas had undetectable p63. We did not detect p63 expression in a variety of endocrine tumors, germ cell neoplasms, or melanomas.

One of the interesting new findings in this study was the presence of a p63-positive subset of non-Hodgkin's B-cell lymphomas and a p63-positive population of lymphocytes in morphologically normal lymph nodes. The expression of p63 has been detected by Western blot in peripheral blood lymphocytes (8), but to the best of our knowledge, no study of p63 in lymphoid malignancies has been reported. Higher levels of protein expression were found in tumor lymphocytes compared with normal lymphoid tissues. There are no data, as yet, on the function of p63 in these cells; thus, this represents an avenue for future investigation.

We observed that DLCLs most commonly express high levels of p63. Yang *et al.* (19) speculated that because p63 lies close to the translocation break point found in some DLCLs, genomic instability in this area may lead to the dysregulation of p63. However, this does not explain why we see a predominance of the TA isoforms in the small group of tumors analyzed by RT-PCR.

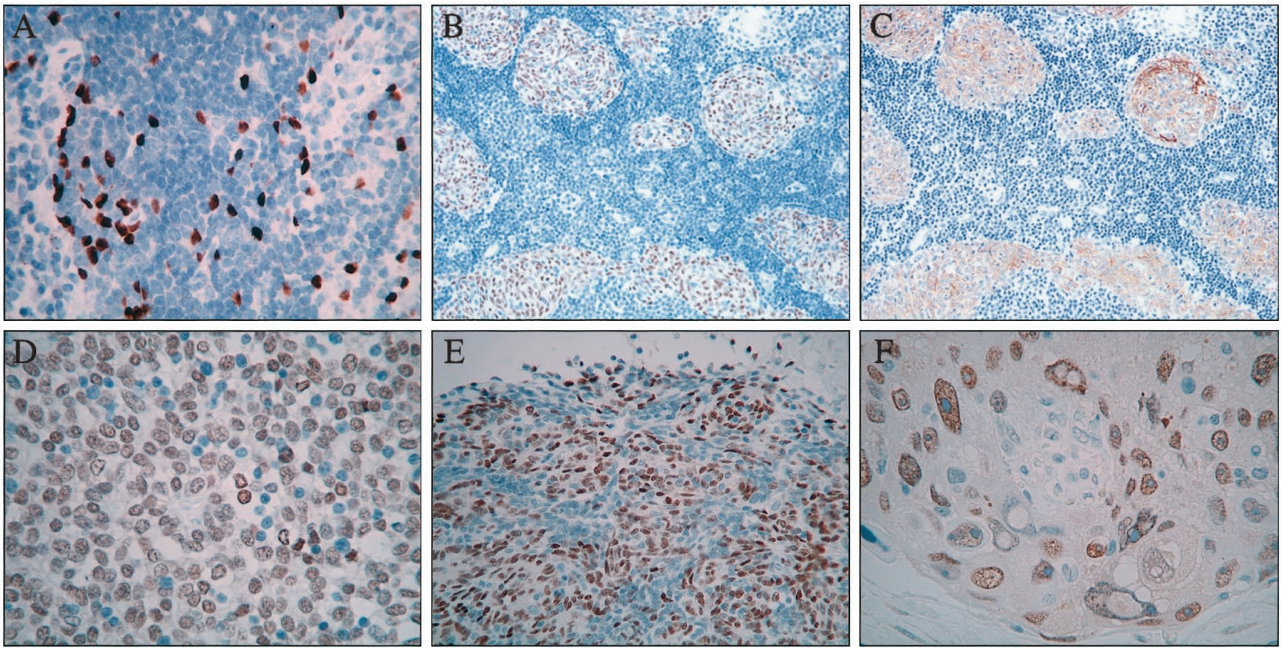

Fig. 4 Representative photomicrographs of immunophenotypes of p63 in normal thymus and thymomas, obtained using the anti-p63 4A4 monoclonal antibody. Strong p63 nuclear staining is observed in a population of cells identified as the epithelial elements of the thymus (*A*; see below). We also observed p63 immunostaining in the neoplastic component of various thymomas, including invasive (*B* and *D*), spindle cell (*E*), and thymic carcinoma (*F*). Consecutive normal thymus (not shown) and thymoma (*B* and *C*) sections were stained with p63 (*B*) and the anticytokeratin AE1/AE3 monoclonal antibody cocktail (*C*), revealing that the p63-expressing cells were also costained for cytokeratins and thus are considered of epithelial nature. Original magnifications: ×100 for *B* and *C*; ×200 for *D* and *E*; ×400 for *A* and *F*.

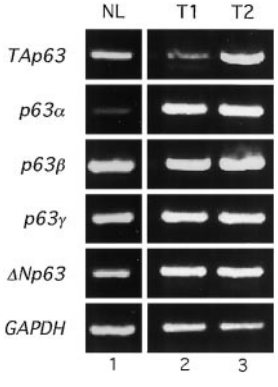

Fig. 5 Expression of p63 isotypes in normal thymus and thymoma. RT-PCR analysis for p63 using isoform-specific primers. *Lane 1*, normal thymus (*NL*); *Lanes 2* and *3*, thymomas (*T1* and *T2*). *GAPDH* was used as an endogenous standard. A representative result from three experiments is shown. RT-PCR analysis with one primer was negative for all samples (data not shown).

Additionally, p63 expression was defined in a series of thymomas. We did not observe any correlation between p63 expression and clinical aggressiveness because p63 was uniformly expressed in the epithelial component in all cases examined. Others have reported a correlation between p53 expression and malignancy in thymomas (25). No lymphoid or thymic abnormalities have been described in p63-null mice (2, 3). However, in humans, heterozygous germline p63 mutations have been described in EEC syndrome, in which a single case has been described with thymic hypoplasia (26). Another case study examined a sporadic incident of EEC syndrome of complete form of the anomaly with late onset of NHL (27). Interestingly, the NHL was of the diffuse large-cell type, where we observed intense p63 immunoreactivity.

In contrast to *p53*, a prototypical tumor suppressor gene, somatic mutations of the *TP63* gene are very rare, whereas germline mutations in *TP63* have been reported for patients with limb mammary syndrome, split-hand/split-foot malformation, ankyloblepharon-ectodermal dysplasia-clefting syndrome, and EEC syndrome (28–31). It appears that alterations of *TP63* are epigenetic in nature. Data from this and other studies regarding p63 overexpression in tumor cells support an important role for p63 in human primary tumors, including squamous and transitional cell carcinomas, as well as certain lymphomas and thymomas. In this context, we might speculate that when certain p63 isoforms are expressed, such as dominant-negative ΔNp63, they could bind to and inhibit transactivation by p53 and TAp63 (19). Alternatively, these ΔNp63 isoforms, by binding to specific promoter elements, could block the transcription of otherwise critical genes, such as those involved in the apoptotic response. Rather than inactivating tumor suppressor activities (*e.g.*, p53), altered patterns of p63 expression could be driving the transcription of oncogenic proteins, such as hMDM2, a target reported for TAp63γ (32, 33). Further studies, integrating mechanistic approaches, are needed to elucidate the role of p63 in human cancer.

ACKNOWLEDGMENTS

We thank Maria Dudas for technical assistance. Critical comments on the manuscript were made by Drs. Paola Capodieci, Kenan Onel, and David Polsky.

REFERENCES

1. Yang, A., and McKeon, F. p63 and p73: p53 mimics, menaces, and more. Nat. Rev. Mol. Cell. Biol., *1:* 199–207, 2000.

2. Mills, A. A., Zheng, B., Wang, X. J., Vogel, H., Roop, D. R., and Bradley, A. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature (Lond.), *398:* 708–713, 1999.

3. Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C., and McKeon, F. p63 is essential for regenerative proliferation in limb, craniofacial, and epithelial development. Nature (Lond.), *398:* 714–718, 1999.

4. Parsa, R., Yang, A., McKeon, F., and Green, H. Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. J. Investig. Dermatol., *113:* 1099–1105, 1999.

5. Signoretti, S., Waltregny, D., Dilks, J., Isaac, B., Lin, D., Garraway, L., Yang, A., Montironi, R., McKeon, F., and Loda, M. p63 is a prostate basal cell marker and is required for prostate development. Am. J. Pathol., *157:* 1769–1775, 2000.

6. Bjorkqvist, A. M., Husgafvel-Pursiainen, K., Anttila, S., Karjalainen, A., Tammilehto, L., Mattson, K., Vainio, H., and Knuutila, S. DNA gains in 3q occur frequently in squamous cell carcinoma of the lung, but not in adenocarcinoma. Genes Chromosomes Cancer, *22:* 79–82, 1998.

7. Bockmuhl, U., Schwendel, A., Dietel, M., and Petersen, I. Distinct patterns of chromosomal alterations in high- and low-grade head and neck squamous cell carcinomas. Cancer Res., *56:* 5325–5329, 1996.

8. Hall, P. A., Campbell, S. J., O'Neill, M., Royston, D. J., Nylander, K., Carey, F. A., and Kernohan, N. M. Expression of the p53 homologue p63α and δNp63α in normal and neoplastic cells. Carcinogenesis (Lond.), *21:* 153–160, 2000.

9. Hibi, K., Trink, B., Patturajan, M., Westra, W. H., Caballero, O. L., Hill, D. E., Ratovitski, E. A., Jen, J., and Sidransky, D. *AIS* is an oncogene amplified in squamous cell carcinoma. Proc. Natl. Acad. Sci. USA, *97:* 5462–5467, 2000.

10. Nylander, K., Coates, P. J., and Hall, P. A. Characterization of the expression pattern of p63α and δNp63α in benign and malignant oral epithelial lesions. Int. J. Cancer, *87:* 368–372, 2000.

11. O'Connell, J. T., Mutter, G. L., Cviko, A., Nucci, M., Quade, B. J., Kozakewich, H. P., Neffen, E., Sun, D., Yang, A., McKeon, F. D., and Crum, C. P. Identification of a basal/reserve cell immunophenotype in benign and neoplastic endometrium: a study with the p53 homologue p63. Gynecol. Oncol., *80:* 30–36, 2001.

12. Quade, B. J., Yang, A., Wang, Y., Sun, D., Park, J., Sheets, E. E., Cviko, A., Federschneider, J. M., Peters, R., McKeon, F. D., and Crum, C. P. Expression of the p53 homologue p63 in early cervical neoplasia. Gynecol. Oncol., *80:* 24–29, 2001.

13. Wang, T. Y., Chen, B. F., Yang, Y. C., Chen, H., Wang, Y., Cviko, A., Quade, B. J., Sun, D., Yang, A., McKeon, F. D., and Crum, C. P. Histologic and immunophenotypic classification of cervical carcinomas by expression of the p53 homologue p63: a study of 250 cases. Hum. Pathol., *32:* 479–486, 2001.

14. Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M. J., Sauter, G., and Kallioniemi, O. P. Tissue microarrays for high-throughput molecular profiling of tumor specimens [see comments]. Nat. Med., *4:* 844–847, 1998.

15. Hoos, A., Urist, M. J., Stojadinovic, A., Mastorides, S., Dudas, M. E., Leung, D. H. Y., Kuo, D., Brennan, M. F., Lewis, J. J., and Cordon-Cardo, C. Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. Am. J. Pathol., *158:* 1245–1251, 2001.

16. Cordon-Cardo, C., and Richon, V. M. Expression of the retinoblastoma protein is regulated in normal human tissues. Am. J. Pathol., *144:* 500–510, 1994.

17. MacCallum, D. E., Hupp, T. R., Midgley, C. A., Stuart, D., Campbell, S. J., Harper, A., Walsh, F. S., Wright, E. G., Balmain, A., Lane, D. P., and Hall, P. A. The p53 response to ionising radiation in adult and developing murine tissues. Oncogene, *13:* 2575–2587, 1996.

18. Fuchs, E. Epidermal differentiation: the bare essentials. J. Cell Biol., *111:* 2807–2814, 1990.

19. Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D., and McKeon, F. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol. Cell, *2:* 305–316, 1998.

20. Osada, M., Ohba, M., Kawahara, C., Ishioka, C., Kanamaru, R., Katoh, I., Ikawa, Y., Nimura, Y., Nakagawara, A., Obinata, M., and Ikawa, S. Cloning and functional analysis of human p51, which structurally and functionally resembles p53 [see comments; published erratum appears in Nat. Med., *4:* 982, 1998]. Nat. Med., *4:* 839–843, 1998.

21. Senoo, M., Seki, N., Ohira, M., Sugano, S., Watanabe, M., Inuzuka, S., Okamoto, T., Tachibana, M., Tanaka, T., Shinkai, Y., and Kato, H. A second p53-related protein, p73L, with high homology to p73 [published erratum appears in Biochem. Biophys. Res. Commun., *250:* 536, 1998]. Biochem. Biophys. Res. Commun., *248:* 603–607, 1998.

22. Sun, T-T., Eichner, R., Schermer, A., Cooper, D., Nelson, W. G., and Weiss, R. A. The transformed phenotype. *In:* The Cancer Cell, Vol. 1, pp. 169–176. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1984.

23. De Laurenzi, V., Rossi, A., Terrinoni, A., Barcaroli, D., Levrero, M., Costanzo, A., Knight, R. A., Guerrieri, P., and Melino, G. *p63* and *p73* transactivate differentiation gene promoters in human keratinocytes. Biochem. Biophys. Res. Commun., *273:* 342–346, 2000.

24. Gaiddon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. Mol. Cell. Biol., *21:* 1874–1887, 2001.

25. Fukiwake, N., Kase, S., Yamazaki, K., Yano, T., and Sugimachi, K. Correlation between clinical aggressiveness of thymic epithelial tumors and expression of tumor suppressor gene products (*p53*, *p27*). Fukuoka Igaku Zasshi, *90:* 339–341, 1999.

26. Frick, H., Munger, D. M., Fauchere, J. C., and Stallmach, T. Hypoplastic thymus and T-cell reduction in EECUT syndrome. Am. J. Med. Genet., *69:* 65–68, 1997.

27. Ogutcen-Toller, M., Gulen, O., Okten, G., and Elbistan, M. Non-Hodgkin's lymphoma in a patient with ectrodactyly ectodermal dysplasia-clefting syndrome. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod., *90:* 124–125, 2000.

28. Celli, J., Duijf, P., Hamel, B. C., Bamshad, M., Kramer, B., Smits, A. P., Newbury-Ecob, R., Hennekam, R. C., Van Buggenhout, G., van Haeringen, A., Woods, C. G., van Essen, A. J., de Waal, R., Vriend, G., *et al*. Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome. Cell, *99:* 143–153, 1999.

29. Ianakiev, P., Kilpatrick, M. W., Toudjarska, I., Basel, D., Beighton, P., and Tsipouras, P. Split-hand/split-foot malformation is caused by mutations in the *p63* gene on 3q27. Am. J. Hum. Genet., *67:* 59–66, 2000.

30. McGrath, J. A., Duijf, P. H., Doetsch, V., Irvine, A. D., de Waal, R., Vanmolkot, K. R., Wessagowit, V., Kelly, A., Atherton, D. J., Griffiths, W. A., Orlow, S. J., van Haeringen, A., Ausems, M. G., Yang, A., *et al*. Hay-Wells syndrome is caused by heterozygous missense mutations in the SAM domain of p63. Hum. Mol. Genet., *10:* 221–229, 2001.

31. Wessagowit, V., Mellerio, J. E., Pembroke, A. C., and McGrath, J. A. Heterozygous germline missense mutation in the *p63* gene underlying EEC syndrome. Clin. Exp. Dermatol., *25:* 441–443, 2000.

32. Ikawa, S., Nakagawara, A., and Ikawa, Y. *p53* family genes: structural comparison, expression, and mutation [see comments]. Cell Death Differ., *6:* 1154–1161, 1999.

33. Roth, J., and Dobbelstein, M. Failure of viral oncoproteins to target the p53-homologue p51A. J. Gen. Virol., 80: 3251–3255, 1999.