Over-Expression of $p45^{SKP2}$ in Kaposi's Sarcoma Correlates with Higher Tumor Stage and Extracutaneous Involvement but Is Not Directly Related to $p27^{KIP1}$ Down-Regulation

R. M. Penin, M.D., M. T. Fernandez-Figueras, M.D., L. Puig, M.D., J. Rex, M.D., C. Ferrandiz, M.D., A. Ariza, M.D.

Departments of Pathology (RMP, MTF-F, AA) and Dermatology (JR, CF), Hospital Universitari Germans Trias i Pujol, Barcelona, Spain; and Department of Dermatology (LP), Hospital de la Santa Creu i Sant Pau, Autonomous University of Barcelona, Barcelona, Spain

F-Box protein $p45^{SKP2}$ is the substrate-specific receptor of ubiquitin-protein ligase SCF/$p45^{SKP2}$ and is involved in the degradation of $p27^{Kip1}$ through the ubiquitin/proteasome pathway. In addition, $p45^{SKP2}$ facilitates proteolysis of other molecules related to the cell cycle, is frequently over-expressed in transformed cells, and induces S phase in quiescent cells. The aim of this study was to determine whether $p45^{SKP2}$ expression is altered in aggressive lesions of Kaposi's sarcoma and its relation to $p27^{KIP1}$down-regulation. We performed immunohistochemistry using antibodies directed to $p45^{SKP2}$, $p27^{KIP1}$, and Ki67 on paraffin blocks corresponding to 47 cases of Kaposi's sarcoma (8 macules, 10 plaques, 12 tumors, and 15 extracutaneous lesions). $p45^{SKP2}$ nuclear over-expression was present in all Kaposi's sarcoma stages, being significantly increased in skin tumors (mean ± 95% confidence interval: 39.2 ± 18.8) and extracutaneous lesions (25.8 ± 17.3) as compared with macules (18.9 ± 8.2) and plaques (29.2 ± 12.0; $P = .0199$). On the other hand, Kaposi's sarcoma progression was associated with a decrease in $p27^{KIP1}$ expression and Ki67 immunoreactivity was independent of disease stage. No statistically significant differences were found in regard to patients' sex and human immunodeficiency virus status and regression analysis failed to show a correlation among $p45^{SKP2}$, $p27^{KIP1}$ and Ki67 immunostaining scores. These findings suggest that $p45^{SKP2}$ is involved in Kaposi's sarcoma progression, not only by promoting the degradation of $p27^{KIP1}$ but also through other mechanisms still unknown.

KEY WORDS: Cell cycle, Kaposi's sarcoma, Ki67, $p27^{KIP1}$, $p45^{SKP2}$, Proteasome, Ubiquitin.

Mod Pathol 2002;15(11):1227–1235

Cell cycle progression requires the coordinated performance of a series of regulating molecules that orchestrate cycle transitions through either mitogenic or antiproliferative signals (1).

The two crucial mechanisms used by cells to control the protein levels required in each step of the cycle are protein synthesis and degradation. Disruption of these two mechanisms leads to abnormal cell proliferation and oncogenesis, particularly if the derangement results in loss of control at the G1-S transition.

One of the many cell-cycle regulating molecules is cyclin A, whose role as an S-phase propeller is the consequence of its ability to complex with its catalytic partner cyclin-dependent kinase 2 (CDK2). Subsequently, the latter acts as a modulator of the behavior of effector proteins such as the Kip/Cip family CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$, transcription factor E2F-1, DNA replication and repair factor proliferating cell nuclear antigen (PCNA), and S phase kinase-associated proteins 1 and 2 ($p19^{SKP1}$ and $p45^{SKP2}$), among others (2).

In recent years, the mechanisms of protein degradation have attracted much attention and intense efforts have been made to elucidate the intricacies of the machinery involved in the ubiquitin proteasomal pathway, which plays a paramount role in the degradation of short-lived regulatory proteins involved in the cell cycle (3). The two main steps followed by the ubiquitin proteasomal pathway are attachment of multiple ubiquitin molecules to a

VOL. 15, NO. 11, P. 1227, 2002 Printed in the U.S.A.
Date of acceptance: August 15, 2002.
Address reprint requests to: Maria Teresa Fernandez-Figueras, M.D., Department of Pathology, Hospital Universitari Germans Trias i Pujol, Ctra. de Canyet, s/n, 08916 Badalona, Barcelona, Spain; e-mail: mtferfig@ns.hugtip.scs.es; fax: 34-93-4978843.

DOI: 10.1097/01.MP.0000036589.99516.D6

protein substrate and degradation through the 26S-proteasome complex. Transference of ubiquitin to the substrate requires at least the collaboration of an ubiquitin-activating enzyme (E1) and an ubiquitin-conjugating enzyme (E2). Oftentimes, however, improvement of substrate recognition requires the cooperation of a third component termed ubiquitin–protein ligase (E3).

Recently, a novel class of E3 ubiquitin ligases named SKP1/CDC53(cullin)/F-box protein complex (SCF) has been described. This E3 class is involved in the degradation of several key cell-cycle regulatory proteins such as $p27^{KIP1}$, $p21^{CIP1}$ (4), E2F-1 (5), cyclin A (2), cyclin D (4), and cyclin E (6). The SCF complex consists of the invariable components $p19^{SKP1}$, cullin-1 (Cul1 or CDC53) and regulator of cullins/RING box protein (Roc1/Rbx1) as well as a variable component known as F-box proteins. The latter include $p45^{SKP2}$, which by binding to $p19^{SKP1}$ is responsible for substrate specificity (7). The SCF core complex is localized to the cytoplasm until $p45^{SKP2}$ is expressed in late G1 and moves the SCF/$p45^{SKP2}$ complex into the nucleus (Diagram 1), where it promotes the ubiquitination of selected proteins. Nevertheless, a frequent splice variant named C-terminal variant ($p45^{SKP2}$-CTV) is found in the cytoplasm of various cell lines, in most cases associated with the usual form of $p45^{SKP2}$. Probably owing to this cytoplasmic mislocation, $p45^{SKP2}$-CTV is unable to achieve proper ubiquitination of the selected proteins (8). The C terminal domain may contain a cytoplasmic retention domain that is dominant over the nuclear localization signal of $p45^{SKP2}$ failing to bind $p19^{SKP1}$ (9).

The fact that $p27^{KIP1}$ is rapidly degraded during late G1 phase in most cell types suggests that this cyclin-kinase inhibitor plays an important role in cell-cycle control, especially in regard to the G–S transition. In various tumors decreased CDK inhibitor $p27^{KIP1}$ levels are associated with a poor prognosis (8–12). The low levels of $p27^{KIP1}$ tumor suppressor may be due to either transcriptional and translational reduction, as in prostatic hyperplasia (11), or posttranscriptional protein degradation by the ubiquitin proteasomal pathway, as in numerous malignant neoplasms. Tumor-specific mutations in the $p27^{KIP1}$ gene, on the contrary, seem to be exceptional events.

Recent studies have demonstrated that $p27^{KIP1}$ degradation (Diagram 1) is mediated by the SCF/$p45^{SKP2}$ complex and by ubiquitin-independent processing during progression from G1 to S phase (6). $P45^{SKP2}$ specifically interacts with $p27^{KIP1}$ only when the CDK inhibitor is phosphorylated by cyclin E-CDK2, thereby promoting the ubiquitination and degradation of $p27^{KIP1}$. $P45^{SKP2}$ is frequently overexpressed in transformed cells, induces S phase in quiescent cells (13), and is a suspected proto-oncogene in human tumors. In fact, there are recent reports of increased levels of $p45^{SKP2}$ in association with reduced $p27^{KIP1}$ levels in epithelial neoplasms (13–16). On the other hand, cyclin E expression is a periodic event that reaches maximal levels at the G1-S transition and cyclin E degradation is mediated by ubiquitin-dependent proteolysis. Both CDK2-associated and free forms of cyclin E appear to be targets for ubiquitination and rapid degradation, whereas binding of $p45^{SKP2}$ is one of the events involved in the proteolysis of free (but not of CDK2-associated) cyclin E (6). Apart from their potential action as mediators of ubiquitin-dependent cyclin A proteolysis, $p19^{SKP1}$ and $p45^{SKP2}$ may also directly regulate the kinase activity of cyclin A-CDK2 (17).

Kaposi's sarcoma is an angiohyperplastic disease mediated by inflammatory cytokines and angiogenic growth factors in a setting of human herpesvirus type 8 infection. In advanced stages Kaposi's sarcoma may behave as a multifocal neoplasm whose various lesions display monoclonality (18). Immunosuppression is a triggering factor for Kaposi's sarcoma development, as demonstrated by the common occurrence of AIDS-associated Kaposi's sarcoma, which usually exhibits a much more aggressive behavior than classic Kaposi's sarcoma. In the skin, the organ primarily targeted by Kaposi's sarcoma, lesions are clinicopathologically classified into macules, plaques and tumors in agreement with their progression in severity (19). In aggressive cases, usually of the AIDS-associated Kaposi's sarcoma form, the lesions may also involve extracutaneous locations.

We have previously demonstrated that decreased immunoreactivity for the cell-cycle regulator $p27^{KIP1}$ correlates with higher stage and extracutaneous involvement in Kaposi's sarcoma (20). To the best of our knowledge, studies of $p45^{SKP2}$ expression have never been performed in Kaposi's sarcoma. Thus, the aim of this study was to determine whether $p45^{SKP2}$ expression is altered in aggressive Kaposi's sarcoma lesions and explore the possible relation of $p45^{SKP2}$ expression to $p27^{KIP1}$ downregulation and other variables such as Ki67 expression, gender, and human immunodeficiency virus infection.

MATERIALS AND METHODS

Human Tissue Samples

Cutaneous and extracutaneous Kaposi's sarcoma biopsy paraffin blocks were retrieved from the files of the Department of Pathology of Hospital Germans Trias i Pujol, Badalona, Barcelona, Spain. Paraffin blocks of extracutaneous cases of Kaposi's sarcoma were also provided by the Departments of

Pathology of Hospital de la Santa Creu i Sant Pau and Hospital Prínceps d'Espanya, Barcelona, Spain. We evaluated 47 cases of Kaposi's sarcoma, of which 35 corresponded to AIDS-associated Kaposi's sarcoma and 17 to classic Kaposi's sarcoma. One of the 17 classic Kaposi's sarcoma cases followed an aggressive course and showed early extracutaneous involvement. The 47 Kaposi's sarcoma cases provided 32 skin biopsy specimens (8 macules, 10 plaques, and 12 tumors) and 15 biopsy specimens from extracutaneous locations (6 from lymph nodes, 4 from oral mucosa or conjunctiva, 2 from the gastrointestinal tract [including the 1 case of aggressive classic Kaposi's sarcoma], 1 from the larynx, 1 from the lung, and 1 from soft tissues).

The stage of cutaneous Kaposi's sarcoma cases was determined by histopathologic study of hematoxylin-eosin stained sections. Macular stage lesions consisted of a superficial or mid-dermal proliferation of collagen-dissecting jagged capillary vessels that disposed themselves around normal dermal structures. In some instances the newly formed vessels were confluent, but the spindle-cell component was always inconspicuous. A diagnosis of plaque stage Kaposi's sarcoma was made when the lesion consisted of a proliferation of malformed vascular channels that dissected collagen fibers and contained only isolated spindle-shaped cells or small groups of them. Cases were classified as nodular or tumor phase Kaposi's sarcoma when the entire lesion or most of it showed a compact proliferation of spindle-shaped cells with an intersecting fascicle-like pattern alleviated only by some inflammatory cells, erythrocytes, or telangiectatic spaces. All tissue specimens had been fixed in neutral-buffered formalin and routinely processed.

Antibodies and Immunohistochemical Studies

Immunohistochemistry studies were performed using polyclonal anti-full-length SKP2 p45 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA, diluted 1:500 with phosphate-buffered saline (PBS)), anti-p27 protein mouse monoclonal antibody, clone 1B4, (Novocastra, Newcastle, UK, diluted 1:40 with phosphate-buffered saline (PBS)), and NCL-Ki67-MM1 mouse monoclonal antibody (Novocastra, diluted 1:50 with phosphate-buffered saline (PBS)). Five-micron-thick sections were deparaffinized, hydrated, immersed in buffered citrate and autoclaved. Afterwards, the sections were incubated for 30 minutes in rabbit serum. Incubations with primary antibodies were carried out for 22 hours at room temperature. Slides were washed and incubated with biotinylated rabbit anti-mouse Ig antibodies at a 1:700 dilution and then incubated in PBS/6% hydrogen peroxide for 15 minutes at room temperature before avidin-biotin peroxidase complex addition (Dakopatts, Glostrup, Denmark). The chromogen 3, 3'-diaminobenzidine tetrachloride (Serva, Heidelberg, Germany) was applied, and counter-staining was performed with Harris hematoxylin. A non-immune mouse serum was used as a negative control in this protocol.

Both elongated cells lining abnormal blood vessels and spindle-shaped cells unrelated to vessels were considered to be Kaposi's sarcoma neoplastic cells. On evaluating $p45^{SKP2}$ staining intensity, a sub-population of cells with strongly positive nuclei could be easily distinguished. The percentages of tumor cells showing $p45^{SKP2}$ positive nuclei, $p45^{SKP2}$ intensely positive nuclei, $p27^{KIP1}$ positive nuclei and Ki67 positive nuclei were independently assessed by two researchers (RMP and MTFF) in 500 tumor cells. In macule and plaque stage lesions, where neoplastic cells were less abundant, 20 fields were evaluated. At least 200 cells were counted in every case. The quotients (positive tumor cells/total number of tumor cells counted) were converted to percentages and rounded to the nearest integer. The arithmetic mean of both observers' scores was used for statistical evaluation. All cases in which inter-observer variation exceeded 10% were jointly re-evaluated. In addition, the intensity of cytoplasmic stain for $p45^{SKP2}$ was jointly evaluated using the following score: 4, strong stain in at least 50% of cells; 3, strong stain in 25 to 50% of cells or moderate in more than 80% of cells; 2, strong stain in 5 to 25% of cells or moderate in 5 to 80% of cells; 1, moderate or strong stain in less than 5% of cells or weak in more than 5% of cells; 0, absent or weak stain in less than 5% of cells.

Statistical Study

The statistical significance of differences observed between classic Kaposi's sarcoma and AIDS-associated Kaposi's sarcoma lesions and between plaque lesions and tumor lesions in regard to $p45^{SKP2}$, $p27^{KIP1}$, and Ki67 immunostaining scores, Kaposi's sarcoma clinical-epidemiological type, sex of patient, lesion location and clinicopathological type was determined using analysis of variance. When variances were not homogeneous or samples were not normally distributed, the Kruskal-Wallis test was used. In addition, $p27^{KIP1}$ expression was compared in the same groups using the cut-off described above and the Fisher's exact test for differences between proportions. Differences between groups were considered to be statistically significant when the p value was less than .05. The Pearson correlation coefficient with 95% confidence limits was used to test the strength of association between $p27^{KIP1}$, $p45^{SKP2}$ and Ki67 expressions.

Diagram 1. General model of $p45^{SKP2}$–$p27^{KIP1}$–cyclin/CDK2 interaction. The SKP1/CDC53(cullin)/F-box protein complex (SCF) is involved in the degradation of several key cell-cycle regulatory proteins such as $p27^{KIP1}$, $p21^{CIP1}$, cyclin A, cyclin D, and cyclin E. The SCF core complex is localized to the cytoplasm until $p45^{SKP2}$ is expressed in late G1 and moves the SCF/$p45^{SKP2}$ complex into the nucleus, where it promotes the ubiquitination of selected proteins. $p27^{KIP1}$ degradation is mediated by the SCF/$p45^{SKP2}$ complex and by ubiquitin-independent processing during progression from G1 to S phase (6). The association between loss of $p27^{KIP1}$ protein and uncontrolled proliferation of cancer cells is congruent with the function of $p27^{KIP1}$ as a negative regulator of cyclins E and A, which in complex with CDK2 drive cells into the S-phase (15). $P45^{SKP2}$ is frequently over-expressed in transformed cells, induces S phase in quiescent cells, and is a suspected proto-oncogene in human tumors. It has been postulated that low levels of $p27^{KIP1}$ in aggressive human cancers may be caused by increased expression of $p45^{SKP2}$ that targets $p27^{KIP1}$ for ubiquitin-mediated degradation.

TABLE 1. Summary of Mean Scores of $p45^{SKP2}$, $p27^{KIP1}$, and Ki-67 Immunostaining in Cutaneous and Extracutaneous Kaposi's Sarcoma Lesions Showing Increasing Severity and Progression

	Macules	Plaques	Tumors	Extracutaneous	*P*
$p45^{SKP2}$ nuclear	18.9 ± 8.2	29.2 ± 12.0	39.2 ± 18.8	25.8 ± 17.3	**.0199**
$p45^{SKP2}$ intense nuclear	3.7 ± 2.1	4.8 ± 3.7	11.1 ± 12.7	7.4 ± 5.8	**.0126**
$p45^{SKP2}$ cytoplasmic	0.6 ± 0.7	0.8 ± 0.6	1.8 ± 1.0	1.8 ± 1.4	.0154
$p27^{KIP1}$ nuclear	86 ± 12.8	74.3 ± 26.5	55.4 ± 26.0	45.3 ± 24.3	**.0012**
Ki-67 nuclear	17.6 ± 15.9	12.0 ± 6.6	17.4 ± 10.7	15.9 ± 5.7	.4124

The score is the percentage of positive neoplastic cells; results are given ±95% confidence interval.

RESULTS

The results are summarized Tables 1, 2, and 3. We have found $p45^{SKP2}$ nuclear expression in all stages of Kaposi's sarcoma. The average percentage of neoplastic cells expressing $p45^{SKP2}$ in their nuclei was significantly increased in skin tumors (mean ± 95% confidence interval: 39.2 ± 18.8) and extracutaneous lesions (25.8 ± 17.3) as compared with macules (18.9 ± 8.2) and plaques (29.2 ± 12.0; $P = .0199$) (Table 1). The staining in some nuclei was distinctly intense, allowing separate quantification. The corresponding percentages were significantly increased in skin tumors (11.1 ± 12.7) and extracutaneous lesions (7.4 ± 5.8) with respect to macules (3.7 ± 2.1) and plaques (4.8 ± 3.7; $P = .0126$). The differences between macules and plaques were not significant and, when lesions in both stages were put together (Table 2), the differences between groups became even more significant ($P = .0052$). Interestingly, $p45^{SKP2}$ expression in extracutaneous Kaposi's sarcoma lesions was lower than in cutaneous tumors but higher than in plaques. As regards $p45^{SKP2}$ cytoplasmic stain, we observed a marked tendency toward an increase in the advanced stages (Table 3), but this trend did not reach statistical significance ($P = .0557$ when macules and plaques were grouped together) owing to the non-continuous nature of the score we used. Application of the Kruskal-Wallis test to a non-continuous variable was not possible, but the corresponding means and results are shown in Tables 1 and 2 just for the purpose of comparison with nuclear staining scores.

We have found Kaposi's sarcoma progression to be associated with a decrease in $p27^{KIP1}$ expression, thus confirming the results of our previous study (20) of a different set of cases. On the other hand, Ki67 expression levels were independent of the disease stage, as already demonstrated by a previous study of ours (20). Regression analysis showed no statistical correlation between $p45^{SKP2}$ overexpression and loss of $p27^{KIP1}$. In some Kaposi's sarcoma skin lesions a reduced expression of $p27^{KIP1}$ paralleled a high nuclear and cytoplasmic expression of $p45^{SKP2}$ (Fig. 1), whereas in others high $p45^{SKP2}$ levels were associated with $p27^{KIP1}$ preservation (Fig. 2). Similar findings regarding $p45^{SKP2}$ and $p27^{KIP1}$ expression were also observed

TABLE 2. Summary of Mean Scores of $p45^{SKP2}$, $p27^{KIP1}$, and Ki-67 Immunostaining in Cutaneous and Extracutaneous Kaposi's Sarcoma Lesions Showing Increasing Severity and Progression

	Macules-Plaques	Tumors	Extracutaneous	*P*
$p45^{SKP2}$ nuclear	24.6 ± 11.5	39.2 ± 18.8	25.8 ± 17.3	**.0304**
$p45^{SKP2}$ intense nuclear	4.3 ± 3.1	11.1 ± 12.7	7.4 ± 5.8	**.0052**
$p45^{SKP2}$ cytoplasmic	0.7 ± 0.8	1.8 ± 1.0	1.8 ± 1.4	.0060
$p27^{KIP1}$ nuclear	79.5 ± 21.8	55.4 ± 26.0	45.3 ± 24.3	**.0012**
Ki-67 nuclear	14.5 ± 11.6	17.4 ± 10.7	15.9 ± 5.7	.2930

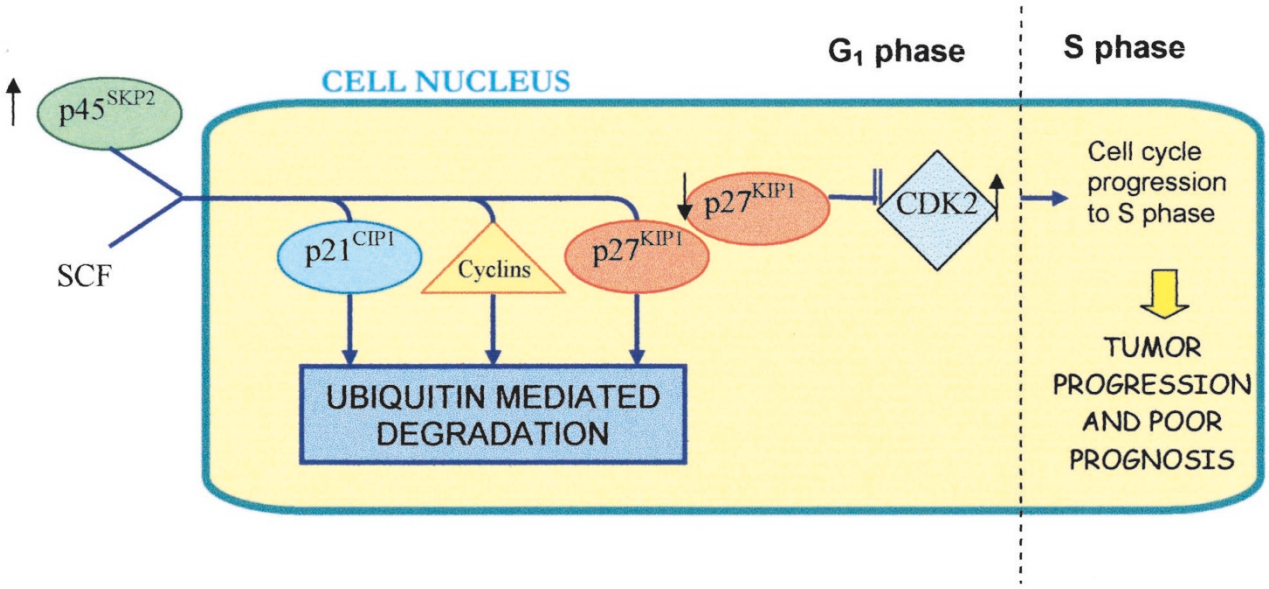
The score is the percentage of positive neoplastic cells; results are given ±95% confidence interval.

Lesions corresponding to cutaneous macules and plaques are grouped together.

TABLE 3. Comparison of the Number of Cases with Cytoplasmic $p45^{SKP2}$ Immunostaining Scores (0 to 4) in Kaposi's Sarcoma Lesions Showing Increasing Severity and Progression

	Macules	Plaques	Tumors	Extracutaneous	Total
0	4	4	0	2	10
1	3	4	7	6	20
2	1	2	4	3	10
3	0	0	2	1	3
4	0	0	1	3	4
	8	10	14	15	47

$p45^{SKP2}$ cytoplasmic staining intensity was graded as follows: 4, strong stain in at least 50% of cells; 3, strong stain in 25 to 50% of cells or moderate in more than 80% of cells; 2, strong stain in 5 to 25% of cells or moderate in 5 to 80% of cells; 1, moderate or strong stain in less than 5% of cells or weak in more than 5% of cells; 0, absent or weak stain in less than 5% of cells.



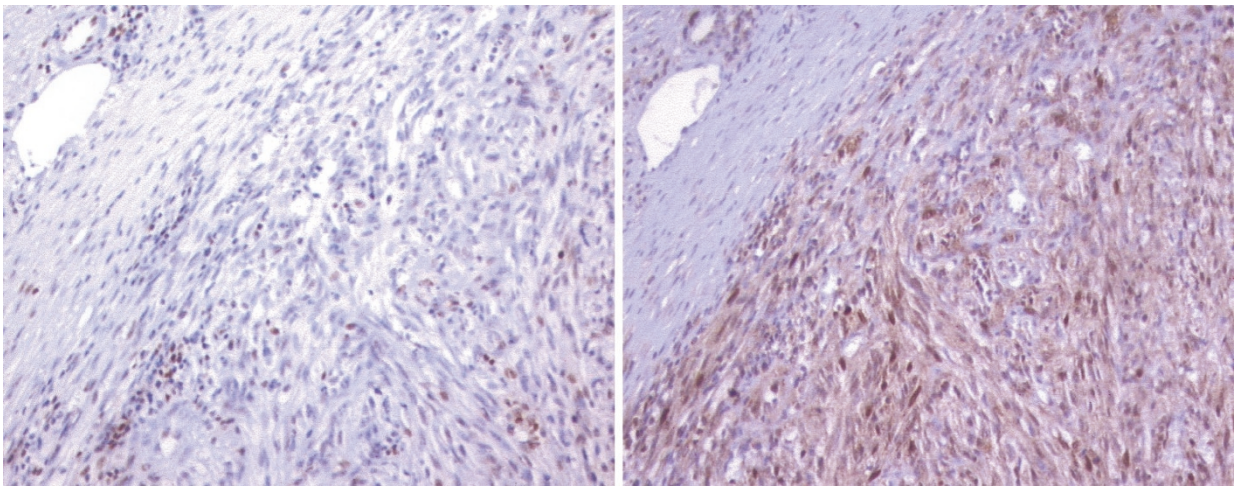


FIGURE 1. Classic Kaposi's sarcoma tumor lesion with marked $p27^{KIP1}$ down-regulation (***left***) and $p45^{SKP2}$ over-expression in the same area (***right***). Slight cytoplasmic stain for $p45^{SKP2}$ is also present.

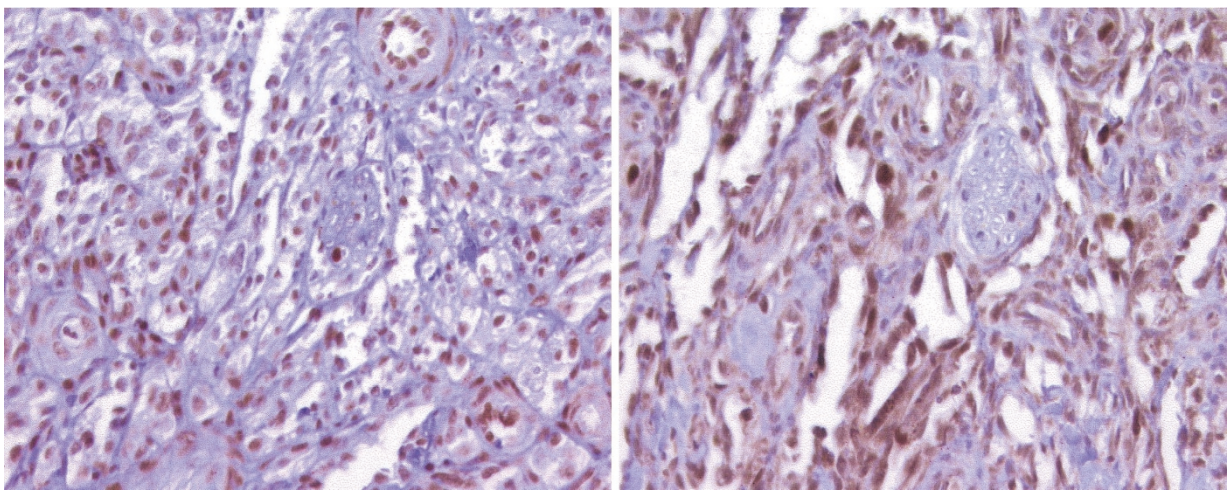


FIGURE 2. Example of over-expression of both $p27^{KIP1}$ (***left***) and $p45^{SKP2}$ (***right***) in a classic Kaposi's sarcoma tumor lesion.

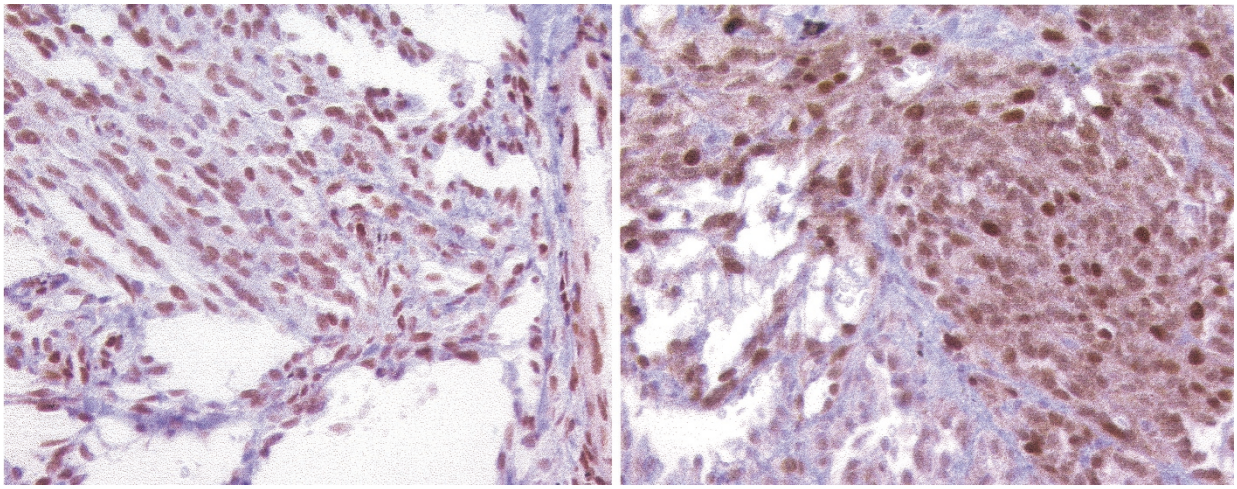


FIGURE 3. Example of over-expression of $p27^{KIP1}$ (***left***) and $p45^{SKP2}$ (***right***) in a Kaposi's sarcoma extracutaneous lesion (lung). This case also displays strong cytoplasmic positivity and variable nuclear intensities of $p45^{SKP2}$ staining.

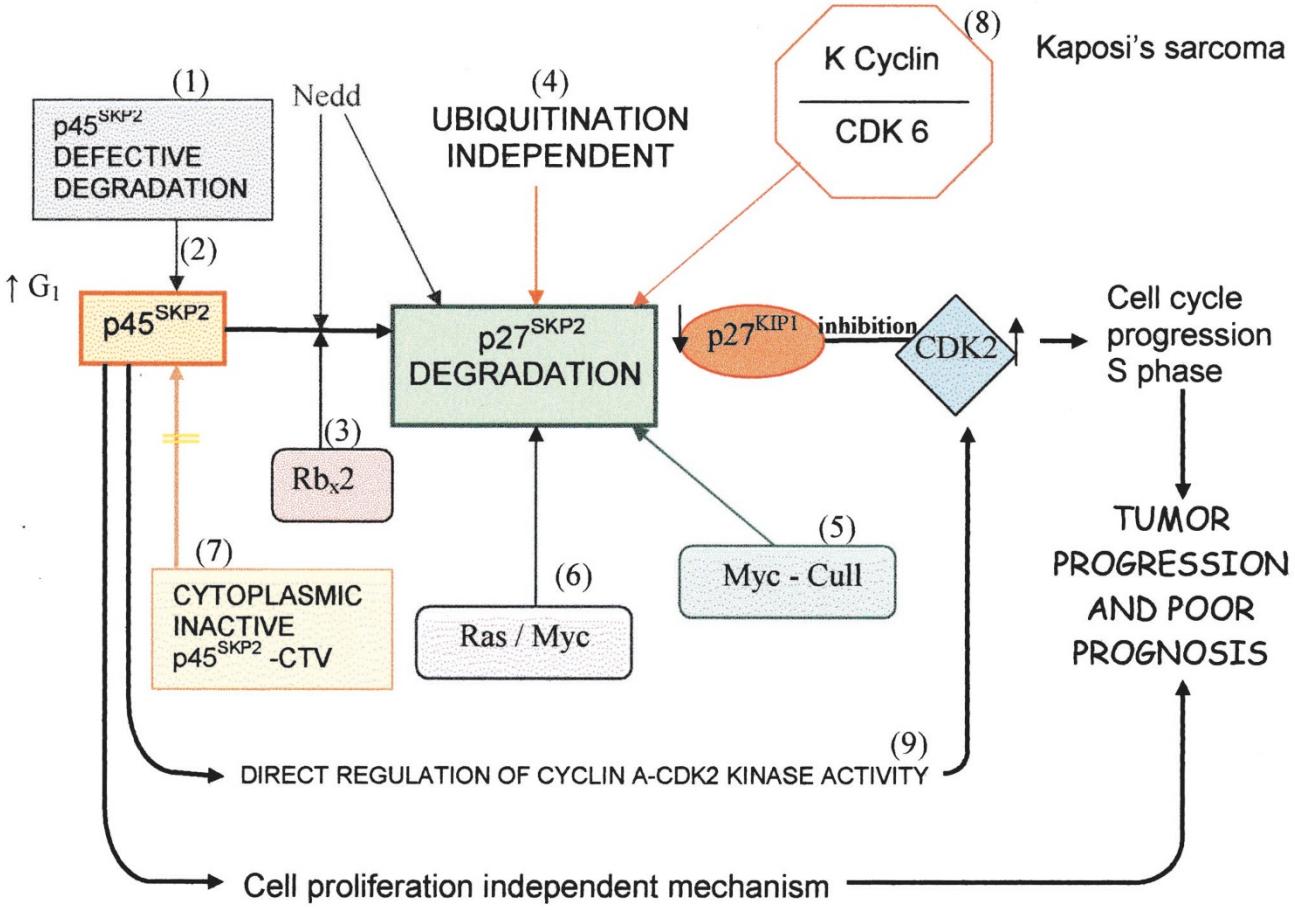


Diagram 2. Putative explanations for the lack of inverse correlations between $p45^{SKP2}$ and $p27^{KIP1}$ expressions in Kaposi's sarcoma. 1: Increased $p27^{KIP1}$ degradation may be the result of a defective ubiquitination and degradation of $p45^{SKP2}$ by either SCF/$p45^{SKP2}$ action during the G0-G1 phase (23) or an alternative pathway independent of cell-cycle phase (24). 2: Nedd8 enhances SCF/$p45^{SKP2}$ effect on $p27^{KIP1}$ (27) and may regulate $p27^{KIP1}$ turnover independently of $p27^{KIP1}$ phosphorylation (28). 3: Rbx2 is also able to bind $p45^{SKP2}$ and mediate $p27^{KIP1}$ degradation (29). 4: $p27^{KIP1}$ is also processed rapidly by an ubiquitination-independent mechanism that exhibits higher activity in the S phase than during the G0-G1 phase (30). 5: Increased $p27^{KIP1}$ proteolysis in Kaposi's sarcoma may be caused by *Myc* oncogenic activation leading to Cul1 over-expression (25) or 6: by a proteolytic pathway activated by mitogens through *Ras* and *Myc* (26). 7: Increased expression of the inactive $p45^{SKP2}$–CTV splice variant (8) might provide an explanation for nuclear $p45^{SKP2}$ expression levels being lower in extracutaneous Kaposi's sarcoma lesions than in cutaneous Kaposi's sarcoma tumors with similar cytoplasmic $p45^{SKP2}$ expression. 8: human herpesvirus type 8-encoded K cyclin, which is resistant to $p27^{KIP1}$ CDK inhibition, would facilitate $p27^{KIP1}$ phosphorylation and down-regulation, thus enabling activation of endogenous cyclin/CDK2 complexes (31, 32). 9: $p45^{SKP2}$ may also directly regulate cyclinA-CDK2 kinase activity (17) and contribute to the malignant phenotype without affecting proliferation (14).

in some extracutaneous Kaposi's sarcoma lesions (Fig. 3), while in some aggressive lesions both molecules were poorly expressed. Moreover, whereas the maximum degree of $p27^{KIP1}$ down-regulation was observed in extracutaneous Kaposi's sarcoma lesions and then in cutaneous Kaposi's sarcoma tumors, the highest degree of $p45^{SKP2}$ expression corresponded to cutaneous Kaposi's sarcoma tumors and then to extracutaneous Kaposi's sarcoma lesions.

Immunohistochemical expression of $p45^{SKP2}$ was not correlated with Ki67 proliferation index, and no statistically significant differences were found in regard to patients' sex and human immunodeficiency virus status (results not shown).

DISCUSSION

Numerous studies have shown that reduced levels of $p27^{KIP1}$ protein, an inhibitor of cyclin-dependent kinases, are associated with a more aggressive course and a poorer prognosis in a large variety of carcinomas. Moreover, in the case of colon (10, 15), breast (12) and prostate cancers (11) low $p27^{KIP1}$ expression provides independent prognostic information. The association between loss of $p27^{KIP1}$ protein and uncontrolled proliferation of cancer cells is congruent with the function of $p27^{KIP1}$ as a negative regulator of cyclins E and A, which in complex with their catalytic partner CDK2 drive cells into the S-phase (15). In the normal cell cycle, the G0/G1 phase is characterized by high $p27^{KIP1}$ levels and low $p45^{SKP2}$ levels. Subsequently, during the S-phase, $p45^{SKP2}$ levels increase and $p27^{KIP1}$ is rapidly degraded, thus allowing the promotion of cell proliferation by the conjoint action of cyclin E/CDK2 and cyclin A/CDK2 (15). $P27^{KIP1}$ appears to belong to a recently recognized class of tumor suppressors in which reduced protein expression is usually not caused by genetic change. Recent studies have identified the machinery involved in $p27^{KIP1}$degradation as an SCF type ubiquitin ligase complex that contains $p45^{SKP2}$ as the specific substrate-recognition unit. Levels of $p45^{SKP2}$ are rate-limiting for the degradation of $p27^{KIP1}$ (21), and it has been postulated that low levels of $p27^{KIP1}$ in aggressive human cancers may be caused by increased expression of $p45^{SKP2}$ that targets $p27^{KIP1}$ for ubiquitin-mediated degradation. Levels of $p45^{SKP2}$ expression correlate directly with malignancy grade and inversely with $p27^{KIP1}$ levels in human lymphomas (22), colorectal carcinomas (15) and oral squamous cell carcinomas (14, 16). In the normal cell cycle, $p45^{SKP2}$ levels are very low in the G0/G1 phase, increase in the S-phase, and decline afterwards (2). High levels of $p45^{SKP2}$ are not due just to increased proliferation, despite the direct correlation observed in lymphomas (22), inasmuch as the percentage of cells expressing high levels of $p45^{SKP2}$ in colorectal carcinoma greatly exceeds the percentage of cells expected to be in the S-phase in a randomly dividing population (15). Increased $p45^{SKP2}$ protein levels do not always correlate with increased cell proliferation (as assayed by Ki67 staining), which suggests that $p45^{SKP2}$ alterations may contribute to the malignant phenotype without affecting proliferation (14). Our results indicate that the aforementioned observations also apply to Kaposi's sarcoma, although the picture seems to be somewhat more complicated in this neoplasm. Specifically, the findings in need of alternative explanatory hypotheses in Kaposi's sarcoma are the lack of an inverse correlation between $p27^{KIP1}$ and $p45^{SKP2}$ levels and the apparent paradox of $p45^{SKP2}$ expression, which in extracutaneous Kaposi's sarcoma lesions happens to be lower than in cutaneous Kaposi's sarcoma tumors but higher than in Kaposi's sarcoma macules/plaques.

Increased $p27^{KIP1}$ degradation (Diagram 2) may be the result of a defective ubiquitination and degradation of $p45^{SKP2}$ by either SCF/$p45^{SKP2}$ action during the G0-G1 phase (23) or an alternative pathway independent of cell-cycle phase (24). In addition to $p45^{SKP2}$ over-expression, increased $p27^{KIP1}$ proteolysis in Kaposi's sarcoma and other neoplasms may be caused by *Myc* oncogenic activation leading to Cul1 over-expression (25). $P27^{KIP1}$ control may be also achieved by a second proteolytic pathway that is activated by mitogens through *Ras* and *Myc* and is operative during the G1 phase (26).

Nedd8, an ubiquitin-like protein expressed in proliferating cells, acts on Cul1, enhances SCF/$p45^{SKP2}$ effect on $p27^{KIP1}$ (27), and may regulate $p27^{KIP1}$ turnover independently of $p27^{KIP1}$ phosphorylation (28). Also able to bind $p45^{SKP2}$ and mediate $p27^{KIP1}$ degradation is Rbx2, which is the product of the sensitive-to-apoptosis gene (SAG) and the second member of the RING box protein family (29). In parallel with its ubiquitin-dependent degradation, $p27^{KIP1}$ is processed rapidly by an ubiquitination-independent mechanism that exhibits higher activity in the S phase than during the G0-G1 phase (30).

On the other hand, increased expression of a $p45^{SKP2}$ splice variant that localizes to the cytoplasm and fails to direct cyclin D1 (and supposedly $p27^{KIP1}$) ubiquitination and degradation (8) might provide an explanation for our finding that nuclear $p45^{SKP2}$ expression levels are lower in extracutaneous Kaposi's sarcoma lesions than in cutaneous Kaposi's sarcoma tumors with similar cytoplasmic $p45^{SKP2}$ expression levels.

The fact that Kaposi's sarcoma is related to human herpesvirus type 8 infection suggests a plausible explanation for the lack of an inverse correlation

between $p27^{KIP1}$ and $p45^{SKP2}$ expression levels in this neoplasm. Specifically, human herpesvirus type 8-encoded K cyclin, which is resistant to the actions of $p16^{INK4A}$, $p21^{CIP1}$ and $p27^{KIP1}$ CDK inhibitors, would bypass a $p27^{KIP1}$-imposed G1 arrest by facilitating $p27^{KIP1}$ phosphorylation and down-regulation and thus enabling activation of endogenous cyclin/CDK2 complexes (31, 32). Indeed, the occurrence of a $p27^{KIP1}$-phosphorylating CDK6 complex in cell lines derived from primary effusion lymphoma and Kaposi's sarcoma may well indicate that virally induced $p27^{KIP1}$ degradation takes place in human herpesvirus type 8-related tumors (32).

Acknowledgments: *We thank Dr Xavier Matias-Guiu, Department of Pathology, Hospital de la Santa Creu i Sant Pau, for providing the paraffin blocks corresponding to three extracutaneous Kaposi's sarcoma cases and to Dr Roger Bernat, Department of Pathology, Hospital Prínceps d'Espanya, for providing the paraffin block corresponding to an extracutaneous Kaposi's sarcoma case. This work was supported in part by CYCIT grant SAF 97/0220 and DURSI grant 2001SGR00400.*

REFERENCES

1. Cordón-Cardo C. Mutation of cell cycle regulators. Biological and clinical implications. Am J Pathol 1995;147:545–60.
2. Lisztwan J, Marti A, Sutterluty H, Gstaiger M, Wirbelauer C, Krek W. Association of human CUL-1 and ubiquitin-conjugating enzyme CDC34 with the F-box protein p45 (SKP2): evidence for evolutionary conservation in the subunit composition of the CDC34-SCF pathway. EMBO J 1998; 17:368–83.
3. Harper JW. Protein destruction: adapting roles for Cks proteins. Curr Biol 2001;11:431–5.
4. Yu Z, Gervais JL, Zhang H. Human CUL-1 associates with the SKP1/SKP2 complex and regulates $p21^{CIP/WAF1}$ and cyclin D proteins. Proc Natl Acad Sci USA 1998;95:11324–9.
5. Marti A, Wirbelauer C, Scheffner M, Krek W. Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation. Nat Cell Biol 1999; 1:14–9.
6. Nakayama KI, Hatakeyama S, Nakayama K. Regulation of the cell cycle at the G_1-S transition by proteolysis of cyclin E and $p27^{Kip1}$. Biochem Biophys Res Commun 2001;282:853–60.
7. Schulman BA, Carrano AC, Jeffrey PD, Bowen Z, Kinnucan ER, Finnin MS, *et al.* Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. Nature 2000;408: 381–6.
8. Ganiatsas S, Dow R, Thompson A, Schulman B, Germain D. A splice variant of Skp2 is retained in the cytoplasm and fails to direct cyclin D1 ubiquitination in the uterine cancer cell line SK-ut. Oncogene 2001;20:3641–50.
9. Jordan RCK, Bradley G, Slingerland J. Reduced levels of the cell-cycle inhibitor $p27^{Kip1}$ in epithelial dysplasia and carcinoma of the oral cavity. Am J Pathol 1998;152:585–90.
10. Thomas GV, Szigeti K, Murphy M, Draetta G, Pagano M, Loda M. Down-regulation of p27 is associated with development of colorectal adenocarcinoma metastases. Am J Pathol 1998;153:681–7.
11. Cordón-Cardo C, Koff A, Drobnjak M, Capodieci P, Osman I, Millard SS, *et al.* Distinct altered patterns of $p27^{KIP1}$ gene expression in benign prostatic hyperplasia and prostatic carcinoma. J Natl Cancer Inst 1998;90:1284–91.
12. Gillett CE, Smith P, Peters G, Lu X, Barnes DM. Cyclin-dependent kinase inhibitor $p27^{Kip1}$ expression and interaction with other cell cycle-associated proteins in mammary carcinoma. J Pathol 1999;187:200–6.
13. Sutterlüty H, Chatelain E, Marti A, Wirbelauer C, Seften M, Müller U, *et al.* $P45^{SKP2}$ promotes $P27^{Kip1}$ degradation and induces S phase in quiescent cells. Nat Cell Biol 1999;1:207–14.
14. Gstaiger M, Jordan R, Lim M, Catzavelos C, Mestan J, Slingerland J, *et al.* Skp2 is oncogenic and overexpressed in human cancers. Proc Natl Acad Sci USA 2001;24:98:5043–8.
15. Hershko D, Bornstein G, Ben-Izhak O, Carrano A, Pagano M, Krausz MM, *et al.* Inverse relation between levels of p27 (Kip1) and of its ubiquitin ligase subunit Skp2 in colorectal carcinomas. Cancer 2001;91:1745–51.
16. Kudo Y, Kitajima S, Sato S, Miyauchi M, Ogawa I, Takata T. High expression of S-phase kinase-interacting protein 2, human F-box protein, correlates with poor prognosis in oral squamous cell carcinomas. Cancer Res 2001;61:7044–7.
17. Yam CH, Ng RW, Siu WY, Lau AW, Poon RY. Regulation of cyclin A-Cdk2 by SCF component Skp1 and F-box protein Skp2. Mol Cell Biol 1999;19:635–45.
18. Rabkin CS, Janz S, Lash A, Coleman AE, Musaba E, Liotta L, *et al.* Monoclonal origin of multicentric Kaposi's sarcoma lesions. N Engl J Med 1997;336:988–93.
19. Chor PJ, Santa Cruz DJ. Kaposi's sarcoma. A clinicopathologic review and differential diagnosis. J Cutan Pathol 1992; 19:6–20.
20. Fernández-Figueras MT, Puig L, Penin RM, Mate JL, Bigatà X, Ariza A. Decreased immunoreactivity for cell-cycle regulator $p27^{Kip1}$ in Kaposi's sarcoma correlates with higher stage and extracutaneous involvement. J Pathol 2000;191:387–93.
21. Carrano AC, Eytan E, Hershko A, Pagano M. Skp2 is required for ubiquitin-mediated degradation of the Cdk inhibitor p27. Nat Cell Biol 1999;1:193–7.
22. Latres E, Chiarle R, Schulman BA, Pavletich NP, Pellicer A, Inghirami G, *et al.* Role of the F-box protein Skp2 in lymphomagenesis. Proc Natl Acad Sci 2001;98:2515–20.
23. Wirbelauer C, Sutterluty H, Blondel M, Gstaiger M, Peter M, Reymond F, *et al.* The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. EMBO J 2000;19:5362–75.
24. Dow R, Hendley J, Pirkmaier A, Musgrove EA, Germain D. Retinoic acid-mediated growth arrest requires ubiquitylation and degradation of the F-box protein Skp2. J Biol Chem 2001;276:45945–51.
25. O'Hagan RC, Ohh M, David G, de Alboran IM, Alt FW, Kaelin WG Jr, *et al.* Myc-enhanced expression of Cul1 promotes ubiquitin-dependent proteolysis and cell cycle progression. Genes Dev 2000;14:2185–91.
26. Malek NP, Sundberg H, McGrew S, Nakayama K, Kyriakides TR, Roberts JM. A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase. Nature 2001;413:323–7.
27. Morimoto M, Nishida T, Honda R, Yasuda H. Modification of cullin-1 by ubiquitin-like protein Nedd8 enhances the activity of SCF(skp2) toward p27(kip1). Biochem Biophys Res Commun 2000;270:1093–6.
28. Podust VN, Brownell JE, Gladysheva TB, Luo RS, Wang C, Coggins MB, *et al.* A Nedd8 conjugation pathway is essential for proteolytic targeting of p27Kip1 by ubiquitination. Proc Natl Acad Sci USA 2000;97:4579–84.
29. Duan H, Tsvetkov LM, Liu Y, Song Y, Swaroop M, Wen R, *et al.* Promotion of S-phase entry and cell growth under serum

starvation by SAG/ROC2/Rbx2/Hrt2, an E3 ubiquitin ligase component: association with inhibition of p27 accumulation. Mol Carcinog 2001;30:37–46.
30. Shirane M, Harumiya Y, Ishida N, Hirai A, Miyamoto C, Hatakeyama S, *et al*. Down-regulation of p27(Kip1) by two mechanisms, ubiquitin-mediated degradation and proteolytic processing. J Biol Chem 1999;274:13886–93.
31. Ellis M, Chew YP, Fallis L, Freddersdorf S, Boshoff C, Weiss RA, *et al*. Degradation of p27Kip cdk inhibitor triggered by Kaposi's sarcoma virus cyclin-cdk6 complex. EMBO J 1999; 18:644–53.
32. Mann DJ, Child ES, Swanton C, Laman H, Jones N. Modulation of $p27^{Kip1}$ levels by the cyclin encoded by Kaposi's sarcoma-associated herpesvirus. EMBO J 1999;18:654–63.