

## RBM3-Regulated Genes Promote DNA Integrity and Affect Clinical Outcome in Epithelial Ovarian Cancer<sup>1,2</sup>

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### Abstract

The RNA-binding motif protein 3 (RBM3) was initially discovered as a putative cancer biomarker based on its differential expression in various cancer forms in the Human Protein Atlas (HPA). We previously reported an association between high expression of RBM3 and prolonged survival in breast and epithelial ovarian cancer (EOC). Because the function of RBM3 has not been fully elucidated, the aim of this study was to use gene set enrichment analysis to identify the underlying biologic processes associated with RBM3 expression in a previously analyzed EOC cohort (cohort 1,  $n = 267$ ). This revealed an association between RBM3 expression and several cellular processes involved in the maintenance of DNA integrity. RBM3-regulated genes were subsequently screened in the HPA to select for putative prognostic markers, and candidate proteins were analyzed in the ovarian cancer cell line A2780, whereby an up-regulation of Chk1, Chk2, and MCM3 was demonstrated in siRBM3-treated cells compared to controls. The prognostic value of these markers was assessed at the messenger RNA level in cohort 1 and the protein level in an independent EOC cohort (cohort 2,  $n = 154$ ). High expression levels of Chk1, Chk2, and MCM3 were associated with a significantly shorter survival in both cohorts, and phosphorylated Chk2 was an adverse prognostic marker in cohort 2. These results uncover a putative role for RBM3 in DNA damage response, which might, in part, explain its cisplatin-sensitizing properties and good prognostic value in EOC. Furthermore, it is demonstrated that Chk1, Chk2, and MCM3 are poor prognostic markers in EOC.

*Translational Oncology (2011) 4, 212–221*

### Introduction

Epithelial ovarian cancer (EOC) is the fifth most common cause of cancer-related death in women and carries the highest mortality rate of gynecological malignancies in the western world. In 2008, it was estimated that 21,650 new ovarian cancer cases would be diagnosed in the United States and that 15,520 would die of the disease [1]. The poor ratio of survival to incidence in EOC is related to the high percentage of cases that are diagnosed at an advanced stage and the lack of effective therapies for advanced refractory disease. Adjuvant systemic chemotherapy for ovarian cancer is empiric and initial treatment involves paclitaxel-platinum-based regimens, which continue

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<sup>1</sup>This study was supported by grants from the Knut and Alice Wallenberg Foundation, the Swedish Cancer Society, Gunnar Nilsson's Cancer Foundation, and the Research funds of Malmö University Hospital. The UCD Conway Institute is funded by the Programme for Third-Level Institutions, as administered by the Higher Education Authority of Ireland. The authors have no conflicts of interest to declare.

<sup>2</sup>This article refers to supplementary materials, which are designated by Tables W1 and W2 and are available online at [www.transonc.com](http://www.transonc.com).

Received 25 January 2011; Revised 5 April 2011; Accepted 6 April 2011

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DOI 10.1593/tlo.11106

to show improved outcomes compared with other cytotoxic agents such as gemcitabine, topotecan, and liposomal doxorubicin [2]. Despite aggressive surgery and chemotherapy, most patients relapse within 3 to 5 years, and the median time to relapse is 15 months after diagnosis [3]. Such poor statistics indicate the urgent need for the development of new diagnostic, prognostic, and predictive biomarkers, which are essential for the development of personalized therapeutic regimens for ovarian cancer patients [4].

RNA-binding proteins with RNA-binding motifs (RBM) are involved in many aspects of RNA processing and regulation of gene transcription [5,6]. The RNA-binding motif protein 3 (RBM3) protein has been shown to bind to both DNA and RNA [7]. We initially described RBM3 as a putative cancer biomarker based on its differential expression in various cancer forms in the Human Protein Atlas (HPA) ([www.proteinatlas.org](http://www.proteinatlas.org)) [8,9]. Within this context, we described RBM3 as a prognostic biomarker in breast cancer, which is associated with an improved survival, particularly in estrogen receptor-positive tumors [10]. We subsequently reported an association between RBM3 messenger RNA (mRNA) and protein expression and good prognosis in two independent EOC cohorts and demonstrated that RBM3 expression conferred sensitivity to cisplatin *in vitro* [11].

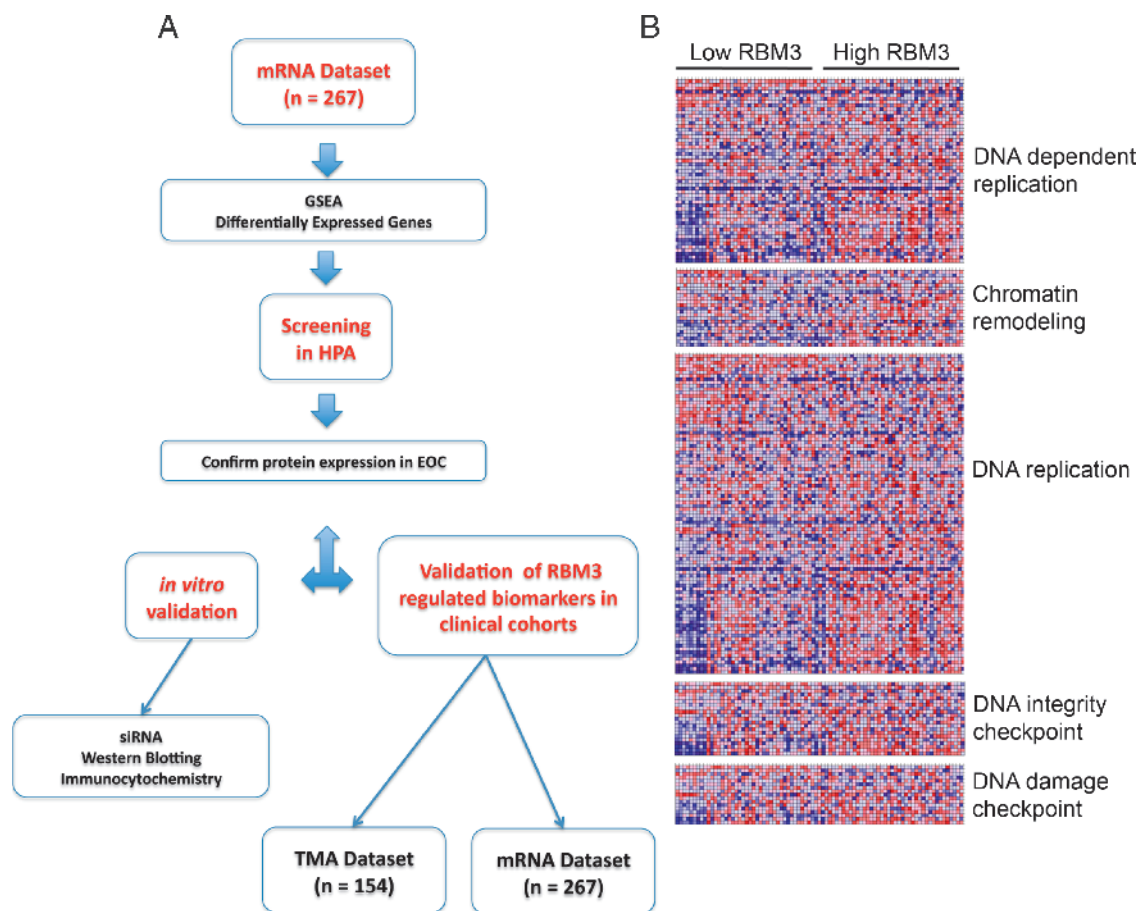
These data suggest that RBM3 may play a key role in both breast and ovarian tumorigenesis and progression; however, its exact function is still to be fully elucidated. The aim of this study was to identify the

underlying biologic processes associated with RBM3 expression in EOC and use this approach to identify new prognostic and predictive biomarkers. Our secondary objective was to improve understanding of the molecular mechanisms underlying the prognostic value of RBM3 in EOC. This approach involved the integration of transcriptomic and antibody-based proteomic data whereby gene set enrichment analysis (GSEA) was performed in a cohort of 267 EOC cases from a publicly available data set [12], in which we have previously demonstrated that high RBM3 expression levels independently predict a prolonged survival [11]. The HPA was then screened to select promising EOC biomarker candidates identified from the aforementioned GSEA. These biomarkers were subsequently validated *in vitro* and in an independent EOC tissue microarray (TMA). This method, schematically described in Figure 1A, highlights a novel approach to biomarker discovery whereby transcriptomic and proteomic data can be integrated to identify new biomarkers.

## Materials and Methods

### Patients

**Cohort 1.** Cohort 1 is composed of 285 cases of serous and endometrioid carcinoma of the ovary, fallopian tube, and peritoneum.



**Figure 1.** Identification of cellular processes associated with RBM3 expression in EOC. (A) Flowchart illustrating a novel approach to biomarker discovery whereby transcriptomic and proteomic data can be integrated to identify new biomarkers. (B) GSEA demonstrated that increased RBM3 expression was associated with a number of processes including DNA-dependent replication  $P < .01$ , chromatin remodeling  $P < .05$ , DNA replication  $P < .01$ , DNA integrity checkpoint  $P < .05$ , and DNA damage checkpoint  $P < .05$ .

The cohort has been described previously [12]. Most patients underwent laparotomy for staging and debulking and, subsequently, received first-line platinum/taxane-based chemotherapy. In most cases, tumor tissue was excised at the time of primary surgery, before the administration of chemotherapy. Eighteen patients who received neoadjuvant platinum-based chemotherapy were excluded from this study; hence, the total number of patients examined was 267. Optimal debulking was defined as less than 1 cm (diameter) residual disease, and suboptimal debulking was more than 1 cm (diameter) residual disease. Recurrence-free survival (RFS) was defined as the time interval between the date of diagnosis and the first confirmed sign of disease recurrence based on GCIg definitions. Overall survival (OS) was defined as the time interval between the date of histological diagnosis and the date of death from any cause. Median follow-up was 29 months (range = 0-214 months).

RNA was extracted from tumors and hybridized to Affymetrix U133 Plus 2 arrays as previously described [12]. Complete expression data were downloaded from GEO ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)) (Accession GSE9899). R package "Affy" ([www.bioconductor.org](http://www.bioconductor.org)) was used to normalize the CEL files using the Robust Multichip Average (RMA) method [13]. For RBM3 analysis, normalized gene expression values were extracted from the data set and used without modification. Tumor samples were classified using a previously published method [14].

**Cohort 2.** This cohort is a merge of all incident cases of EOCs in the large, population-based prospective cohort studies Malmö Diet and Cancer Study [18] ( $n = 101$ ) and Malmö Preventive Medicine Study [19] ( $n = 108$ ) until December 31, 2008, and has been described previously [11]. Thirty-five patients participated in both studies, and archival tumor tissue could be retrieved from 154 of the total number of 174 cases. After a median follow-up of 2.65 years (range = 0-21 years), 105 patients (68.2%) were dead and 49 (31.8%) were alive. All tumors were reevaluated regarding histological subtype and histological grade. Information regarding clinical stage was obtained from the medical charts, following the standardized classification of tumor staging of the International Federation of Gynecology and Obstetrics. Information on residual disease after surgery was not available. Standard adjuvant therapy was platinum-based chemotherapy from the 1990s, given in combination with paclitaxel.

Ethical permission was obtained from the ethics committee at Lund University (reference no. 447-07 and 35/08), whereby informed consent was deemed not to be required other than by the opt-out method.

### Human Protein Atlas TMAs

Tissue microarrays containing triplicate 1-mm cores of 48 different types of normal tissue, duplicate 1-mm cores of 216 different cancer tissues, and a cell microarray including 47 different cell lines and 12 patient cell samples were generated as previously described [15,16].

### GSEA and Selection of Interesting Genes

The microarray data set was downloaded from the GEO Web site (<http://www.ncbi.nlm.nih.gov/geo>). Data were analyzed using Bioconductor 1.9 (<http://bioconductor.org>) running on R 2.6.0 [17]. Probe set expression measures were calculated using the Affymetrix package's RMA default method [18]. The function GeneSetTest from the limma package [19] was used to assess whether each sample had a tendency to be associated with an up-regulation or down-regulation of RBM3. All samples were ranked on this enrichment, from the most

significant to the least significant. The top and bottom 50 samples were extracted from the data set and given the names of "high-RBM3" and "low-RBM3," respectively. Differential gene expression was assessed using the signal-to-noise ratio. Gene set enrichment was performed using GSEA software (<http://www.broadinstitute.org/gsea/index.jsp>) as previously described [20,21]. Heat maps were drawn using expression data showing the probes that mapped to the biologic processes of DNA dependent DNA replication, chromatin remodeling, DNA replication, DNA integrity checkpoint, and DNA damage checkpoint.

### Cell Lines and Reagents

The human ovarian cancer cell line A2780 (received as a gift from Prof R. Brown, Imperial College, London) was maintained in RPMI-1640 supplemented with glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin in a humidified incubator of 5% CO<sub>2</sub> at 37°C.

### Real-time Quantitative Polymerase Chain Reaction and Western Blot Analysis

Total RNA isolation (RNeasy; QIAgen, Hilden, Germany), complementary DNA synthesis (Reverse Transcriptase Kit; Applied Biosystems, Warrington, United Kingdom), and real-time quantitative polymerase chain reaction (QPCR) analysis with SYBR Green PCR master mix (Applied Biosystems) were performed as previously described [22,23]. Quantification of expression levels was done using the comparative C<sub>t</sub> method, normalization according to housekeeping genes *HMBS*, *YWHAZ*, and *UBC*. Primer sequences are given in Table W1. All primers were designed using Primer Express (Applied Biosystems).

For immunoblot analysis, cells were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride) and supplemented with protease inhibitor cocktail Complete Mini (Roche, Basel, Switzerland). For Western blot analysis, 20 to 50 µg of protein was separated on 15% SDS-PAGE gels and transferred onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The membranes were probed with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science, Alesbury, United Kingdom) and visualized using the enhanced chemiluminescence detection system (ECL) and ECL films (Amersham Pharmacia Biotech). RBM3 was detected by the mouse monoclonal anti-RBM3 antibody (AAb030038; Atlas Antibodies AB, Stockholm, Sweden) diluted 1:500 in blocking solution (5% bovine serum albumin, 1× PBS, 0.1% Tween 20). Dilutions of the investigative antibodies are given in Table W2. Membranes were stripped and reprobed with an anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000, to provide a loading control.

### TMA Construction

Before TMA construction, all cases were histopathologically reevaluated on hematoxylin and eosin-stained slides. Areas representative of cancer were then marked, and TMAs were constructed as previously described [24]. In brief, two to four 1.0-mm cores were taken from each tumor and mounted in a new recipient block using a semiautomated arraying device (TMArrayer; Pathology Devices, Inc, Westminster, MD).

### Immunohistochemistry and Analysis of Staining

For immunohistochemical analysis, 4- $\mu$ m TMA sections were automatically pretreated using the PT-link system (DAKO, Copenhagen, Denmark) and then stained in a Techmate 500 (DAKO) with the mouse monoclonal anti-RBM3 antibody (AAb030038) diluted 1:5000 and MCM3 (HPA 004789) diluted 1:1000 from Atlas Antibodies. The following antibodies were purchased from Cell Signaling Technologies (Danvers, MA): Chk1 (mouse monoclonal, clone 2G1D5, no. 2360) diluted 1:100, Chk2 (mouse, monoclonal, clone 1C12, no. 3440,) diluted 1:2000, pSer345-Chk1 (rabbit monoclonal, no. 2348) diluted 1:150, and pT68-Chk2 (rabbit polyclonal no. 2661) diluted 1:50.

Chk1, Chk2, MCM3, and phosphorylated Chk1 and Chk2 were mainly expressed in the nuclei, and both the fraction of positive cells and staining intensity were taken into account using a semiquantitative scoring system as described previously for the assessment of RBM3 staining [14]. Nuclear fraction (NF) was categorized into four groups, namely 0 (0%-1%), 1 (2%-25%), 2 (26%-75%), and 3 (>75%) and nuclear staining intensity (NI) denoted as 0 to 2, whereby 0 = negative, 1 = intermediate, and 2 = moderate to strong intensity. A combined nuclear score (NS) of  $NF \times NI$ , which had a range of 0 to 6, was then constructed.

### Cell Pellet Arrays

Cell lines were fixed in 4% formalin and processed in gradient alcohols. Cell pellets were cleared in xylene and washed multiple times in molten paraffin. Once processed, cell lines were arrayed in duplicate 1.0-mm cores using a manual tissue arrayer (Beecher, Inc, Sun Prairie, WI), and immunohistochemistry was performed on 5- $\mu$ m sections using the same antibodies as for immunohistochemistry with the following dilutions: RBM3, 1:1000; Chk1, 1:100; Chk2 and MCM3, 1:2000; pSer345-Chk1 and pT68-Chk2, 1:50.

### Small Interfering RNA-Mediated Knockdown of RBM3 Gene Expression

Transfection with small Interfering RNA (siRNA) against RBM3 (Applied Biosystems, Carlsbad, CA) or control siRNA (Applied Biosystems) was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with a final concentration of 50 nM siRNA. All siRNA experiments were performed using three independent RNA oligonucleotides (nos. 58, 59, and 60) targeting RBM3.

### Statistics

Spearman  $\rho$  tests were used for comparison of Chk1, Chk2, and MCM3 expression with RBM3 expression and relevant clinicopathological characteristics. Kaplan-Meier analysis, using mean expression value to dichotomize data, and log-rank test were used to illustrate differences in RFS and overall survival (OS) according to *CHK1*, *CHK2*, and *MCM3* gene expression and OS according to Chk1, Chk2, and MCM3 protein level. Cox regression proportional hazards models were used to estimate the impact of RBM3 expression on RFS and OS in both univariate and multivariate analyses, adjusted for stage and differentiation grade (both cohorts) and volume of residual tumor (0 vs >0) in cohort 1. Patients who had received neoadjuvant chemotherapy in cohort 1 ( $n = 18$ ) were excluded from the survival analyses. All calculations were performed using SPSS version 15.0 (SPSS, Inc, Chicago, IL). All statistical tests were two-sided, and  $P < .05$  was considered statistically significant. Experimental data are expressed as mean  $\pm$  SEM of at least three independent experiments.

Statistical significance of differences between means was determined by Student's  $t$  test.

## Results

### Identification of Cellular Processes Associated with RBM3 Expression in EOC

In an attempt to profile the effect of RBM3 expression in EOC, we used gene expression data from a previously described cohort [12] to compare the gene profiles of treatment-naïve tumors with high RBM3 mRNA levels to those tumors showing no or low RBM3 expression. Comparison of the 50 tumors expressing the highest levels of RBM3 mRNA to the 50 tumors expressing the lowest levels of RBM3 mRNA using GSEA demonstrated that increased RBM3 expression was associated with a number of processes including DNA-dependent replication, DNA replication, chromatin remodeling, and DNA integrity checkpoint (Figure 1B). Low RBM3 mRNA expression was associated with a variety of different processes including cAMP G protein signaling, transcription factor activity, and the protein kinase cascade.

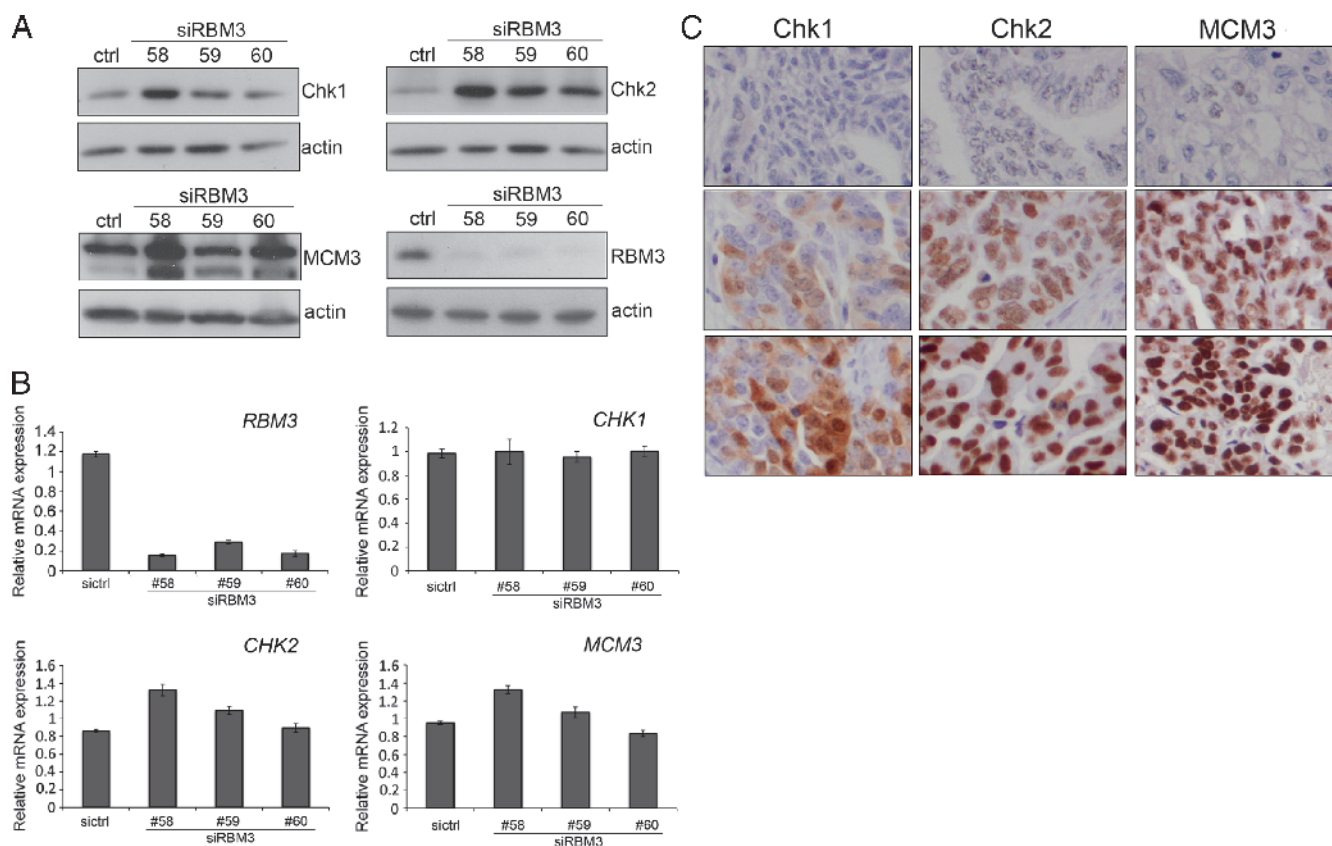
### Validation of Selected Candidates by Western Blot Analysis and Real-time QPCR in siRBM3-Treated A2780 Ovarian Cancer Cells

The HPA platform was then used to select the most promising biomarkers identified from the GSEA for further validation. As increased RBM3 was associated with an improved prognosis and cisplatin sensitivity, we concentrated on cellular processes associated with high RBM3 mRNA expression. From the list of differentially expressed genes associated with DNA-dependent replication, DNA replication, chromatin remodeling, and DNA integrity checkpoint, we selected corresponding proteins with a differential expression pattern in EOC in the HPA, with available validated antibodies. In total, 28 genes were selected for an initial validation in the human ovarian cancer cell line A2780 (Table W2). Of these 28 genes, 3 were chosen for further validation; the DNA damage checkpoint kinases (*CHK1* and *CHK2*) and minichromosome maintenance protein 3 (*MCM3*). These markers were chosen because they play a role in DNA integrity, and we have previously shown that RBM3 sensitizes A2780 cells to the DNA-damaging agent cisplatin.

To demonstrate an association between RBM3 and the selected candidates, A2780 cells were transfected with RBM3-specific siRNA followed by Western blot analysis. siRNA-mediated knockdown of RBM3 resulted in an increase in Chk1, Chk2, and MCM3 protein expression (Figure 2A). Real-time QPCR demonstrated that siRNA-mediated knockdown of RBM3 did not alter transcription of the *CHK1*, *CHK2*, and *MCM3* genes, suggesting that RBM3 may silence translation of these proteins (Figure 2B). Evaluation of Chk1, Chk2, and MCM3 protein expression in EOC tumor tissue demonstrated nuclear expression of all three proteins, with Chk1 also expressed occasionally in the cytoplasm (Figure 2C).

### Survival Analysis of RBM3-Regulated Biomarkers in EOC

Cohort 1 ( $n = 267$ ) was used to examine the prognostic value of *CHK1*, *CHK2*, and *MCM3* at the mRNA level, and immunohistochemistry was performed on a TMA consisting of 154 prospectively collected EOC cases (cohort 2) using antibodies against the corresponding proteins. As visualized in Table 1, the relationship between RBM3 and the candidate biomarkers demonstrated a negative correlation



**Figure 2.** Downregulation of RBM3 affects the expression of MCM3, Chk1, and Chk2. The expression of MCM3, Chk1, Chk2, and RBM3 were examined by (A) Western blot analysis and (B) reverse transcription–PCR in A2780 cells 48 hours after transfection of cells with three different siRNAs targeting RBM3 (nos. 58, 59, and 60). Data shown are mean ± SEM of four, for siRBM3 nos. 58 and 59, and three for siRBM3 no. 60, independent experiments performed in triplicate. (C) Immunohistochemical staining of Chk1, Chk2, and MCM3 in EOC tumors denoted as negative, intermediate, and strong.

between RBM3 and *MCM3*, *CHK1*, and *CHK2* at the mRNA level in cohort 1 but not at the protein level in cohort 2. *MCM3*, *CHK1*, and *CHK2* correlated significantly with each other and with a lower differentiation grade in both cohorts. *MCM3* expression was associated with a more advanced clinical stage in both cohorts, and the same was seen for *CHK1* in cohort 1, whereas *CHK2* mRNA or protein levels were not significantly associated with clinical stage.

Kaplan-Meier analysis demonstrated an association between high *MCM3* mRNA and protein expression and a significantly reduced RFS, but not OS, in cohort 1 and a reduced OS in cohort 2 (Figure 3A). Cox univariate analysis confirmed the association between increased *MCM3* mRNA expression and decreased RFS (hazard ratio [HR] = 1.98, 95% confidence interval [CI] = 1.18-3.31, *P* = .010) in cohort 1 and increased MCM3 protein expression (NS > 3) and poor OS in cohort 2 (HR = 1.82, 95% CI = 1.09-3.04, *P* = .022). However, multivariate Cox regression analysis did not confirm *MCM3* as an independent prognostic marker in either cohort (Table 2).

Kaplan-Meier analysis revealed an association between high *CHK1* mRNA levels and a reduced RFS in cohort 1 (Figure 3B), which was further confirmed by Cox univariate analysis (HR = 2.05, 95% CI = 1.33-3.15, *P* = .001) (Table 2). Cox multivariate analysis demonstrated that *CHK1* was not an independent predictor of RFS (HR = 1.37, 95% CI = 0.84-2.24, *P* = .203) in cohort 1 (Table 2), and *CHK1* mRNA expression was not associated with OS (Figure 3B). In cohort 2, Chk1 protein expression (NS > 0) was associated with a

**Table 1.** Associations between Chk1, Chk2, and MCM3 and Patient and Tumor Characteristics in Cohorts 1 and 2.

Cohort	1			2		
	CHK1	CHK2	MCM3	Chk1	Chk2	MCM3
Age						
<i>R</i>	0.102	0.069	0.168	-0.009	0.038	0.126
<i>P</i>	.1	.265	<b>.006</b>	.914	.652	.139
<i>n</i>	263	263	263	141	145	140
Differentiation grade						
<i>R</i>	0.329	0.186	0.323	0.328	0.270	0.286
<i>P</i>	<b>&lt;.001</b>	<b>.003</b>	<b>&lt;.001</b>	<b>&lt;.001</b>	<b>.001</b>	<b>.001</b>
<i>n</i>	263	260	260	141	145	140
Clinical stage						
<i>R</i>	0.13	0.108	0.141	0.129	0.116	0.181
<i>P</i>	<b>.035</b>	.080	<b>.022</b>	.141	.185	<b>.04</b>
<i>n</i>	263	263	263	131	133	130
RBM3						
<i>R</i>	-0.247	-0.192	-0.253	0.126	0.074	0.074
<i>P</i>	<b>&lt;.001</b>	<b>.002</b>	<b>&lt;.001</b>	.138	.38	.386
<i>n</i>	263	263	263	140	143	140
<i>CHK1/Chk1</i>						
<i>R</i>		0.440	0.599		0.451	0.462
<i>P</i>		<b>&lt;.001</b>	<b>&lt;.001</b>		<b>&lt;.001</b>	<b>&lt;.001</b>
<i>n</i>		263	263		139	137
<i>CHK2/Chk2</i>						
<i>R</i>		0.440	0.412		0.451	0.509
<i>P</i>		<b>&lt;.001</b>	<b>&lt;.001</b>		<b>&lt;.001</b>	<b>&lt;.001</b>
<i>n</i>		263	263		139	139

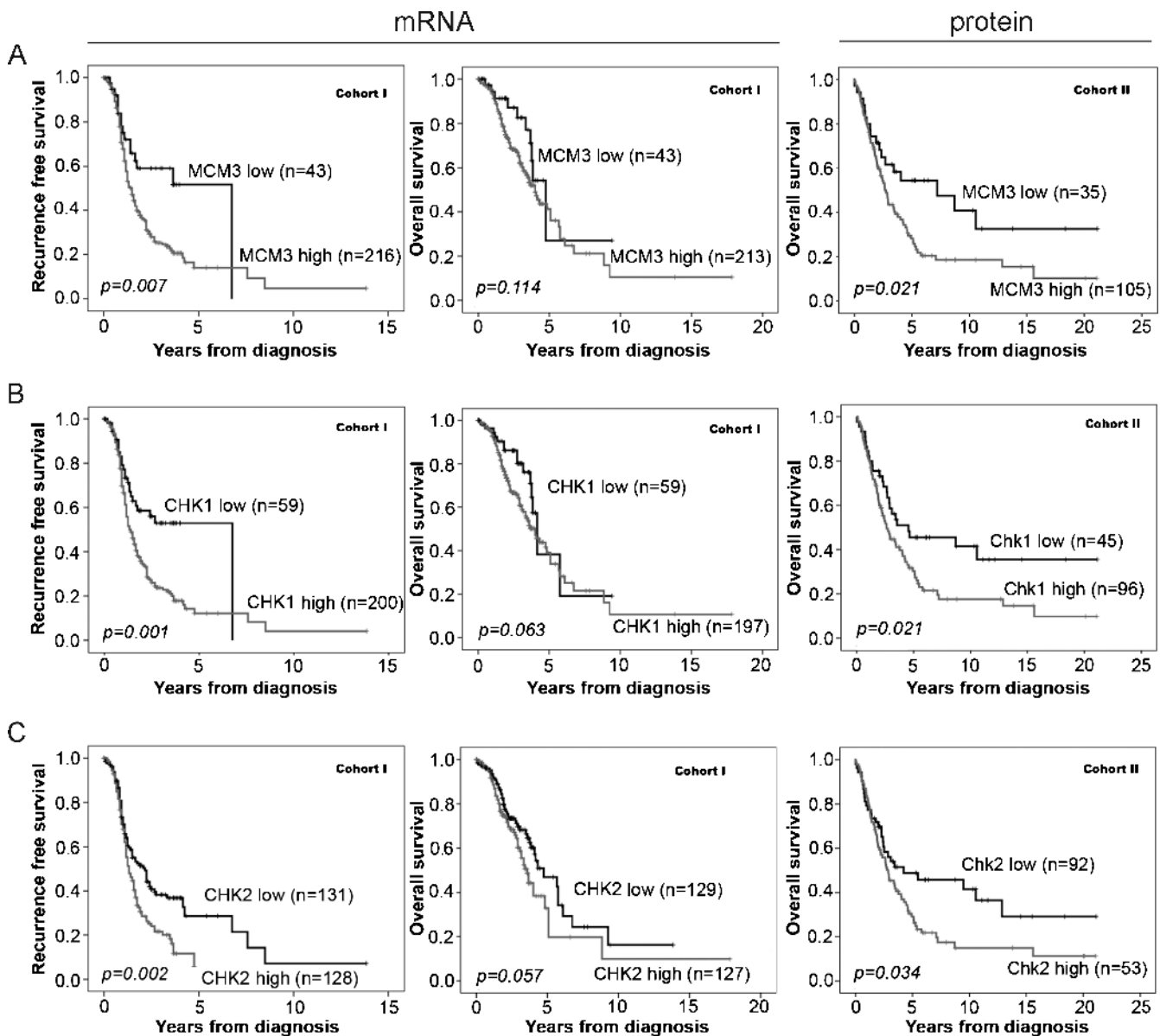
*n* indicates number of tumor samples; *R*, Spearman correlations coefficient. *P* < .005 in bold.

reduced OS (Figure 3B) confirmed by Cox univariate analysis (HR = 1.70, 95% CI = 1.08-2.68,  $P = .023$ ). However, multivariate analysis did not confirm Chk1 protein expression as an independent predictor of OS in cohort 2 (HR = 1.23, 95% CI = 0.71-2.10,  $P = .47$ ) (Table 2).

High levels of Chk2, both mRNA and protein levels, were associated with an impaired survival in EOC (Figure 3C). Cox univariate analysis confirmed the association between increased *CHK2* mRNA expression and RFS in cohort 1 (HR = 1.61, 95% CI = 1.19-2.19,  $P = .002$ ) and Chk2 protein expression (NS > 0) and OS in cohort 2 (HR = 1.59, 95% CI = 1.03-2.47,  $P = .036$ ). Multivariate Cox regression analysis confirmed the association between high *CHK2* mRNA expression and poor outcome in cohort 1 (HR = 1.52, 95% CI = 1.08-2.13,  $P = .015$ ); however, this was not replicated at the protein level in the cohort 2 (HR = 1.21, 95% CI = 0.97-1.97,  $P = .448$ ) (Table 2).

**Down-regulation of RBM3 Generates an Increase in Phosphorylation of Chk1**

The protein kinases Chk1 and Chk2 are crucial checkpoint proteins activated in response to DNA damage by signals from ATM and ATR leading to cell cycle arrest and DNA repair through activation of a complex signaling network [25–27]. Taken in the context of a previous study by Sureban et al. [28], who demonstrated that down-regulation of RBM3 in the human colon adenocarcinoma cell line HCT116 resulted in activation of DNA damage response by phosphorylation of the checkpoint proteins Chk1 and Chk2 [28], we hypothesized that RBM3 activates Chk1 and Chk2 in EOC. To address this issue, we examined the phosphorylation of Chk1 at Ser-345 and Chk2 at Thr-68 by Western blot analysis in A2780 cells transfected with siRNA targeting RBM3. A slightly higher level of pSer345-Chk1, but not pThr68-Chk2, was observed in the siRBM3 transfected A2780 cells (Figure 4A), indicating activation of the



**Figure 3.** Increased mRNA (cohort 1) and protein expression (cohort 2) of MCM3, Chk1, and Chk2 are associated with an impaired survival. Kaplan-Meier analysis of RFS and OS according to (A) *MCM3*, (B) *CHK1*, and (C) *CHK2* mRNA levels in cohort 1. Kaplan-Meier analysis of OS according to immunohistochemical (A) MCM3, (B) Chk1, and (C) Chk2 staining in cohort 2.

**Table 2.** Cox Univariate and Multivariate Analyses of RFS of Chk1, Chk2, and MCM3 According to mRNA Expression (Cohort 1) and Protein Expression (Cohort 2).

Cohort 1 (mRNA)			Cohort 2 (Protein)		
	HR (95% CI)	P		HR (95% CI)	P
<b>MCM3</b>			<b>MCM3</b>		
Univariate			Univariate		
Low	1.00		Low	1.00	
High	1.98 (1.18-3.31)	.01	High	1.82 (1.09-3.04)	.022
Multivariate			Multivariate		
Low	1.00		Low	1.00	
High	2.39 (1.30-0.72)	.383	High	1.05 (0.60-1.83)	.871
<b>Chk1</b>			<b>Chk1</b>		
Univariate			Univariate		
Low	1.00		Low	1.00	
High	2.05 (1.33-3.15)	.001	High	1.70 (1.08-2.68)	.023
Multivariate			Multivariate		
Low	1.00		Low	1.00	
High	1.37 (0.84-2.24)	.203	High	1.22 (0.71-2.10)	.47
<b>Chk2</b>			<b>Chk2</b>		
Univariate			Univariate		
Low	1.00		Low	1.00	
High	1.61 (1.19-2.19)	.002	High	1.59 (1.03-2.46)	.036
Multivariate			Multivariate		
Low	1.00		Low	1.00	
High	1.52 (1.08-2.13)	.015	High	1.21 (0.74-1.97)	.448

Multivariate analysis included adjustment for age (continuous), stage (I-II vs III-IV), grade (1-2 vs 3), and residual disease (none vs any, only available for cohort 1).

checkpoint proteins by down-regulation of RBM3 in the absence of DNA damage. This suggests that RBM3 may restrain a checkpoint response in the absence of DNA damage by regulating the protein levels of Chk1 and Chk2 to maintain a relative low cellular level of the phosphorylated and total proteins in absence of a DNA damage stimulus.

**Phosphorylated Chk2 on Thr-68 Is Associated with an Impaired Survival**

Having demonstrated that RBM3 regulates Chk1 and Chk2 protein expression *in vitro* and that both Chk1 and Chk2 are associated with an impaired survival in EOC, we next sought to examine the relationship between phosphorylated Chk1 and Chk2 and prognosis. pSer345-Chk1 and pThr68-Chk2 were thus assessed using immunohistochemistry in cohort 2 (Figure 4B).

RBM3 protein expression did not correlate with phosphorylated Chk1 or Chk2; however, there was a significant correlation between pSer345-Chk1 and pThr68-Chk2 ( $R = 0.298, P < .001$ ). Neither pSer345-Chk1 nor pThr68-Chk2 was associated with any clinicopathological parameters (data not shown). Kaplan-Meier analysis demonstrated no prognostic significance of pSer345-Chk1; however, pThr68-Chk2 positivity (NS > 0) was associated with a reduced OS ( $P = .047$ ; Figure 4C). Cox univariate analysis confirmed the association between pThr68-Chk2 and a reduced OS (HR = 1.62, 95% CI = 1.00-2.63,  $P = .049$ ); however, this did not remain significant in multivariate analysis.

**Discussion**

We previously reported an association between RBM3 and a prolonged survival in breast cancer and EOC [10,11]. In the present study, we identified differentially expressed genes in EOC tumors with high versus low RBM3 expression, aiming to discover novel prognostic EOC biomarkers and to gain a deeper understanding of the function of RBM3. GSEA revealed an association between RBM3 expression

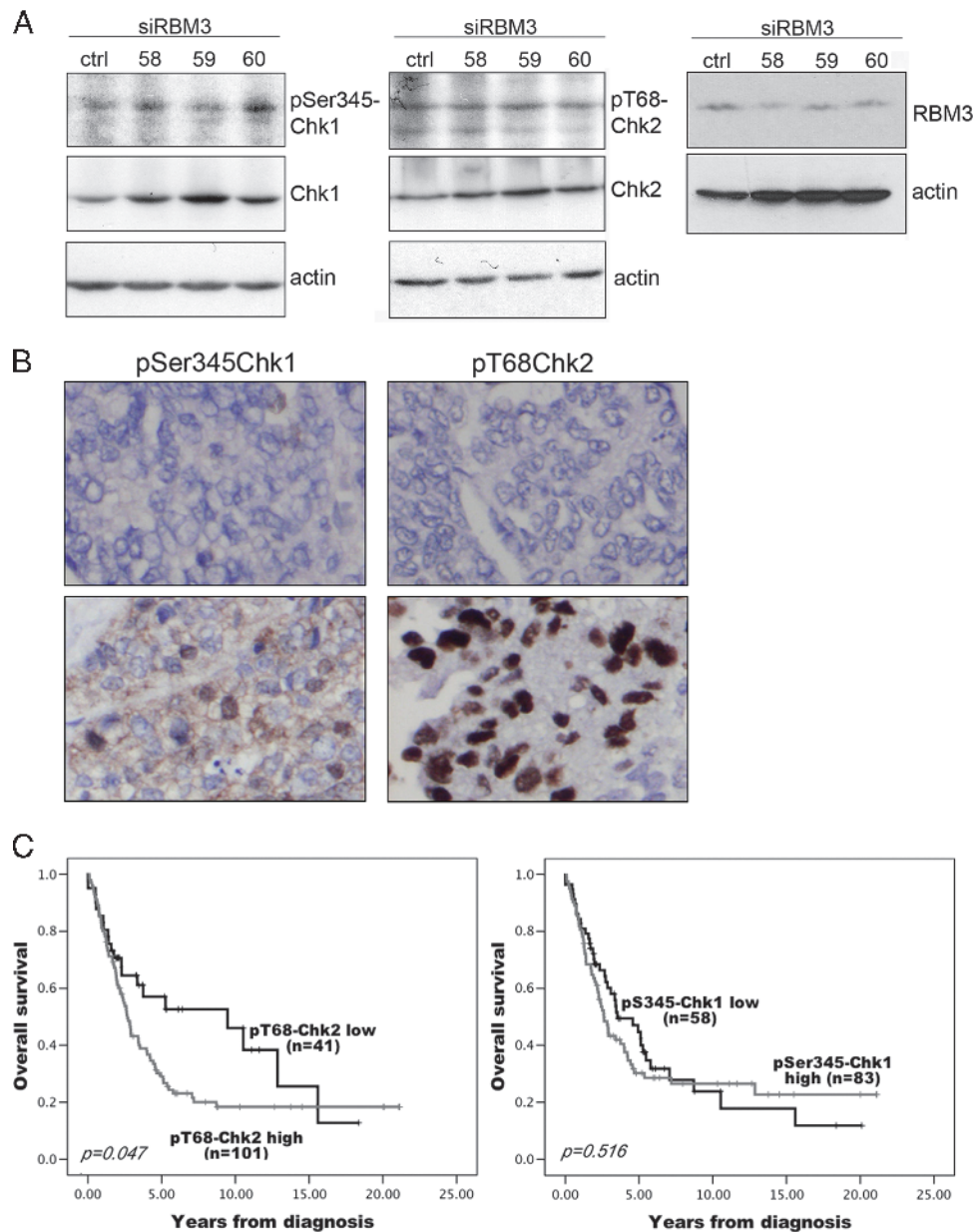
and a number of cellular processes involved in the maintenance of DNA integrity including regulation of DNA replication, DNA replication, chromatin remodeling, and DNA integrity checkpoint. In the light of previous findings demonstrating a relationship between RBM3 and cisplatin sensitivity [11], these results suggest that RBM3 may be involved in the cellular response to DNA damage. Further investigations are, however, required to prove this hypothesis and to determine the exact function of RBM3 in this context.

The unearthing of RBM3 as a putative prognostic tissue biomarker in EOC was the result of an antibody-based approach, using the HPA as a discovery tool [9], followed by further validation in clinically well-annotated tumor samples from two independent EOC cohorts [11]. In this study, we used an integrated transcriptomic and proteomic approach, based on tumor samples from the same clinical cohorts, to identify novel putative EOC biomarkers among RBM3-associated genes and their corresponding proteins. Our results provide, to our knowledge, the first description of an association between high expression of Chk1, Chk2, and MCM3 and poor prognosis in EOC patients.

Chk1 has previously been associated with tumor grade and cell proliferation in breast cancer [29], and *CHK2* mutations have been frequently studied in the context of hereditary breast cancer [30]. The negative correlation demonstrated between RBM3 and DNA damage checkpoint proteins Chk1 and Chk2 *in vitro* suggests that RBM3 might be involved in DNA damage response. These serine/threonine protein kinases play crucial roles in maintaining genomic stability by mediating the signaling cascade initiated by the checkpoint proteins ATM and ATR in response to DNA damage leading to DNA repair, cell cycle arrest, or apoptosis [27]. Chk1 and Chk2 are phosphorylated by ATM and ATR in response to DNA damage, and once activated, they can phosphorylate downstream targets and control cell cycle progression by regulating the activities of Cdc25 phosphatases [31–33], p53 [34], and DNA repair factors [35]. Traditionally, the signaling network has been divided into two major protein kinase pathways: ATM activating Chk2 in response to double-stranded breaks and ATR operating together with Chk1 in response to single-stranded breaks and stalled replication forks during the S phase. The negative correlation between RBM3 and Chk1/Chk2 further emphasizes that RBM3 expression may predict response to platinum-based chemotherapy by silencing these important regulators of cellular DNA damage response. Inhibition of Chk1 and ATR was recently shown to generate the greatest impact on cisplatin response in ovarian cancer cell lines as illustrated in an RNAi screen [36], which might, in part, explain the cisplatin-sensitizing effect of RBM3 that we have previously described [11]. Inhibition of Chk1 has been reported to sensitize tumor cells to chemotherapy in various cell lines [37–39], and several Chk1 inhibitors have been developed and evaluated in clinical trials [40].

MCM proteins are key components of the DNA replication licensing system essential for maintenance of precise chromosome duplication [41,42]. Disruption of genetic stability has been reported to be a consequence of deregulation of the MCM complexes in yeast, and abnormal expression of MCM proteins has been observed in human cancers. A high expression of MCM3 protein has been reported to be associated with an impaired survival in malignant glioma [43], medulloblastoma [44], and malignant melanoma [45].

The negative association between RBM3 and *CHK1, CHK2,* and *MCM3* genes seen in cohort 1 was not replicated at the protein level in cohort 2, which could potentially be explained by the smaller number of patients in the latter. However, *in vitro* experiments showed a clear inverse association between RBM3 and Chk1, Chk2 and MCM3 at



**Figure 4.** The impact of RBM3 on phosphorylation of Chk1 and Chk2 and the association of pSer345-Chk1 and pT68-Chk2 with OS in EOC. The impact of RBM3 on checkpoint response was examined in siRBM3-transfected cells by Western blot analysis using antibodies against (A) pSer345-Chk1 and pT68-Chk2. (B) Immunohistochemical staining of pSer345-Chk1 and pT68-Chk2 in EOC tumors. (C) Kaplan Meier analysis of OS according to immunohistochemical staining of pS345-Chk1 and pT68-Chk2 in cohort 2 in strata defined as high versus low expression.

the protein level, whereby siRNA-mediated down-regulation of RBM3 in A2780 cells resulted in an obvious increase in Chk1, Chk2, and MCM3 48 hours after transfection, in contrast to a nonsignificant alteration at the mRNA level. Considering the fact that RBM3 is a RNA-binding protein [46], it could be speculated that RBM3 binds and destabilizes the transcripts of *CHK1*, *CHK2*, and *MCM3* in RBM3 high tumors, hence the inverse relationship observed *in vivo*. In addition, a reason to why we did not detect a significant increase in the mRNA levels of Chk1, Chk2, and MCM3 in response to a down-regulation of RBM3 *in vitro* might be that RBM3 primarily acts at the translational rather than the transcriptional level. RBM3 has indeed been reported to be involved in translation contributing to an enhanced rather than suppressed global translation [47–49]. Another hy-

pothesis is that RBM3 indirectly contributes to low levels of some checkpoint proteins by enhanced translation of proteins involved in the turnover of these proteins. Additional, more detailed investigations are required to gain further mechanistic insight into how RBM3 affects the levels of Chk1, Chk2, and MCM3. A limitation to this study is that the *in vitro* experiments have been performed on only one cell line, and future studies should include additional cell models.

Although the functional role of RBM3 in DNA damage requires further investigation, our data indicate a possible suppressive role of RBM3 on the checkpoint response in the absence of DNA damage, illustrated by the observed increased phosphorylation of Chk1 on silencing of RBM3 in the A2780 cells. In line with this observation, down-regulation of RBM3 in colorectal cancer cell lines led to



activation of both Chk1 and Chk2 [28]. Immunohistochemical analysis revealed a negative prognostic value for pT68-Chk2 but not for pS345-Chk1 in cohort 2. The negative prognostic value observed for pT68-Chk2-expressing tumors could be because these tumors have an activated checkpoint response and are thus undergoing pressure for selection of a mutated, more aggressive, phenotype [50,51]. Along this line, it could be speculated that an attenuated DNA damage response imposed by RBM3 could explain the association with a good prognosis observed in RBM3 high breast cancers, irrespective of adjuvant chemotherapy [10].

In conclusion, we have, for the first time, revealed a link between RBM3 in DNA damage response. In addition, three novel potential biomarkers in EOC have been identified: MCM3, Chk1, and Chk2. The negative correlation between RBM3 and Chk1 and Chk2 protein levels *in vitro* might, in part, explain the positive effect of RBM3 on cisplatin response observed in ovarian cancer cell lines. Further investigations are required to understand the mechanisms behind the observed findings and to explain the function of RBM3, particularly its association with a good prognosis in EOC and other cancer forms.

## Acknowledgments

The authors thank Elise Nilsson for excellent technical assistance.

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**Table W1.** Sequences of Primers Used in Real-time QPCRs.

Gene	Forward (5'-3')	Reverse (5'-3')
<i>HMBS</i>	GGC AAT GCG GCT GCA A	GGG TAC CCA CGC GAA TCA C
<i>YWHAZ</i>	TGG GAA CAA GAG GGC ATC TG	CCA CCA CTG CAT CAA ATT CAT G
<i>UBC</i>	ATT TGG GTC GCG GTT CTT G	TGC CTT GAC ATT CTC GAT GGT
<i>RBM3</i>	CIT CAG CAG TTT CGG ACC TA	ACC ATC CAG AGA CTC TCC GT
<i>CHK1</i>	CAACTTGCTGTGAATAGAGTAACTGAAGA	ACAGTCTACGGCAGCCTTCAT
<i>CHK2</i>	GTGTGAATGACAACACTACTGGTTTGG	TTCITTTTCAGCAGTGGTTCATC
<i>MCM3</i>	ACCAGGAATTTATCAGAGCAAAG	CAGGTCATTCACATTGACAATCAG

**Table W2.** Selected Genes from the HPA for Further Validation by Western Blot in A2780 Cells.

Gene Name	Ab ID	Dilutions
<i>BNIP3</i>	ABCAM 28506	1:600
<i>HELLS/LSH</i>	SC-46665	1:500
<i>RSF1</i>	Upstate 05-727	N/A
<i>SMARCC1</i>	HPA024352	1:3000
<i>NASP</i>	HPA028136	1:250
<i>NBN</i>	HPA001429	1:250
<i>NF2</i>	HPA003097	1:250
<i>ATR</i>	SC-1887	1:1000
<i>WRNIP1</i>	HPA031752	1:100
<i>CDK2</i>	BD	1:500
<i>RAD1</i>	HPA006692	1:500
<i>NEK11</i>	HPA016908	1:1000
<i>HUS1</i>	HPA026787	1:100
<i>CCNA2</i>	SC-751	1:500
<i>CHK2</i>	Cell Signaling no. 3440	1:1000
<i>TERF2IP</i>	HPA006719	1:100
<i>POLA1</i>	HPA002947	1:250
<i>DKC1</i>	HPA001022	1:500
<i>MCM5</i>	SEROTEC MCA1860	N/A
<i>TERF2</i>	HPA001907	N/A
<i>UPF1</i>	HPA019587	N/A
<i>ORC2L</i>	Abnova H00004999-M01	N/A
<i>MSH6</i>	HPA028376	1:300
<i>MSH2</i>	BD	1:500
<i>MCM3</i>	HPA004789	1:300
<i>RFC4</i>	SC-28301	1:500
<i>CHK1</i>	Cell Signaling, no. 2360	1:1000

N/A indicates not analyzed.