The Role of RNA-Binding Motif 3 in Epithelial Ovarian Cancer: A Biomarker Discovery Approach

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For my Family
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List of Papers

This thesis is based on the following papers, referred to in the text by their respective Roman numerals.

Paper I
Expression of the RNA-binding protein RBM3 is associated with a favourable prognosis and cisplatin sensitivity in epithelial ovarian cancer.
*J Transl Med. 2010; 8:78*

Paper II
RBM3-regulated genes promote DNA integrity and affect clinical outcome in epithelial ovarian cancer.
*Submitted*

Paper III
Zarrizi R, Ehlén Å, Jirström K, Alvarado-Kristensson M.
Expression of the RNA binding protein RBM3 alters cell cycle progression.
*Manuscript*

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Abbreviations

2DE  two-dimensional electrophoresis
53BP1  p53-binding protein-1
A-T  ataxia-telangiectasia
AREs  AU-rich elements
ATM  ataxia telangiectasia mutated
ATR  ataxia telangiectasia mutated and Rad3-related
BER  base excision repair
CA125  serum cancer antigen 125
Cdns  cyclin-dependent protein kinases
CTR1  copper-transporter-1
DIGE  difference gel electrophoresis
DNA  deoxyribonucleic acid
DNA-PK  DNA-dependent protein kinase
DSB  double-strand breaks
EOC  epithelial ovarian cancer
ER  estrogen receptor
ERCC1  excision repair cross-complementing-1 protein
GCR  global genomic repair
GSEA  Gene Set Enrichment Analysis
GST  glutathione-S-transferases
HE4  human epididymis protein 4
HPA  The Human Protein Atlas
HR  homologous recombination
ICLs  interstrand crosslinks
IHC  immunohistochemistry
IRES  internal ribosome entry sites
MDC1  mediator of DNA-damage checkpoint-1
MMR  mismatch repair
MRN  multiprotein complex Mre11-Rad50-Nbs1
MS/MS  tandem mass spectrometry
NER  nucleotide excision repair
NHEJ  non-homologous end joining
ORC  origin recognition complex
OS  overall survival
PARP  poly (ADP-ribose) polymerase
PCNA  proliferating cell nuclear antigen
PI3K  lipid kinase phosphatidylinositol 3-kinase
PIKKs  phosphatidylinositol 3-kinase like protein kinase family
PRA  replication protein A
PRC  pre-replicative complex
RNA  ribonucleic acid
PrEST  protein epitope signature tags
RFS  recurrence free survival
RNAPII  RNA polymerase II
RRM  RNA-recognition motifs
RPPAs  reverse phase protein arrays
SDS-PAGE  second dimension on a polyacrylamide gel
SSB  single-strand breaks
ssDNA  single-strand DNA
TCR  transcription-coupled repair
TFIIF  ten-component transcription factor
TGCT  testicular germ cell tumors
TMAs  tissue microarrays
UTRs  untranslated regions
UV  ultra violet light
XPA  complementation group A protein
XPC  XP complementation group C protein
XPF  XP complementation group F protein
XRP  xeroderma pigmentosum
Ovarian Cancer

Each year epithelial ovarian cancer (EOC) claims the lives of more than 100,000 women worldwide, making it the most lethal gynaecological malignancy and the fifth most common cause of cancer-related death in women in the Western world [1, 2]. EOC is often referred to as the ‘silent’ killer, with a large number of deaths associated with the disease. The poor ratio of survival to incidence 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. Today, the five year survival rate in EOC is only 45% [1], but if detected at an early stage, while the tumor is still limited to the ovary, the cure rate is up to 90% with conventional surgery and chemotherapy [3]. However, the symptoms of EOC are often vague and overlap with other more common gastrointestinal and gynaecological diseases, making it difficult to detect [4] and only 20% of all ovarian cancers are diagnosed at an early stage [3]. At the molecular level, EOC is a highly heterogenous disease. More than 90% of ovarian cancers originate from the epithelial surface of the ovary, the rest consists of rare germ cells tumors (teratomas and dysgerminomas), ovarian sarcomas, granulosa cell tumors, thecomas and Leydig and Sertoli cell tumors [2].

Genetic alterations in ovarian cancers
Accumulations of genetic abnormalities will eventually lead to tumor formation [5] and at least 30 oncogenes and tumor suppressor genes have been implicated in EOC [2, 3]. Increased expression of oncogenes caused by gene amplification, mutations and hypomethylation, or inactivation of tumor suppressor genes by deletions of large chromosome regions, will drive altered cellular signaling, resulting in increased proliferation, inhibition of apoptosis, increased motility and subsequently invasion of adjacent tissues [6]. It is far beyond the scope of this thesis to review all known genetic alterations that have been implicated in EOC, however, because of the well characterized important role of the tumor suppressor genes BRCA1, BRCA2 and p53 in the cellular response to DNA damage, the impact of these genes in EOC will be briefly discussed here.

Women carrying germline mutations in either BRCA1 or BRCA2 have an increased risk to develop tumors in the breast and ovaries [7] Inherited mutations in cancer
predisposition genes, predominantly *BRCA1* and *BRCA2*, account for 10-15% of all ovarian cancers [3, 8]. The tumor suppressor proteins BRCA1 and BRCA2 are crucial components of the homologous recombination DNA repair machinery, essential for the cell to handle DNA double-stranded breaks (DSB) [9, 10]. While a single genetic event might be sufficient for activation of an oncogene, both alleles of a tumor suppressor gene must normally be inactivated to lose the function of the corresponding protein and, consequently, contribute to transformation. In women with a germline mutation in *BRCA1* or *BRCA2* the remaining normal allele is often inactivated through loss of heterozygosity, which leads to total loss of function of the protein and subsequently to chromosomal rearrangement and genetic instability due to an impaired capacity to repair DNA damage [9, 10]. Somatic mutations in *BRCA1* and *BRCA2* genes are rare and it was originally thought that these genes played a limited role in the development of sporadic ovarian cancer. However, it is today known that alterations of *BRCA1* and *BRCA2* expression occur in sporadic ovarian cancer via loss of heterozygosity and transcriptional silencing through hypermethylation [11, 12]. Interestingly, sporadic ovarian cancer exhibits gene expression profiles similar to *BRCA1* or *BRCA2*-associated cancers, as demonstrated by a cDNA microarray analysis comparing the gene expression in ovarian cancers with germline mutations in either of the two *BRCA* genes with the gene expression in patients with sporadic ovarian cancer [13]. This analysis revealed that sporadic ovarian cancer could be segregated into two categories resembling the gene profile in hereditary ovarian cancer with either *BRCA1* or *BRCA2* mutations, suggesting *BRCA* genes to be important in sporadic ovarian cancer as well as in hereditary cancer and that deregulation of *BRCA* genes contributes to a specific pattern of gene expression [13, 14]. Transcriptional silencing of *BRCA1* appears to be much more common than silencing of *BRCA2* [11], and reduced expression of *BRCA1* has also been found in sporadic breast cancer. Although *BRCA1* is ubiquitously expressed in all tissues it appears that the predisposition for inactivation of the protein is higher in ovarian and breast epithelium compared to other tissues. The role of BRCA protein dysfunction in the progression of sporadic ovarian and breast cancer is not fully understood and it remains to be elucidated whether the breast and ovaries are more susceptible to somatic mutations of BRCA1 or more dependent on proper BRCA1 signaling than other tissues. It has been suggested that BRCA1 influences steroid production and thereby exerts its tumor suppressive activity [12]. Several studies have shown a survival advantage of patients with germline mutations in the *BRCA* genes over patients with sporadic ovarian cancer, due to an increased sensitivity to chemotherapy inducing DNA DSBs [12]. Several studies have demonstrated a hypersensitivity of BRCA1 and BRCA2 deficient cells to DNA-crosslinking agents like the platinum compound cisplatin due to inefficient DNA repair capacity [15, 16]. Similar to platinum compounds, cells with defects in the DNA homologous repair signaling pathways show high sensitivity to poly(ADP-ribose) polymerase (PARP) inhibitors [17, 18]. Although BRCA1/2 mutant ovarian cancer cells show a strong initial response to platinum compounds, they will ultimately develop resistance to the...
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drug. Despite numerous investigations, the underlying mechanisms behind cisplatin resistance are still largely unknown. It has been reported that a second mutation in the BRCA genes can restore the wild type open reading frame of the genes and, thus, the cellular capacity of DNA repair, which will eventually render the cells resistant to both platinum compounds and PARP inhibitors [19-21].

TP53, 'the guardian of the genome' is the most frequently mutated gene in human cancer and one of the most well studied proteins in the context of tumor formation and progression. In response to different kinds of stress the transcriptional activity of p53 leads to growth arrest, senescence or apoptosis [22, 23]. To ensure rapid response to DNA damage, the TP53 gene is constantly transcribed, however in the absence of DNA damage p53 is quickly degraded by the proteasome. The ubiquitin ligase MDM2 is the protein mainly responsible for p53-regulation, in that by binding to p53 it forces p53 into an inactive configuration as well as marks it for degradation [24]. Stabilization and accumulation of p53 occurs in response to DNA damage as a consequence of activation of ATM, ATR and the checkpoint proteins Chk1 and Chk2, that all are capable of disruption of the interaction between MDM2 and p53 by phosphorylation of MDM2 [25, 26].

**Treatment of EOC with platinum compounds**
Standard treatment of advanced EOC involves surgical debulking followed by adjuvant chemotherapy with a combination of a platinum compound (cisplatin or carboplatin) and taxane [27]. In the mid 1960s the biological effect of the platinum-based compound cisplatin was accidentally discovered in bacteria, and cisplatin was eventually approved as a anti-cancer drug by the US Food and Drug Administration.
(FDA) in 1978 [28]. Since then, cisplatin has had a major impact on cancer therapy, particularly in the treatment of testicular and ovarian cancer [28]. Cisplatin acts by forming covalent bonds with purine DNA bases which causes cross-linking of DNA and results in activation of several signal transduction pathways involved in DNA-damage repair, cell cycle arrest and apoptosis [28-30]. The major DNA lesions induced by cisplatin are intrastrand DNA crosslinks between two guanines or guanine and adenine. Interstrand crosslinks (ICLs) between the two DNA strands are less frequent, counting for 5% of all cisplatin lesions [31]. The major DNA repair pathway known to remove cisplatin lesions from DNA is the nucleotide-excision repair (NER).

Despite an initial response to cisplatin treatment, many patients with EOC develop resistance to the drug and relapse within a few years [32]. Several mechanisms have been implicated in cisplatin resistance, i.e. decreased drug uptake, insufficient DNA-binding of the drug, increased DNA-repair of cisplatin adducts and failure of induction of apoptosis, reviewed in [28, 30, 33]. Cisplatin is highly polar and enters the cell either via the plasma-membrane transporter involved in copper homeostasis, the copper-transporter-1 (CTR1) or by passive diffusion. In contrast to the multidrug resistance mechanism, with increased activation of efflux pumps, it is rather a decreased uptake than increased efflux that causes resistance [33]. Active cisplatin has a high affinity for sulphur molecules and can be maintained in the cytoplasm by interaction with species high in sulphur-containing amino acids, like cysteine and methionine, resulting in less cisplatin reaching the nucleus and the DNA. A correlation between cisplatin resistance and the level of glutathione-S-transferases (GSTs) has been observed [28]. The main reason for cisplatin resistance is believed to be related to an increased capacity of the cell to remove and repair the induced DNA adduct. The hypersensitivity of testicular carcinomas to platinum derivatives is thought to be related to DNA repair-deficiency [28, 31]. The DNA repair mechanisms will be discussed in detail in a later section.

**Biomarkers in EOC**

The poor prognosis of EOC is largely related to late diagnosis of the disease and the absence of successful screening tools. Thus, there is an urgent need for reliable biomarkers of early diagnosis, i.e. a biological molecule detectable in blood or tissue as a sign of abnormalities, with high sensitivity and specificity for EOC, that can be used for screening purposes and monitoring of relapse. In addition, prognostic and treatment biomarkers are needed in order to improve individualized treatment of EOC patients.

The most common EOC biomarker in clinical use today is serum cancer antigen 125 (CA125). CA125 is a high molecular weight mucinous glycoprotein that has been extensively evaluated as a potential biomarker for early detection and, hence, screening tool for EOC [34, 35]. Up to 80% of all EOC cases express CA125 and
assessment of its serum levels have been used for monitoring treatment response, as alterations have been demonstrated to correspond to regression or progression of the disease [3]. However, there are limitations to the use of CA125 as a marker of disease progression as approximately 20% of women diagnosed with ovarian cancer have tumors that do not express CA125, and these patients will be left without any reliable marker for monitoring the progression of the disease [35]. Regarding its potential use as a screening tool, CA125 is lacking appropriate sensitivity since 50% of early stage ovarian cancers do not show an increase in serum levels of CA125. Furthermore, there are also limitations to the specificity of CA125 towards EOC, as many benign gynecological diseases and other, non-ovarian, malignancies may generate elevated levels of CA125 [35]. In addition, serum levels of CA125 vary according to menopausal status, with decreasing levels in postmenopausal women [3]. In addition to CA125, elevated levels of human epididymis protein 4 (HE4) have been detected in the blood of ovarian cancer patients [35]. HE4 has not been detected in patients with benign gynecological diseases, thereby showing a higher specificity than CA125. It has been demonstrated that HE4 is expressed in over half of the EOC cases lacking CA125 expression suggesting that, by using a combination of the two markers, the progression of the disease can be monitored in over 90% of all EOC patients [35].

Although multiple new biomarkers have been identified and reported lately, only CA125 and HE4, have been approved by the United States FDA for monitoring of the progression of EOC [35]. Notably, in contrast to the situation in e.g. breast cancer, no biomarkers of prognosis or treatment prediction in EOC have been yet incorporated into clinical protocols.
Cancer Biomarker Discovery

Disease biomarkers are valuable tools for a proper diagnostics, prognostication and/or treatment prediction and for monitoring disease progression/relapse. In the field of cancer, major efforts and investments have been made over the past 15 years in the search for new cancer biomarkers. However, despite numerous research reports describing new promising biomarkers, very few of these have proven to be clinically useful, mainly due to lack of specificity of the biomarker [36, 37]. For an optimal biomarker, a simple measurement should be an indicator of a certain disease and guide the physician in the clinical decision-making. According to the National Institute of Health, the official definition of a biomarker is: “A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (http://www.everythingbio.com/glos/definition.php?ID=3716). There are many criterions a protein or molecule must fulfill before it can be approved for clinical use as a cancer biomarker. For a molecule to be suitable for population screening and early detection it must be expressed and released at detectable levels already at a very early stage in the tumor formation process. Moreover, the biomarker must show a high specificity towards cancer and not be affected by other non-malignant abnormalities to avoid false positive results. In addition, it is important to evaluate whether serum levels of the marker are affected by individual characteristics such as age, sex, lifestyle, drugs and exercise. For the optimal cancer screening test, in addition to the mentioned requirements, aspects of cost and overdiagnosis must be taken into consideration. Overdiagnosis means a detection of cancer that would never have led to any symptoms during a person’s lifetime, thus rendering an increase in the total healthcare costs without providing any health benefits. In fact, overdiagnosis and false-positive screening very often cause unnecessary suffering to the patient due to psychological stress and side-effects from superfluous treatment [38].

Cancer is a heterogenous disease and the complexity of disease progression and treatment response is highly dependent on the combination of genetic changes in individual tumors. Therefore, the establishment of an appropriate panel of biomarkers will potentially aid to identify life-threatening cancers already at an early stage and to distinguish these highly aggressive tumors from more slowly growing tumors, with the ultimate aim to design optimal cancer therapies for individual patients. Due to recent
advancements in high-throughput genomic and proteomic screening technologies, visionary terms like “individualized cancer therapy” and “personalized medicine” seem like achievable goals. The “–omics” revolution has generated an enormous amount of data contributing to a deeper understanding of the molecular basis of tumorigenesis, disease progression and therapeutic response that eventually will facilitate the discovery of new specific and informative biomarkers helpful in therapeutic decisions [39]. Biomarker development has been described to involve five phases, where the first is the discovery phase, followed by a clinical assay and validation stage, next an evaluation in retrospective studies, then a prospective screening evaluation and, lastly, incorporation into randomized control trials [36].

**Microarray techniques**

The introduction of microarray techniques has been revolutionary for molecular biology research, allowing for investigations of cellular processes from a global perspective. Since the mid-1990s, DNA microarrays have been a widely applied technique in cancer research and the uncovering that biological and clinical tumor properties can be associated with a specific gene expression pattern have had direct consequences on diagnosis, prognosis and cancer treatment [40]. In the search for ovarian cancer biomarkers, gene expression profiles in ovarian cancer and normal ovaries have been compared. Several microarray studies have been performed with the aim to identify gene expression profiles related to ovarian cancer but the overlap between the different identified gene signatures is quite poor [36]. However, the serum ovarian cancer biomarker discussed above, HE4, was discovered and identified using cDNA microarrays [36].

**Protein profiling**

The proteome represents the entire set of proteins in an organism and a comprehensive overview of the active processes and cellular signaling within the organisms can be obtained by examination of the proteome. The analysis of the proteome is more complex than for the genome due to the many post-translational modifications a protein can be subjected to; i.e. phosphorylation, acetylation and glycosylation [39]. These modifications will influence the localization, activation and function of the protein. Today, several proteomic technology platforms are being applied in the field of biomarker discovery and the proteome can be examined by traditional separation techniques like two-dimensional electrophoresis (2DE) and multi-dimensional liquid chromatography coupled to single or tandem mass spectrometry (MS/MS), or microarray techniques, i.e. antibody microarrays and reverse phase protein arrays (RRPAs) [39]. The common goal is to identify disease-associated signatures by comparison of the proteome between two or more conditions, for example malignant and non-malignant samples or treated versus non-treated patients in the search for potential biomarkers for cancer diagnosis and prognosis. In 2-DE analysis and multi-dimensional liquid chromatography the proteins in a complex biological sample are
first separated, followed by identification with mass spectrometry. In 2-DE analysis the proteins are separated according to the electric charge by isoelectric focusing in the first dimension, followed by separation according to size in the second dimension on a polyacrylamide gel (SDS-PAGE) [41]. Reproducibility has been shown to be significantly improved by incorporating fluorescent cyanine dyes (Cy3 and Cy5) into the proteins before analysis, the technique is called difference gel electrophoresis (DIGE) [41]. The main limitations with 2DE techniques are the large amount of protein samples required and the difficulties in detection of moderate and low abundant proteins in complex samples [42]. In multi-dimensional liquid chromatography the proteins are separated with chromatography in at least two dimension, for example reverse-phase chromatography followed by ion-exchange chromatography. Although conventional proteomic tools have been successful in identifying proteins and signaling pathways, there are limitations due to sample complexity, sensitivity and selectivity, leading to the development of new techniques like antibody microarrays for global protein profiling.

**Antibody-based proteomics**

Antibody-based methods are frequently used in proteomics and antibody-based microarrays is a rapidly developing technique with promising potential for global high-throughput analysis of the entire proteome [39]. Antibody arrays are a screening tool for proteins, analogous to DNA microarrays, where antibodies rather than oligonucleotides are printed onto a solid surface [43]. There are two categories of antibody-based arrays; single capture array or dual antibody arrays. The single capture array is a direct labeling method requiring labeling of the proteins prior to the incubation on the microarray to enable detection of the bound proteins whereas the dual antibody array is based on a sandwich method requiring two separate antibody binding reactions, whereby the protein capture on the microarray is detected by a set of secondary labeled antibodies [39]. A third antibody-based array is the reverse-phase protein array (RPPA), in which the protein lysate is immobilized on a solid surface and the array is probed with labeled antibodies [39].

For detection of proteins in biological tissue samples, immunohistochemistry (IHC) is a widely applied method and commonly used as a diagnostic tool for pathologists. The method is based on specific binding of antibodies to antigen in a tissue section. In brief, the tissue is fixed in formalin (4% formaldehyde) immediately after surgical removal to preserve the morphology of the tissue, followed by dehydration and clearing with xylene before the tissue is embedded in paraffin blocks. Thin sections of the paraffin embedded tissue is mounted onto glass slides followed by deparaffinization and antigen retrieval for exposure of the epitopes before labeling with antibody against the protein of interest. The advantage of IHC as a detection technique is that it provides information not only about the expression level but also about the morphology of the tissue and subcellular localization of the protein. IHC has been criticized as a semi-
quantitative method, with lack of reproducibility of manual scoring of the staining intensity. Automated IHC scoring, for which various algorithms are currently being evaluated, might provide a solution to a more accurate quantification, ensuring reproducibility without any scoring discordances [39].

The development of tissue microarrays (TMAs) by Kononen et al. [44] has had a major impact in the field of biomarker discovery, enabling simultaneous IHC staining of multiple of tissue specimens. A TMA consists of hundreds of tissue cores (0.6-2.0 mm) assembled in the same paraffin block [44]. The access of specific antibodies is fundamental to all antibody-based methods and also the main concern and limitation in the effort to generate high-throughput methods for global protein profiling of the entire proteome. Hence, the main challenge today is to generate validated antibodies with a wide range of specificity targeting all gene products in the human genome.

![Figure 2. The tissue microarray technique. A tissue microarray (TMA) consists of hundreds of different tissue cores assembled in the same paraffin block, in this image breast cancer samples stained with an anti-RBM3 antibody.](image)

**The Human Protein Atlas**

The Human Protein Atlas (HPA) is a publicly accessible database providing expression profiles of human proteins in normal and malignant tissues. The HPA research project started in Sweden in 2003 with the vision to systematically explore the human proteome by applying antibody-based proteomics [45]. The long-term goal is to generate validated specific antibodies to all gene products in the human genome in a high-throughput manner (http://www.proteinatlas.org) [46, 47]. An additional goal is to generate a public web-based protein atlas providing information about protein expression and localization patterns in normal and cancerous human tissues, that could serve as a resource for biomedical research [48]. Today, seven years later, 13 150 antibodies have been produced, targeting approximately half of the genome and the HPA database consists of expression profiles of more than 13 000 antibodies corresponding to more than 10 000 human genes (http://www.proteinatlas.org) [49].
Generation of mono-specific antibodies

Within the HPA project, mono-specific antibodies are produced in a high-throughput manner, applying a strategy based on the use of recombinant protein epitope signature tags (PrEST) as antigens [50]. Mono-specific antibodies are obtained by affinity-purification of polyclonal antibodies generated towards recombinant PrEST proteins, whereby the mono-specific antibodies bind a single epitope, like monoclonal antibodies. This strategy enables large-scale production of antibodies towards a single epitope avoiding the time-consuming screening step required for the production of monoclonal antibodies. The PrESTs are designed to contain 100-150 amino acids in a region of the protein showing low homology to other human proteins, up to four PrESTs are designed for each gene [47]. PrEST gene fragments are cloned into an expression vector to produce recombinant PrEST proteins fused to a dual tag facilitating purification. The recombinant PrEST protein is used as an antigen towards which polyclonal antibodies are generated, affinity purification using the PrEST as ligands will finally produce mono-specific antibodies [51]. Continuous quality controls are performed during the entire process to ensure specificity of the antibody, like DNA sequencing of all cloned PrESTs, determination of the mass of the produced recombinant PrEST proteins with
mass spectrometry and, finally, the binding specificity of the generated antibodies are verified on protein microarrays containing various PrESTs. Approved monoclonal antibodies are further analyzed by Western blotting. For the examination of protein expression patterns in human normal and malignant tissue, IHC techniques are applied on TMAs containing 48 normal tissues, 216 human tumors representing the 20 most common forms of human cancer and 47 human cell lines [52, 53]. Furthermore, the subcellular localization of each protein is analyzed in three human cell lines using immunofluorescence [54] and epitope mapping is performed on a selection of HPA antibodies [55].

**The Human Protein Atlas as a tool for biomarker discovery**

For biomarker discovery, the HPA database can serve as an important screening tool for the discovery of potential biomarkers as it provides information about protein expression in a variety of tumor types represented by biopsies from 12 patients. This makes it possible to perceive a protein signature for each cancer type and to identify and select proteins with biomarker potential for extended analysis. The HPA is a high-throughput project and the expression profiles presented in the atlas ought to be considered as valuable screening information and as a starting point for further investigations. Proteins with potential biomarker properties identified in the HPA must be further validated in clinically well-annotated tumors from extended patient cohorts [56].

The protein investigated in this thesis, RBM3, was found to exhibit a differential expression pattern in the HPA. First, it was further evaluated in two large breast cancer cohorts, whereby its nuclear expression was found to be associated with a significantly improved survival [57]. In this thesis, the potential utility of RBM3 as a biomarker in epithelial ovarian cancer was evaluated in **Paper I**. Next, in **Paper II**, we applied the HPA to select and further explore the prognostic value of RBM3 associated proteins.
RNA-Binding Proteins and Post-Transcriptional Regulation

Cellular processes are governed by a hierarchy of selective protein interactions tightly regulated by dynamic changes in protein expression and localization. The processes of transcription and translation are highly regulated to obtain an optimal protein balance in the cell [58]. However, it becomes more and more evident that post-transcriptional and -translational events are crucial complements to maintain proper cellular functions. Post-translational modifications of proteins have been extensively investigated and are essential for activation, subcellular localization, interactions and stabilization of proteins [59]. Post-transcriptional modifications including mRNA processing, localization and stabilization of RNAs are less explored but their crucial role in the maintenance of proper gene expression becomes more and more evident [60]. Defects in different post-transcriptional mechanisms are linked to oncogenesis as a consequence of aberrant gene expression [60-62]. Conserved sequence elements, most often AU-rich elements (AREs), in the untranslated regions (UTRs) of the mRNA play an important role in mRNA stabilization. Proteins containing specific RNA-recognition motifs (RRM) reversibly bind to these sequence elements, resulting in either stabilization or destabilization of the mRNA [63-65]. The family of RNA binding proteins is steadily increasing but for the majority, the function and targets remain unclear. RNA-binding proteins influence the structure and interaction of RNAs and are important for their biogenesis, stability, function, transport and cellular localization [60]. A number of genes associated with cancer progression and inflammation, such as COX-2, c-myc, p53, VEGF, have been reported to be regulated by RNA binding proteins [60]. Many RNA-binding proteins have been coupled to stress conditions and recent reports demonstrate some key proteins in the DNA damage response machinery to be regulated at the post-transcriptional level [66, 67]. Furthermore, following DNA damage, translational reprogramming permits the synthesis of subsets of mRNAs crucial for DNA repair or apoptosis [68].

The RNA-binding protein RBM3
The RNA-binding motif protein 3, RBM3, was initially identified in a human fetal brain tissue cDNA library [69]. The RBM3 gene maps to Xp11.23 and encodes two alternatively spliced RNA transcripts. RBM3 expression has been observed in various human tissues with high levels in Sertoli cells of normal testes [69]. RBM3 contains one RNA-recognition motif (RRM) and is able to bind to both DNA and
The synthesis of RBM3 is an early event in response to mild hypothermia as demonstrated by a dramatic increase in both the mRNA and protein levels of RBM3 upon a decrease of the temperature from 37°C to 32°C in the mouse Sertoli cell line [66, 71]. The cold-shock proteins are crucial in tissues normally exposed to lower temperatures, like the skin and testes, and play an important role in the spermatogenesis [71]. The physiological effect of a cold-shock response is similar to what has been observed for heat–shock: an increased denaturation of proteins, a decrease in cell cycle progression, disruption of the cytoskeleton, changes in membrane permability with alterations in the fatty acid composition, and transcriptional and translational changes resulting in a general reduction of protein synthesis [66]. Mechanistically, the cold-shock response is poorly understood. One immediate effect of stress conditions is a general decrease in protein synthesis due to repression of normal cap-dependent translation and a switch towards translation initiated via internal ribosome entry sites (IRES) [66]. Cold-inducible proteins containing a single RRM adjacent to a glycine-rich region have been suggested to act as mRNA chaperones to facilitate translation during stress and enhance translation upon recovery of normal physiological conditions [72, 73].

However, in vivo, mammalian tissue temperatures almost never decrease to levels as low as 32°C, and most probably the cold-shock proteins are involved in other functions under normal physiological conditions [66]. In addition to hypothermia, RBM3 and CIRP have been demonstrated to be induced by a wide range of conditions causing cellular stress, like glucose deprivation [74], hypoxia [75], exposure to microgravity [76] and UV radiation, the latter only for CIRP [66, 77], indicating a more global stress related function of these proteins that were originally found to protect against hypothermia. Upon cellular stress induced by UV radiation, CIRP stabilizes several transcripts, in particular the replication protein A (RPA2), important for single stranded DNA break repair by binding to the 3'-UTR of the mRNA [66]. In response to UV radiation CIRP becomes phosphorylated by the GSK3beta kinase and is translocated to the cytoplasm, where it increases translation by interacting with the translational machinery [66]. Cells with reduced levels of CIRP are more sensitive to UV induced DNA damage suggesting CIRP to stabilize transcripts responsible for stress response. Recently it was demonstrated that CIRP can bind and stabilize the ataxia telangiectasia mutated and Rad3-related (ATR) mRNA, thus generating an increase in ATR protein levels after UV exposure, and subsequently an increase in active Chk1 [78]. This shows a direct relation between cold-shock proteins and the
DNA damage response machinery. The role of RBM3 in relation to stress conditions is less examined but RBM3 is susceptible for posttranslational modifications by phosphorylation at both serine, threonine and tyrosine residues [79]. However, the role of these phosphorylation events needs to be further investigated. In response to hypoxia both CIRP and RBM3 is are upregulated in a HIF independent manner [75].

**RBM3 expression enhances protein synthesis**

RBM3 promotes translation and enhances global protein synthesis at mild hypothermia and physiological temperatures as demonstrated by overexpression of RBM3 both in murine neuronal cell lines and in primary cultures of rat neurons [79, 80]. During stress, the translational machinery is repressed and RBM3 is thought to act as a back-up mechanism to protect the cells during poor conditions by facilitating translation of a subset of mRNAs for maintenance of a basal level of protein synthesis necessary for survival [66, 74]. The mechanism by which RBM3 contributes to translation is not fully defined. One possible model is that RBM3 binds and stabilizes mRNAs via ARE elements, and it has further been speculated that RBM3 changes the secondary structure of the mRNA, which is thought to enhance the binding of translation initiating factors [66, 80, 81]. A proportion of cytoplasmic RBM3 can bind directly to the ribosome subunit 60S, independent of RNA or newly synthesized polypeptide chains [80], which is thought to facilitate translation. In line with this observation, alterations in the phosphorylation of translational initiation factors have been observed in cells overexpressing RBM3. RBM3 expression has also been identified in neurons in the rat brain, where it is dynamically regulated during development, suggesting a role for RBM3 in translation-dependent processes underlying proliferation and differentiation [81]. Two alternatively spliced isoforms of RBM3 exist in neurons but these do not differ in the ability to enhance protein synthesis [79, 81].

**The role of RBM3 in cancer**

There are several pieces of evidence implicating an important role for RBM3 in cancer, based on both *in vivo* observations and *in vitro* experiments performed in a broad set of cell lines [57, 74, 82-85]. The notion that RBM3 is upregulated in various types of human malignancies like cancers of the breast, ovaries, colon and prostate, is confirmed by numerous independent investigations [57, 74, 84]. In a gene expression study, RBM3 was identified as a gene that was downregulated during malignant melanoma progression [82]. In breast cancer, high nuclear RBM3 expression was found to be significantly associated with a prolonged survival, particularly for estrogen receptor (ER) positive tumors [57]. To conclude from these studies, a high tumor-specific RBM3 expression seems to be a favorable prognostic factor in several cancer forms, which is further supported by the results presented in this thesis, demonstrating that RBM3 expression, both at the gene and protein levels, is significantly associated with a prolonged survival and sensitivity to cisplatin treatment in EOC (Paper I).
Several *in vitro* studies show an association between RBM3 and various processes central to cancer biology, like proliferation [84], apoptosis [84, 85] and angiogenesis [75, 84]. However, the function, interaction partners and targets of RBM3 remain to be uncovered, and the current data regarding RBM3 and its role in cancer associated processes are provided by studies in a wide range of different model system, which must be taken into consideration when interpreting the data. There is now growing evidence from different experimental setups that RBM3 can provide growth advantages by an increased proliferation. In colon cancer cell lines an increased proliferation rate was observed as a consequence of overexpression of RBM3 [84], while an inhibitory effect on cell survival was observed in siRNA experiments targeting RBM3 in prostate cancer cell lines [85]. Downregulation of RBM3 in colon cancer cell lines resulted in growth arrest, increased caspase-mediated apoptosis and mitotic catastrophe with increased levels of phosphorylated Chk1, Chk2 and Cdc25 [84]. In a recent report, siRNA mediated silencing of RBM3 resulted in decreased proliferation and loss of cell viability in the human kidney and leukemic cell lines HEK293 and K562 respectively [74]. Wellman *et al* also demonstrated a stronger immunohistochemical staining of RBM3 in normal intestinal epithelial cells with a high proliferation rate, compared with lowly proliferative skeletal and heart muscles cells, indicating a positive correlation between RBM3 and proliferation during normal conditions [74].

RBM3 has been shown to stabilize and promote translation of transcripts coding for VEGF, interleukin IL-8 and COX-2 proteins, all known to be important proteins in tumor progression coupled to angiogenesis [84]. These results are challenged by observations in kidney cells where neither down- nor upregulation of RBM3 had an effect on the mRNA levels of COX-2 [74].

A positive correlation between the X chromosome related RBM-genes (RBMX, RBM3, RBM10) and the proapoptotic Bax gene has been demonstrated in breast cancer [83]. Bax is a key regulator of caspase-mediated apoptosis and Bax downregulation has been associated to an impaired survival in metastatic breast cancer [86]. In colon cancer cells, silencing of RBM3 was demonstrated to generate an increase in caspase-3 mediated apoptosis [84]. However, downregulation of RBM3 in kidney HEK293 cells failed to induce apoptosis and no cleavage of caspase-3 or cell cycle arrest were observed [74]. The decrease in proliferation generated by silencing RBM3 was thought to be due to a decreased translation rate and a lack of proteins important for cell growth rather than activation of apoptotic pathways [74]. Taken together, the function of RBM3 in the context of cancer progression is not fully understood and needs to be further investigated.
RBM3 as a predictive biomarker in cancer

The discovery of RBM3 as a putative cancer biomarker was based on its differential expression in various cancer forms in the Human Protein Atlas (HPA) (www.proteinatlas.org) [56, 87]. In an initial study, an extended validation of RBM3 protein expression in two large, independent breast cancer cohorts, revealed a significant association with high nuclear RBM3 expression and prolonged survival, particularly in estrogen receptor (ER) positive tumors [57].
The Cell Cycle

Repeated cycles of cell growth and division form the fundamental concept of all living organisms. The core system of the cell cycle is a series of highly regulated events resulting in duplication of the genome and an accurate transmission of the genetic information from one cell to its daughter cell [88]. The genome is duplicated during the S-phase, followed by chromosome segregation and cell division in M-phase. A gap, G1, between each completed cell division and the onset of next round of replication provides time for the cell to grow and to adapt to the environment. During G1 the cell is sensitive to environmental stimuli and is triggered by extracellular signals to enter a new round of cell division [89, 90]. As the cell progresses though the restriction point, it makes the final decision to undergo cell division. In G1, the cell is depending on extracellular signals but as soon as it passes the restriction point it becomes refractory to environmental stimuli and is governed by intracellular signals that force it from one phase to the next in a highly regulated manner [91]. The G2-phase between the replication phase and mitosis serves as a second gap, providing time for preparation of chromosomal segregation in addition to providing time for DNA repair.

The cell cycle progression is governed by a cascade of biochemical switches, depending on the activity of cyclin-dependent protein kinases (Cdks), that drives the cells from one phase into another [92]. The Cdks require association with its regulatory subunits, the cyclins, for activation, as well as a cascade of phosphorylation and dephosphorylation events for full activity [93]. To ensure a faithful cell division, and to push the cell forward precluding re-entering into the previous phases, transient formation of specific Cdk/cyclin complexes govern the cell phase transitions. The cyclins are synthesized and destroyed at specific times during the cell cycle while the Cdks remain at a constant level [94-97].

Cell cycle progression is a highly regulated process and aberrations in the control mechanisms are tightly coupled with genomic instability, one of the hallmarks of cancer [5, 88]. The cell cycle process is a complex system that involves multiple of factors and positive / negative feed-back loops that drive the process forward to cell division. It is far beyond the scope of this thesis to describe the process in detail and therefore only the key concepts will be described briefly. The human cell contains three interphase Cdks (Cdk2, Cdk4 and Cdk6) and one mitotic Cdk (Cdk1) as well as ten
cyclins belonging to four different classes of cyclins (the A-, B, D- and E-type cyclins) [95]. S-phase entry is blocked by the activity of the pocket proteins, Rb, p107 and p130, which suppress transcription factors of the E2F family responsible for expression of genes essential for the replication of DNA [95, 98]. Extracellular mitogenic signals result in transcription of the D cyclins which in complex with Cdk4 and Cdk6, trigger the initiation of the cell cycle process in G1 [90]. The activity of Cdk4/6-cyclin D complexes leads to phosphorylation of Rb, resulting in transcription of cyclin E by activation of E2F [98, 99]. Cyclin E in complex with Cdk2 further phosphorylates the pocket proteins resulting in final dissociation and complete inactivation of these suppressor proteins. The transient increase in E2F activity leads to a high expression of cyclin E and to an increased activity of cyclinE/Cdk2. Cyclin A is synthesized in late G1 and participates in the G1-S transition in complex with Cdk2. Once the cells enter the S-phase, cyclin E is rapidly degraded by ubiquitin-mediated proteolysis. Cyclin A1 further activates Cdk1, which drives the cells through the G2-phase. At G2/M cyclin B becomes the new Cdk1 partner during M-phase [88]. The M-phase consists
of mitosis and cytokinesis. During mitosis the two sister chromatids are segregated into two nascent cells [92, 100]. Cytokinesis is the process of cleavage and occurs after complete mitosis. The Cdk1/cyclin B complex plays a crucial role in mitosis like the Polo and Aurora families of kinases [101]. Activated Cdk1/cyclin B complex phosphorylates a range of proteins, which leads to a variety of morphological changes like chromosome condensation, nuclear envelope breakage, reorganization of various cellular compartments and the formation of the mitotic spindle, that eventually will pull the chromosomes apart. All events are highly regulated and the DNA checkpoints ensure a faithful mitotic process [93, 100]. Activation of the Cdk1/cyclin B complex is required for the onset of mitosis [101].

At the end of the M-phase, the decrease in Cdk1 activity in the end of M-phase allows the pre-replicative complex (PRC) to bind to sites at the chromatin known as replication orgins. The Cdk/cyclin complex during G1 triggers the recruitment of DNA helicases, primases and polymerase to the chromatin causing unwinding of the DNA helix [102]. The family of MCM helicases are highly conserved during evolution and ensures that the chromosomes are replicated only once per cycle [103]. Cdc45 binds to the complex on DNA and associates with DNA polymerase. For faithful replication, it must be ensured that the replication origins are only fired once per cell division [104]. Entry into S-phase is highly dependent on the levels of cyclin E in the cell. Additional control mechanisms to prevent pre-entry into the different phases of the cell cycle are the Cdk- inhibitors. The p21 and p27 members of the Cip/Kip family are potent inhibitors of Cdk2/cyclin E but do also bind the Cdk4/6-cyclin D complex [98, 105, 106]. In quiescent cells, when the cyclin D levels are low, p21 and p27 bind Cdk2/cyclin E and thereby inhibit the entry into S-phase. Mitotic signals stimulate the transcription of cyclin D generating a rapid increase in the cyclin D protein levels, and the Cdk4/5-cyclin D complexes will eventually bind and occupy p21/p27 which will facilitate the activation of the Cdk2/cyclin E complex [105].

Important regulators of the activity of the Cdns are the Cdc25 family of phosphatases that activate the Cdk/cyclin complexes by removing inhibitory phosphorylations. The Cdc25 proteins are direct targets of activated checkpoint proteins generating a block in cell cycle progression in the presence of damaged DNA [107].
DNA damage response - essential for maintenance of genomic integrity

Living organisms are constantly exposed to a number of environmental agents harmful to their genome. Ultra violet (UV) light, ionizing radiation and genotoxic chemicals, as well as byproducts of normal cellular processes, e.g. reactive oxygen and incomplete replication, frequently cause genomic damage that is threatening for cell function and the survival of the organism. If left unrepaired, such DNA damage can induce permanent mutations and chromosomal defects that lead to genomic instability, another hallmark of malignant cells [5, 108]. The key concept in maintenance of genomic stability is the transmission of accurate copies of the genome to the next generation. To ensure faithful cell division in an environment constantly assaulting the DNA, the eukaryotic cells have evolved a complex control mechanism, the DNA damage response, including the checkpoint machinery and the DNA repair pathways, that regulates cell cycle progression and allows mitotic entry first when all chromosomes are accurate duplicated, the environment favorable and no DNA damage present [25, 109]. Several pathways work in a coordinated way to detect and signal the presence of abnormal DNA structures, and to halt cell cycle progression until the damage is repaired, or, in the case of severe damage, the apoptotic machinery is activated. Defects in the DNA damage response are associated with a variety of diseases like immune deficiencies, neurodegenerative disorders and cancer [108]. Many of the proteins involved in cell cycle regulation and DNA damage have been evolutionary conserved from yeast to humans indicating their importance for cell survival of the organisms [109]. Cancer cells do often exhibit defects in the DNA damage response pathways pointing to the global importance of these genes in maintaining genomic stability [6, 108].

DNA damage repair mechanisms
The eukaryotic cell has evolved multiple sophisticated mechanisms to meet the challenge of repair of a wide diversity of DNA lesions induced by both endogenous and exogenous sources of DNA damage. Spontaneous alterations constantly arise in the DNA due to misincorporation of dNTPs during replication, loss of DNA bases due to DNA depurination or deamination of cytosine to uracil [110]. Environmental, physical or chemical stress sources can harm the DNA in many ways and different types of DNA lesions are induced depending on the stress condition. Ionizing radiation causes both single-stranded (SSBs) and double-stranded DNA breaks (DSBs), among
which DSB is the most lethal and mutagenic DNA lesion for the human cell [111, 112]. DSBs can be a life-threatening consequence of unrepaired SSBs undergoing replication [113]. Chemical agents attack the DNA and chemical modifications generate alterations in the DNA structure. The toxicity of many drugs, including cisplatin, is due to the introduction of covalent links between the DNA bases, causing either intrastrand crosslink’s if the bases are in the same DNA strand, or interstrand crosslink’s if the covalent bonds are between two bases in different DNA strands [110]. Other chemical substrates, like the drug etoposide, target the topoisomerase causing both SSBs and DSBs [112]. To combat the threat of damaged DNA, the cell uses a myriad of proteins with different enzymatic properties, i.e helicases, topoisomerases, recombinases, ligases and nucleases, in the attempt to modify and repair the lesion [112, 113]. Loss of function of several of these enzymes is linked to various severe human diseases and often associated with an increased risk of tumor formation [114]. This indicates the importance of the complex network representing the cellular DNA repair mechanism, defending the human cell from environmental genomic attacks.

**DNA repair mechanisms for repair of single and double DNA strand breaks**

Mismatch repair (MMR) proteins detect mispaired DNA bases and replace them with the correct base while a process called base excision repair (BER) takes care of small chemical modifications of bases by excision of the damaged base [110]. Nucleotide excision repair (NER) is the mechanism by which the cell handles crosslinks of the DNA, which is of special interest for this thesis, considering the mode of action of cisplatin [29, 33]. The less frequent interstrand crosslink introduced by cisplatin is repaired by a different mechanism involving the Fanconi anaemia repair pathway [112]. To handle the danger of DSBs the mammalian cell has evolved two distinct repair mechanisms, the rapid but error-prone process of non-homologus end joining (NHEJ) and the more precise process of homologous recombination (HR) [25, 111, 112]. The NHEJ connects and resells the two DNA ends without a template, whereas HR copies a DNA sequence from an intact DNA strand, most often the newly synthesized sister chromatid [25, 111–113]. In brief, the NHEJ process is initiated by the recognition of DSBs by the ku70/80 heterodimer, followed by recruitment and activation of the DNA-dependent protein kinase (DNA-PK). DNA-PK in turn attracts the DNA ligase IV complex that finally seals the DNA ends [25, 111, 113]. Although the NHEJ is an error-prone mechanism it serves its purpose as a rapid emergency mechanism, without any cell cycle phase restrictions, whereas the activity of the high-fidelity HR process is restricted to the S and G2-phases of the cell cycle because of its dependence on the presence of sister chromatids as a template [25, 111, 113]. In addition to its role as a DNA repair mechanism, NHEJ is important in V(D)J recombination when the T-cell receptor and immunoglobulin genes are rearranged [25]. Several HR sub-pathways have been identified, all based on the utility of a template sequence to perform a precise, error-free re-synthesizing of the missing DNA sequence [25, 111]. Crucial factors in the HR pathways are the MRE11-RAD50-NBS1 complex together
with Rad51 and the BRCA proteins [25]. All HR pathways are initiated by a 5’-3’ resection at the DSB end to generate short ssDNA. Rad51-coated ssDNA mediates strand invasion of the sister chromatid, enabling homologous recombination [111]. Loss of function of either BRCA1 or BRCA2 reduces the efficiency of the high-fidelity HR DNA repair process and renders these cells dependent on the error-prone NHEJ process in response to DSB induction, which partly explains the accumulation of genetic changes in these cells [16, 115, 116]. Both BRCA proteins are involved in HR-mediated repair and facilitate the loading of RAD51 onto the ssDNA [16, 115].

**Cisplatin treatment activates the nucleotide excision repair pathway**

The NER process, involving at least 30 proteins, removes DNA adducts introduced by environmental sources like UV light or chemical agents like cisplatin [114]. Important key proteins in the process are the xeroderma pigmentosum (XR) complementation group A protein (XPA), XP complementation group C (XPC) and XP complementation group F (XPF) and the excision repair cross-complementing-1 protein (ERCC1) [114, 117]. The sensitivity to cisplatin has been demonstrated to associate with the cellular capacity to perform the process of NER [30]. The high sensitivity of testis tumor cells to cisplatin has been suggested to be related to the relatively low expression of ERCC1 and XPF, resulting in activation of apoptosis rather than DNA repair upon exposure to cisplatin [28, 31]. Increased levels of ERCC1 and the related XPA protein have been demonstrated to contribute to cisplatin resistance, further supporting an association between NER capacity and cisplatin treatment [30]. The NER repair mechanism is divided into two pathways depending on whether the damage occurs in a transcriptionally active or inactive region; global genomic repair (GCR) or transcription-coupled repair (TCR), respectively [117]. The main difference between the two pathways lies in the recognition of the damage; if the damage occurs in a transcriptionally active region it will be detected by the arrest of the RNA polymerase II (RNAPII), whereas the XPC protein recognizes global DNA adducts anywhere in the genome. DNA damage recognition is followed by recruitment of the ten-component transcription factor (TFIIH) containing DNA helicases that unwind the DNA, RPAs that mark the site of the damage and endonucleases that cleave the DNA around the damaged site. The single stranded gap is immediately re-replicated by proliferating cell nuclear antigen (PCNA) and DNA polymerase, then finally sealed by DNA ligase [114, 117].

Another DNA repair mechanism coupled to cisplatin response is the highly conserved mismatch repair pathway (MMR) [30, 118]. Areas with mismatched bases or structural changes in the DNA helix are recognized, excised and re-synthesized by the MMR process. In contrast to NER, loss of function of the MMR pathway results in cisplatin resistance. MMR mediated repair is inhibited by the interference with platinum compounds which in turn leads to failure in the repair process and eventually apoptosis. The danger with a deficient MMR process is that the aberrant
DNA structure cannot be detected by the MMR proteins, and the cells continue to proliferate even in the presence of DNA damage [30, 118].

**DNA damage signaling**

*Initiation of the DNA damage response by ATM and ATR*

Regulation of cell cycle division, DNA repair and apoptosis are all central processes to ensure genomic integrity and the cell needs to coordinate these pathways to handle the presence of damaged DNA [26]. The complex network of multiple signaling pathways that forms the DNA damage response machinery collectively operates in a cascade of events to detect the damage, and to signal its presence to the effector proteins that finally generate the cellular response. The signaling cascade is based on dynamic protein-protein interactions and timely posttranslational modifications to enhance or reduce the enzymatic activity of the involved protein complexes. In addition to the key players, a myriad of mediator proteins are required to bring the interaction partners in close proximity to each other to enable protein-protein interaction and activation or deactivation of enzymatic activity [26, 109, 119]. Following DNA damage, sensor multiprotein complexes recognize aberrations in the DNA structure and recruit the two master proteins in the DNA damage response; ataxia telengiectasia mutated (ATM) and ATR [26]. ATM and ATR belong to the phosphatidylinositol 3-kinase like protein kinase family (PIKKs) of serine/threonine kinases [25]. The PIKKs contain catalytic domains with motifs similar to the lipid kinase phosphatidylinositol 3-kinase (PI3K), thereby the name, but the PIKKs phosphorylate proteins rather than lipids [25, 120]. ATM and ATR are on the top of a complex network orchestrating various pathways ending with cell cycle arrest, DNA repair or apoptosis [25, 26, 120]. Although ATM and ATR share many features and target overlapping substrates, there are essential differences. ATM is activated mainly in the response to DNA DSBs [121] while ATR is responsible for SSB damage and replication associated damage [122].

*Activation of ATM in response to double stranded DNA breaks*

ATM is responsible for the immediate response to DSB whereas ATR contributes in a later stage to maintain the phosphorylation status of some particular substrates [25]. In response to the presence of DSBs, ATM is rapidly activated through autophosphorylation on Ser1981 causing dissociation of ATM dimers [123]. The exact mechanism behind this initial activation of ATM is not fully understood but it is suggested that ATM senses a change in the chromatin topology caused by a breakage and does not necessarily require physical interaction with the chromatin [123, 124]. ATM is recruited to the site of DSB by the sensor multiprotein complex Mre11-Rad50-Nbs1 (MRN) that binds directly to the site of DNA breakage [125]. The MRN complex attracts ATM and directs it to its substrates. There are mainly three mediators required for ATM function; mediator of DNA-damage checkpoint-1 (MDC1), p53-binding protein-1 (53BP1) and BRCA1, and the presence of these
mediator proteins facilitates the interactions of ATM and its substrates [119, 126, 127]. 53BP1 together with BRCA1 play important roles in the DNA repair via HR and NHEJ [119]. An early event after the induction of DSB is the phosphorylation of histone H2AX [25], these chromatin modification marks the site of DSB and is thought to act as a signal amplifier important for further recruitment of factors that will enhance activation of ATM and execute repair processes [109, 119, 128]. The phosphorylation of H2AX is dependent on ATM and ATR in response to DSB and replication stress respectively [25].

**ATR is activated by single stranded DNA-lesions**

In contrast to ATM, ATR needs physical interaction with an accessory protein named ATRIP for stabilization and activation [129]. The two proteins are depending on each other and it has been suggested that ATRIP should be considered an obligate subunit of ATR [122, 129]. ATR is directed to the site of DNA damage by replication protein A (PRA) that coats ssDNAs [130]. For activation of ATR, the Rad9-Rad1-Hus1 (9-1-1) complex needs to be loaded onto the ssDNA, a process depending on Rad17 [131]. The 9-1-1 complex is very similar in structure to the replicative sliding clamp proliferating cell nuclear antigen (PCNA) and shares the ability to encircle DNA [132, 133]. In addition, it is currently established that the topoisomerase –binding protein TOPBP1 protein is necessary for full kinase activity of ATR [134].

ATR, but not ATM, is essential for viability [129, 135] due to its central role in regulation of replication origin firing and repair of damage replication forks [136]. Mice deficient in ATM are viable but show hypersensitivity to radiation. Loss of function of ATM in humans causes the rare human genomic instability syndrome ataxia telangiectasia (A-T). These patients suffer from neuromotor dysfunction, immunodeficiency and are extremely sensitive to DSB inducing agents [25, 122].

In addition to the PIKK family of proteins two members of the the poly(ADP-ribose) polymerase (PARP) family play crucial roles in the cellular DNA damage response: PARP1 and PARP2. The PARP proteins are activated by both SSB and DSB and are important for the recruitment of crucial initiation complex to the site of DNA damage like the MRN/ATM complex [110]. PARP1 and PARP2 synthesize poly(ADP-ribose) (PAR) chains at the sites of SSB and DSB, and this chromatin modification is believed to attract DNA repair complexes. The PARP proteins have received major attention since the successful development of PARP inhibitors, which have had an important impact on cancer therapy [17].

**The checkpoint proteins Chk1 and Chk2**

The serine/threonine protein kinases Chk1 and Chk2 are substrates of ATM and ATR and play central roles in maintaining genomic stability by mediating the signaling cascade initiated by ATM and ATR [26]. Chk1 and Chk2 have received considerable
The Role of RNA-Binding Motif 3 in Epithelial Ovarian Cancer: A Biomarker Discovery Approach

attention the last decade due to their pivotal role in regulating cell cycle progression [137-139], apoptotic processes [140] and DNA repair mechanisms [141] in the presence of damaged DNA. 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 bulky DNA lesions and stalled replication forks during S-phase. However, today there are several pieces of evidence on the existence of a cross-talk between the two pathways [122, 142]. Chk1 is essential for normal development, demonstrated by embryonic lethality of Chk1-deficient mice and acute lethality of Chk1-deficient embryonic cells [143, 144] while Chk2-deficient mice are viable and fertile but show a tumor-prone phenotype in the exposure to carcinogens [145, 146]. Chk2-deficient cells have demonstrated an increased resistance to ionizing radiation [145].

In addition to the two traditional ATM/Chk2 and ATR/Chk1 pathways, it was recently discovered that a third kinase pathway, p38MAPK/MK, operates downstream of ATM and ATR [67, 119, 147]. The effector protein p38MAPK/MAPKAP-K2 (MK2) and Chk1 share the same substrates and both are capable of inducing a
G2/M checkpoint arrest in response to stress conditions. However, it has also been demonstrated that Chk1 and MK2 operate at different temporal phases during the checkpoint response depending on their subcellular localization. While Chk1 is important for the initiation of the cell cycle arrest, MK2 was shown to be required for a sustained checkpoint response [67]. This illustrates the complexity of the DNA damage pathways and the importance of both initiation and maintenance of the checkpoint response. Interestingly, MK2 was illustrated to act by phosphorylating the RNA-binding protein hnRNP A0, causing it to bind and stabilize the Gadd45alpha mRNA [67]. Consequently, the Gadd45alpha protein level increased in the cytoplasm, where it could bind Cdc25B and Cdc25C and thereby prevent mitotic entry [67]. This not only illustrates the complexity of the DNA damage response, but also adds a level of control to the checkpoint response, namely posttranslational regulation.

**Chk1 and Chk2 contribute to cell cycle arrest by targeting the Cdc25 phosphatases**

Chk1 and Chk2 act to halt cell cycle progression to provide time for the DNA repair mechanisms to operate or, if the damage is too severe, allow the apoptotic system to be activated. Cell cycle delay is obtained by targeting proteins of the Cdc25 family [137-139]. The members of this family of phosphatases are central regulators of cell division and via activation of cyclin dependent kinases (Cdks) they control key transitions between the cell cycle phases [107]. The Cdc25 proteins remove critical inhibitory phosphorylations on Cdk/cyclin complexes, of which the most important is Tyr-15 in the active loop of Cdk1 and Cdk2 [107]. Three isoforms of Cdc25 have been identified in mammalian cells: Cdc25A, Cdc25B and Cdc25C which all act in different phases of the cell cycle. Cdc25A mainly contributes to the G1-S transition by activating the Cdk2-cyclin E and Cdk2-cyclin A complexes but also has a role in G2-M transition by activating Cdk1-cyclin B complexes. Cdc25B and Cdc25C are necessary for the entry into mitosis, whereby Cdc25B promotes the initial activation of Cdk1-cyclin B at the centrosomes in the transition between G2 and M, and Cdc25C activates Cdk1 in the nucleus to drive the final onset of mitosis [107].

Phosphorylation of Cdc25A by Chk1 and Chk2 makes it a target for ubiquitination by the SCF ubiquitin ligase, thereby promoting its proteosomal degradation which causes a delay in the G1/S transition [148]. To delay the onset of mitosis in the presence of DNA damage, Chk1 and Chk2 phosphorylate Cdc25C, thus enabling the binding of the 14-3-3 adaptor that translocates Cdc25C to the cytoplasm [139, 149]. The kinase proteins Wee1 and Myt1 are responsible for the negative regulation of the Cdk1-cyclin B complex to inhibit pre-onset of mitotic entry [95].

**DNA damage checkpoints**

**G1 checkpoint**

The initial defense mechanism against genomic stress in cycling cells is the G1 checkpoint. The G1 checkpoint prevents cells from entering the S phase by inhibiting
the initiation of replication [26, 105]. The phosphorylation of Cdc25A is an immediate, rapid response that does not need transcriptional activity or protein synthesis [26, 107]. Phosphorylation of Cdc25A by Chk1 or Chk2 marks it for degradation preventing the activation of Cdk2/cyclin E and Cdk2/cyclinA complexes. The Chk1/Chk2–Cdc25A checkpoint is rapid and short, and delays the G1/S transition only for a few hours. A more sustained G1 arrest is obtained by activation of the transcriptional activity of p53 [26, 107]. Upon damage, signaling from ATM and ATR leads to an accumulation of p53. p53 can either be directly phosphorylated by ATM and ATR, or indirectly, through the activities of Chk1 and Chk2, resulting in p53 stabilization and transcription of the Cdk inhibitor p21\(^{\text{CIP1/WAF1}}\) [150, 151]. p21 inhibits the activation of the Cdk2/cyclin E(A) and causes cell cycle arrest [105]. In addition, the RB/E2F association is preserved due to absence of active Cdk2/cyclinE that can hyperphosphorylate RB, causing the dissociation of E2F, and, hence repression of the E2F activity, causing a sustained G1 blockade [26, 91, 98, 105]. The pathways of both p53 and RB are commonly deregulated during tumorigenesis due to high mutation rates in these genes [6, 98], thus, defects in the G1-checkpoint response are common in cancer cells, rendering these cells dependent on the G2 checkpoints for DNA damage signaling [26]. Stabilization and activation of p53 in response to DNA damage leads to transcription of a range of p53 transcriptional targets, among which p21\(^{\text{CIP1/WAF1}}\) and the pro-apoptotic Bax gene are the most studied. It has been reported that, in response to DNA damage, p53 favors the transcription of p21\(^{\text{CIP1/WAF1}}\) rather than Bax, due to more efficient binding of p53 to the DNA elements in p21 than Bax [152], resulting in a predominance of induced cell cycle delay over apoptosis. However, it remains mainly unclear what determines the cellular fate in response to DNA damage; apoptosis or DNA repair. Many different signals can generate a stabilization of p53 by using different stabilization molecules and p53 is further subjected to a several posttranslational modifications that affect function and stabilization [23].

S checkpoint
During S-phase the entire human genome, consisting of some three billion base pairs, is faithfully duplicated. High fidelity of replication is absolutely essential for the maintenance of genomic stability and to avoid accumulation of genetic changes, yet another hallmark for cancer [5, 6]. The process of replication is error-prone and the replication forks are vulnerable for structural changes in the DNA and will halt in the presence of structural obstacles, which eventually will lead to DNA breakages if left unrepaired [124]. The cell possesses a robust system to prevent the fatal threat of stalled replication forks in which Chk1 plays a central role, together with ATR, plays a central role to stabilize stalled replication forks, inhibit late origin firing and to activate DNA repair processes. In addition to the risk of DNA replication errors, the cell is under a constant threat from toxic stress that can cause adverse DNA damage even during S-phase [109]. The S-phase checkpoint deals with these dangers and acts
mainly in three processes to protect the cell. Two of these three pathways are directly coupled to the replication machinery and relay mostly on ATR-Chk1 signaling, the replication checkpoint protecting against replication errors and the S-M checkpoint preventing entry into mitosis before the entire genome is replicated. The third checkpoint process, independent of replication, has been called the intra-S-phase-checkpoint and is responsible for detecting and repairing DSB generated outside the active replicons [124]. Notably, none of these three processes require functional p53, the key target in the G1 checkpoint.

The presence of DSB during S-phase, unrelated to the replication process, activates the ATM/Chk2 pathway, causing a delay in S-phase progression due to rapid turn-over of Cdc25A, which inhibits the activation of the Cdk2/cyclin E(A) complexes [107, 124, 153]. An active checkpoint response in the S-phase provides a delay rather than a permanent arrest to promote resumption of replication [26]. Complete replication facilitates high fidelity repair via the process of HR by providing sister chromatids, serving as sequence templates [153]. The localization and activation of ATM at the sites of DSB recruits factors mediating DNA repair.

**Chk1 stabilizes stalled replication forks**

The exposure of ssDNA at the site of stalled replication forks attracts ATR [130]. Translocation of Chk1 to the site of replication forks is mediated by the protein claspin, a crucial event to bring Chk1 in proximity to ATR [154, 155]. ATR subsequently phosphorylates Chk1 at two sites, Ser317 and Ser345 [143, 156]. The interaction between Rad17 and claspin is important for sustained Chk1 activation [157, 158]. A second complex located to the site of replication forks consisting of timeless and timeless-interacting protein is also believed to function, in addition to claspin, to mediate the activation of Chk1 [122]. The phosphorylation of Chk1 triggers its dissociation from the chromatin and it is released into the soluble nuclear, cytoplasmic and centrosome compartments where it exhibits its function leading to cell cycle arrest, repair or apoptosis [122, 149, 159]. A consequence of Cdk2/cyclin E inhibition via the ATR-Chk2-Cdc25A pathway is repression of replication origin firing due to less loading of Cdc45 onto the DNA pre-replication complex, which is required for origin firing and depending on Cdk2 activity [153, 160]. In addition, Chk1 can directly target essential S-phase kinases like Cdc7. Cdc7 is required, together with the MCM proteins, for efficient loading of Cdc45 to replication origins [161].

**G2/M checkpoint**

The G2 checkpoint is a key guardian of the cancer genome where cells that have escaped both the G1 and S checkpoints, or have incurred DNA damage in G2 phase, are prevented from further progression into mitosis [26, 162]. Unlike p53, the G2-checkpoint genes are rarely mutated in cancer, suggesting these genes to be important
for tumor viability [163]. ATR acts through Chk1-Cdc25 to inhibit the activation of the Cdk1/cyclin B complex, the main regulator of the onset of mitosis [88, 107, 164]. Besides inhibition of Cdk1/cyclin B via targeting of Cdc25, Chk1 enhances, by phosphorylation events, the suppressive activity of the kinase Wee1 on Cdk1/cyclin B, hence, emphasizing the repression of Cdk1-cyclin B to prevent the onset of cell division [163, 165]. For a sustained G2/M block Chk1 mediates transcriptional activity of genes which gene products suppress the activity of Cdk1/cyclin B, e.g. the Cdk-inhibitor p21, for sustained G1 arrest, and GADD45, that interrupts the interaction between Cdk1 and cyclin B, facilitating for the 14-3-3 protein to anchor the Cdk1/cyclin B to the cytoplasm via binding to Cdk1 [26].

**Mitotic spindle checkpoint**

Chk1 has been implicated in the mitotic spindle checkpoint and delays the onset of anaphase, *i.e.* the separation of the chromatids, in the presence of defects in the mitotic spindle [166]. It has been described that Chk1 localizes to the kinetochores in response to spindle disruption after treatment with taxol [166]. The mechanism behind the function of Chk1 at the kinetochores and in the mitotic spindle checkpoint still remains to be elucidated but observations point to a connection between Chk1 and Aurora-B, a central kinase for mitotic activity [149]. A recent publication reports an association between Chk1, AuroraA and DNA DSB repair, whereby overexpression of Aurora-A enhances degradation of claspin by Plk1 leading to decreased activity of Chk1 and weakening of the HR capacity [167]. Consequently, the DSB induced by radiation is repaired with the error-prone NHEJ pathway rather than with the high fidelity HR process, which eventually will lead to chromosomal instability. The authors speculate that these results may partly account for the tumorigenic effect of Aurora-A, as Aurora-A is overexpressed in various types of tumors, and suggest a possible beneficial effect of PARP inhibitors in tumors with high levels of Aurora-A [167].

However, there are some question marks regarding the role of Chk1 during mitosis that remain to be elucidated. It has been reported that in mitotic cells, induction of DNA ionization and UV light did not generate phosphorylation of Chk1 at Ser345 [166, 168]. Activation of Chk1 prevents entry into mitosis and it has been demonstrated that, at the onset of mitosis, Chk1 needs to be inactivated and the suppression of Cdk1/cyclin B function must cease in order to drive the cells into mitosis by Cdk1 activity [166]. It has been speculated that Chk1 is phosphorylated and inactivated by Cdk1 in a positive feedback loop [168]. However, this remains to be proven and there are many biochemical details that need to be investigated to further understand the checkpoint machinery during mitosis. It might be that Chk1 is regulated in a non-canonical way at the sites of centrosomes and kinetochores during mitosis and maybe activates a different subset of substrates [166]. Detailed mapping of mitotis-sensitive phosphorylation sites in Chk1 would provide further insights as well as further in-
depth studies regarding the relationship between Chk1 and Aurora A.

**Termination of the checkpoint response**
The cascade of events leading to cell cycle arrest in response to DNA damage has been extensively investigated, however, the underlying mechanisms for recovery and re-entry of the cells into the cell cycle after arrest remain poorly understood [169]. Available data indicate that inactivation of a checkpoint response is an active process that requires specific signaling pathways. A recent report demonstrates the importance of Rad17 degradation for termination of the ATR-Chk1 checkpoint response [170]. Rad17 stabilization was shown to prevent re-entry into the cell cycle and caused prolonged cell cycle arrest [170] after the damage was removed. Immediately after induced DNA damage, Rad17 protein accumulates to facilitate the activation of Chk1. After completion of the checkpoint response, the levels of Rad17 are decreased via ubiquitin-mediated proteasomal degradation by Cdk1/APC leading to dissociation of Chk1 from ATR and discontinuation of the checkpoint [170]. Hence, proteolytic degradation of Rad17 seems to be a mechanism to deactivate the checkpoint signaling after complete checkpoint response. Further, downregulation of Chk1 mediated by p53 activation has been reported to act as a negative feedback loop terminating the checkpoint response [171, 172]. Upon damage, both Chk1 and p53 are activated, and, once activated, p53 represses the transcription of Chk1, resulting in an inactivation of the checkpoint and re-entry of the cells into the cell cycle. This may explain the longer cell cycle arrest observed in p53 deficient cells after exposure to DNA damaging agents [171, 172].

The Polo-kinase-like protein Plk1 has been demonstrated to be essential for the termination of the G2 checkpoint [173]. Plk1 is important for proper cell division and triggers the onset of mitosis [174]. In addition, Plk1 has been suggested to act as a feed-back loop to ensure the irreversibility in mitotic entry, by upregulating the activity of the Cdk1/cyclinB complex as soon as the mitosis is initiated, and by targeting Wee1 for degradation [174]. Plk1 is catalytically inactivated by ATM and ATR signaling during the G2 checkpoint, and prevents mitotic entry of cells with DNA damage. Cells expressing constantly active Plk1 are very sensitive to DNA damage and unable to undergo G2 arrest [175]) whereas Plk1 deficient cells are incapable to enter mitosis after the DNA damage is repaired.

**Phosphorylation of Chk1 and Chk2**
Chk1 can be phosphorylated at multiple sites regulating activation, localization and degradation. Phosphorylation of Chk1 at Ser317 by ATR is a prerequisite for phosphorylation of Ser345-Chk1 [143, 176, 177]. Cells unable to phosphorylate pSer317 are incapable of checkpoint arrest but are viable, while viability is not supported by mutant Ser345-Chk1 [176, 178]. pSer345 is important for preventing mitosis until complete DNA replication is performed in the absence of DNA damage. Mutation of
Ser345 at Chk1 caused mitotic catastrophe, impaired checkpoint response and loss of ability to localize to the cytoplasm [176]. Furthermore, mutation of Ser317 has been shown to result in impaired cell cycle checkpoint response and loss of chromatin release upon stress, while the mutant retained the ability to prevent mitotic catastrophe and to localize to the centrosome [176]. Ser345-Chk1 phosphorylation by ATR is crucial for its activity but it also marks the protein for destruction. Polyubiquitination of Chk1 is triggered by phosphorylation of Ser345 [179].

Chk2 is phosphorylated at multiple sites. In response to DNA damage, ATM phosphorylates Chk2 at Thr68, which has suggested to serve as a starting point for a chain of autophosphorylation events on Thr383 and Thr387 in the active loop, leading to full kinase activity of Chk2 [119]. Increased levels of pT68 have been detected in human lung and breast carcinomas [126].

**DNA damage-independent functions of Chk1 and Chk2**

*Chk1 regulates replication in unperturbed cells*

Many proteins involved in the checkpoint response have been found to associate with chromatin even in the absence of DNA damage and have further been demonstrated to possess a function under normal conditions [180]. It has become increasingly evident that timing and regulation of normal replication during S-phase in unperturbed cells involves the same proteins that are responsible for repressing of cell cycle advancement in the presence of DNA damage or stalled replication forks, *i.e.* ATM, ATM and the checkpoint proteins [181, 182]. An interesting speculation from an evolutionary point of view is that the checkpoint proteins have evolved to prevent introduction of DNA changes in the newly synthesized DNA strands due to normal replication-errors, and that the hyper-activation of checkpoint proteins after excessive DNA damage is an extraordinary activity [183].

The fact that Chk1 is essential for normal development [143, 144] implicates that Chk1 possesses crucial functions uncoupled to DNA damage and thereby contributes to genomic stability in a broader way [182]. Several reports demonstrate that ATR and Chk1 can regulate Cdc25A stabilization by phosphorylation events even in unperturbed cells and, consequently, regulate cell cycle progression [149, 155, 180]. In addition, ATR/Chk1 signaling controls firing of replication origins through the activation of Cdk2/cyclin E(A) complexes [148, 155]. Depletion or inhibition of ATR or Chk1 causes abnormalities in the replication process demonstrated by various independent *in vitro* experiments using different kinds of eukaryotic cells pointing to an impact of ATR and Chk1 in normal replication. In 2004, Shechter and colleagues showed that depletion of ATR caused an increase in origin firing in unperturbed cells [181] and some years later the same observation was made by inhibition of Chk1 [184]. Meanwhile it was demonstrated that loss of function of Chk1, but not Chk2, generated accumulations of both SSBs and DSB [182, 185]. It was further shown that
Chk1 could influence the global replication fork speed with slower progression of the replication fork in chicken DT40 and HeLa cells lacking Chk1 [186]. To summarize, depletion of Chk1 causes increased origin firing and reduced replication fork progress generating DSB and the introduction of DNA damage. Chk1 has been suggested to control origin firing and thereby contribute to the maintenance of replication origin firing, which is believed to be an important mechanism to prevent replication stress in mammalian cells [187].

**Chk1 functions during normal mitosis**

It is now evident that Chk1 regulates entry into mitosis in unperturbed cells as well as under stress conditions. Chk1 is located to the centrosomes during interphase where it is thought to prevent premature activation of CyclinB/Cdk1 through phosphorylation of local Cdc25B and thereby inhibiting premature onset of mitosis [149]. It is suggested but not proved, that phosphorylation of Cdc25B at multiple sites promotes the binding of the adaptor protein 14-3-3 to Cdc25B, preventing substrate binding [149]. Dissociation of Chk1 from the centrosomes in late G2 triggers the onset of mitosis involving activation of Cdc25C and Aurora-A. When the cells are ready for mitosis the Cdk1/cyclin B complex must be activated by Cdc25A(C), and, thus, the negative regulation of Cdc25A(C) by Chk1 needs to be disrupted. It has been speculated that it is Cdk1 itself that phosphorylates and inactivates Chk1 in a positive feedback loop [149, 166, 188].

**Chk2 functions during normal mitosis**

Also the kinase activity of Chk2 was recently reported to be required for a proper and timely spindle assembly [189]. This function was independent of p53 and checkpoint response. The precise mechanism behind the action of Chk2 in spindle assembly has not been elucidated. It has been suggested that BRCA1 is a mitotic target of Chk2 in the absence of DNA damage and that loss of BRCA1 activation leads to spindle misaggregation, as observed in Chk2 deficient cells [189]. However, the mechanism behind the chromosomal defects observed in the absence of Chk2 and BRCA1 needs to be further elucidated.

**Targeting checkpoint proteins in cancer treatment**

Disruption of DNA damage signaling has been proven to be an efficient way of sensitizing cancer cells to DNA damaging agents [18]. Tumor cells often acquire defects in the checkpoint machinery in an early stage of tumor formation, which allows for spontaneous mutations to propagate and promotes the transformation process from a pre-malignant to a malignant state. Loss, mutations or epigenetic silencing of genes involved in the DNA damage response are often observed in cancer and deactivation of checkpoint proteins has been reported to cause genomic instability and predisposition to transformation into non-neoplastic cells [26, 190-192]. However, mutations in one checkpoint gene targeting one DNA damage pathway are generally
not sufficient to drive the transformation process since the cell has various overlapping DNA damage pathways operating in a parallel manner to ensure faithful cell division. Certain combinations of mutations targeting different pathways might be efficient in silencing the control machinery and facilitate growth and malignant transformation. This is illustrated by the fact that Chk2 defects enhance the transformation potential of BRCA1 deficient cells, while BRCA1 loss alone is not sufficient for transformation of cells into a malignant state. The combination of p53 loss and BRCA1 deficiency shows an even higher transformation potential [26].

In the challenge to develop novel efficient cancer drugs, the concept of targeting non-oncogenic properties has been suggested [110]. This model is based on the fact that some pathways are essential for cancer cells but not for normal cells. In the process of transformation the function of several control mechanisms are lost due to mutations which render the tumor cells to relay on one single pathway to avoid lethal cell division, whereas in normal cells most pathways operate in parallel, and the loss of one or a few proteins can often be compensated for [110]. The G1 checkpoint is often inactivated in cancer cells, due to the high mutation rate in the TP53 gene, making the tumor cells dependent on the G2 checkpoint to avoid mitotic entry with lethal DSB [6, 26, 98].

Inhibition of ATM with caffeine has been shown to sensitize cells to chemotherapeutic drugs and ATM inhibitors have been developed but yet not proven to be suitable for clinical use, due to their lack of specificity [193]. Both Chk1 and Chk2 have been plausible targets in cancer therapy due to their central role in DNA damage response. Several lines of evidence suggest that targeting Chk1 or Chk2 in combination with genotoxic drugs will effectively kill the tumor cells in vitro, in particular p53 mutated tumors cells [155, 161, 164, 194, 195]. 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 [196]. Several Chk1 and Chk2 inhibitors have been developed and are currently in clinical trials [18, 189].

The PARP proteins, involved in DNA repair, have received major attention since the successful development of PARP inhibitors, which have had an important impact on cancer therapy [17]. Inhibition of PARP has been demonstrated to be synthetically lethal in BRCA deficient cells. A number of PARP inhibitors are in currently in clinical trial as anti-cancer therapy for BRCA1/2-deficient breast and ovarian cancers [18].

**Checkpoint proteins and cancer development**

*DNA damage response as a first anti-cancer barrier*

Oncogenic activation that provides excessive growth signals is an early event in the transformation process driving normal cells into a cancerous state [5]. DNA hyper-replication as a consequence of hyperproliferative oncogenic stimuli exposes the cell to
replication stress and threatening faithful chromosomal duplication due to increased replication origin firing, re-replication and stalled replication forks [197, 198]. The induction of DNA lesions triggers activation of the checkpoint response, resulting in halted cell cycle progression. Hence, DNA hyper-replication serves as a first anti-cancer barrier to protect pre-cancerous cells from malignant progression in an early state of transformation [190, 191].

Interestingly, tumor cells often exhibit a constitutive activation of DNA damage signaling even in early pre-invasive lesions, before the loss of function of checkpoint that is often proteins that are often found in established tumors [190, 191, 199]. These findings provide evidence in support of the concept that even though the activation of the DNA damage signaling in pre-cancerous cells acts as an anti-cancer barrier inhibiting cell proliferation, it also provides an environment for selection of mutations or epigenetic silencing of checkpoint genes. This will in turn generate clones that are capable to progress into a more malignant phenotype with uncontrolled proliferation, due to the ability to bypass the cellular checkpoint machinery [190, 191, 200]. Loss, mutations or epigenetic silencing of checkpoint genes are frequent events in cancers, causing defects in the checkpoint machinery, which allows for continuous proliferation, accumulation of genetic changes and the progression into a malignant phenotype [190-192].

In contrast to many solid tumors, testicular germ cell tumors (TGCT), derived from precursor germ cells, lack activation of the DNA damage response, even in the pre-invasive i.e. carcinoma in situ, stage [201]. Due to the absence of an activated DNA damage response in the majority of TGCT cells, these cells lack an anti-cancer barrier, and, hence, the pressure for selection of mutant clones is reduced. In contrast to most other solid tumors, TGCT cells show an intact DNA damage response machinery without mutations in the checkpoint genes. For instance, the otherwise commonly lost tumor suppressor, p53, is rarely mutated in TGCT [201]. The reason for these differences between TGCT and other solid tumors is believed to be associated with the biological distinctions between their cells of origin. The TGCT are derived from a germ cell precursor with characteristics of pluripotent cells, and show essential biological differences to somatic cells. In particular, the cell cycle regulation is different in germ cells, that lack the retinoblastoma protein, which makes them more prone to undergo apoptosis than cell cycle arrest in the presence of DNA damage [201]. Furthermore, it has also been speculated that the oncogenic stimulus that drives the transformation of TGCT, is of the nature that it provides a growth advantage but does not cause replication stress, and thereby no activation of DNA damage signaling, in contrast to somatic cells [201]. Due to the intact checkpoint machinery, TGCT cells are very sensitive to DNA damage-inducing chemotherapy [201].
The Present Investigation

Aims
The general objective of this thesis was to identify new prognostic and predictive biomarkers in epithelial ovarian cancer, applying the Human Protein Atlas (HPA) as a biomarker discovery platform [56]. The secondary aim was to study the function of the newly identified biomarkers to improve the understanding of the mechanisms behind the observed clinical associations.

More specifically, this thesis focuses on the RNA binding protein RBM3 and its potential utility as a prognostic and treatment predictive marker in EOC. In addition to high-throughput tissue-based analyses, in vitro studies have been performed to gain a deeper understanding of the function of RBM3 and its role in tumorigenesis.

Background
EOC is the most lethal of all gynaecological malignancies and there is an urgent need to develop new prognostic and treatment predictive biomarkers to obtain an effective individualized treatment regimen. The discovery of the RNA binding protein RBM3 as a putative cancer biomarker was based on its differential expression in various cancer forms in the HPA [56, 87]. Further investigations revealed a significant association between high nuclear RBM3 expression and a significantly improved survival in breast cancer, particularly in estrogen receptor (ER) positive tumors [57]. Several independent investigations, including ours, have observed an up-regulation of RBM3 in various types of malignancies; i.e. cancer of the breast, ovaries, colon and prostate [57, 84]. Published in vitro data are somewhat contrasting in that RBM3 has been suggested to be a proto-oncogene in colorectal cancer cell lines [84], but in a cDNA micro array study, Baldi and colleagues demonstrated that RBM3 is downregulated in metastatic malignant melanoma [82].

In the light of these data, we wanted to elucidate the clinical relevance of RBM3 expression in EOC, and investigate the molecular mechanisms underlying its prognostic value, with the aim to further understand the biological function of RBM3 and to explain the discrepancies in the hitherto published data on RBM3 in the context of cancer.
Results

**RBM3 expression is associated with an improved survival in EOC**

In the search for new cancer biomarkers, RBM3 was discovered to possess properties suitable for a potential biomarker based on its differential expression pattern in several major cancer forms in the HPA. Extended analyses in tumors from two large cohorts of breast cancer revealed a significant association between a high nuclear RBM3 expression and an improved survival in breast cancer, particularly in estrogen receptor (ER) positive tumors [57]. The main aim of this thesis was to identify new potential prognostic and treatment predictive biomarkers in EOC. For this purpose, the prognostic value of RBM3 was examined both at the mRNA level in a cohort of 267 EOC cases (Cohort I) and at the protein level by IHC, in an independent cohort of 154 prospectively collected EOC cases (Cohort II) (Paper I). In both cohorts, high RBM3 expression was significantly associated with an improved survival. Cox multivariate analysis adjusting for age, disease stage, differentiation grade and residual tumor (available only for Cohort I) revealed that RBM3 expression was an independent predictor of recurrence free survival (RFS) and overall survival (OS) in Cohort I and OS in Cohort II (Paper I).

**RBM3 sensitizes A2780 EOC cells to the platinum compound cisplatin**

Since platinum-based chemotherapy is the mainstay of therapy for EOC, we continued to investigate the role of RBM3 in the cellular response to cisplatin treatment in EOC cell lines. We found that cisplatin sensitive A2780 cells showed a higher RBM3 expression, both at the mRNA and protein level, than its cisplatin resistant A2780-Cp70 derivative. These observations raised the question whether RBM3 is implicated in treatment response and, hence, the favorable outcome observed in EOC patients with tumors expressing high levels of RBM3. To clarify this issue, the impact of RBM3 on cisplatin response was investigated by siRNA mediated silencing of RBM3 in A2780 cells (Paper I). These in vitro experiments revealed a decreased cytotoxic effect of cisplatin in siRBM3 transfected cells, demonstrated by an increased survival, a smaller proportion of cells arrested in G2/M, and a trend towards a decreased, however non-significant, percentage of apoptotic cells.

**Identification of RBM3 associated genes uncovers a putative role of RBM3 in DNA damage with a link to MCM3 and the checkpoint proteins Chk1 and Chk2.**

In the search for new prognostic biomarkers in EOC and an improved understanding of the function of RBM3 and the mechanisms contributing to its prognostic and treatment predictive value, we next sought to identify RBM3 associated genes in EOC (Paper II). For this purpose, gene expression profiles of EOC tumors with high versus low RBM3 expression were compared using gene expression data from Cohort I [202]. Gene set enrichment analysis demonstrated that high RBM3 expression was associated with a number of processes including DNA dependent replication, DNA replication, chromatin remodeling and DNA integrity checkpoint. 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.

Next, the HPA was used as a screening tool to select promising biomarker candidates among the RBM3 associated genes for further evaluation. Gene products displaying a differential expression pattern among EOC samples in the HPA were selected for extended analysis regarding their association to RBM3 in the ovarian cancer cell lines A2780 using siRNA mediated silencing of RBM3. In this fashion, we found an inverse association between RBM3 and the proteins Chk1, Chk2 and MCM3. In addition, a negative correlation was observed between RBM3 and telomeric repeat binding factor 2, interacting protein (Terf2IP), also known as Rap1 and a positive correlation was seen between RBM3 and Nbs1, also called Nbn or nibrin (Figure 6). Terf2IP is a member of a protein complex that, together with TRF2, regulates the length of telomeres and protects telomeric ends from a checkpoint response and DNA repair [203, 204]. In addition, a new role for cytoplasmic Terf2IP was recently discovered, whereby Terf2IP was found to regulate NF-kappa B signaling [205]. Elevated levels of cytoplasmic Terf2IP have been found in breast cancer cells compared to normal breast cells [203, 205]. Nbs1 is a central member of the MRN complex important for repair of DNA DSB [206]. Taken together, a plethora of mediator proteins involved in the DNA damage pathways were identified as differentially expressed between RBM3 high and RBM3 low tumors and even more proteins involved in DNA replication, suggesting a possible DNA damage independent role of RBM3.

Despite an evident inverse correlation between RBM3 and Chk1, Chk2 and MCM3 at the protein level in siRBM3 treated A2780 cells, no significant alteration was detected at the mRNA level, as demonstrated by real-time quantitative PCR. However, a trend towards elevated mRNA levels was observed (Paper II).

**High expression of MCM3, Chk1 and Chk2 is associated with an impaired survival in EOC**

The prognostic value of the RBM3 associated genes MCM3, CHEK1 and CHEK2 and their gene products was examined in Cohort I and Cohort II, respectively, revealing an association between high expression of the investigative markers and an impaired survival.
survival, both at the mRNA and protein levels (Paper II). This was confirmed in univariate Cox analysis, however in multivariate analysis, adjusted for established prognostic factors, only high CHEK2 mRNA levels remained an independent prognostic marker. This is, to our knowledge, the first description of an association between these proteins and an impaired survival in EOC patients.

Additionally, we examined the relationship of activated Chk1 and Chk2 and survival by IHC staining of EOC tumors with antibodies against pSer345-Chk1 and pT68-Chk2. This demonstrated phosphorylation of Chk2 to be a prognostic marker for a decreased overall survival, while no association between pSer345-Chk1 and survival was observed (Paper II).

The impact of RBM3 on phosphorylation of Chk1 and Chk2
Having demonstrated that RBM3 has a sensitizes EOC cells to cisplatin treatment

![Figure 7. Relationship between RBM3 and Chk1, Chk2 and MCM3 in EOC cell lines. The expression levels of Chk1, Chk2 and MCM3 were examined in the cisplatin resistant cell line A2780/Cp70 and in the parental cisplatin sensitive cell line A2780 by (A) immunocytochemistry, (B) Western blot analysis and (C) Q-PCR.](image-url)
and an inverse correlation between RBM3 and the checkpoint proteins Chk1 and Chk2, known to be involved in the response to chemotherapeutic drugs, we next sought to examine the relationship between RBM3 and activated Chk1 and Chk2 in the context of cisplatin treatment and DNA damage response. We next examined the levels of Chk1, Chk2 and MCM3 in the cisplatin sensitive and resistant cell lines, A2780 and A2780/Cp70 respectively at both protein and mRNA level. Using both immunocytochemistry and Western blotting we demonstrated increased Chk1 protein expression in the cisplatin resistant A2780/Cp70 cells compared to A2780 cells (Figure 7A-B), hence showing an inverse expression pattern compared to RBM3, which is in line with our previously mentioned findings at the mRNA level in Cohort 1. There was a trend, however not significant, towards higher levels of CHEK1 transcript in A2780/Cp70 cells (Figure 7C). On the contrary, despite altered expression of Chk2 as consequence of RBM3 downregulation in A2780 cells, Chk2 expression was higher in A2780 cells compared to A2780/Cp70 cells, both at protein and mRNA levels (Figure 7). Similar to Chk1, the immunocytochemical staining of MCM3 was clearly more intense in the A2780/Cp70 cell line than in A2780 cells, however the difference was not as convincing in the Western blot analysis, although a slight increase of MCM3 was detected in A2780/Cp70 cells (Figure 7A-B). At the gene expression level, there was no difference in MCM3 expression between the two cell lines, but a trend towards rather to lower expression in A2780/Cp70 was observed (Figure 7C).

In response to cisplatin, we found a distinct increase of pSer345-Chk1 and pT68-Chk2 in the cisplatin sensitive A2780 cells, while no evident difference in the phosphorylation status of Chk1 and Chk2 was observed in the cisplatin resistant A2780/Cp70 cells (Figure 8). In line with a previous study in the adenocarcinoma cell line HCT116 [84], we observed a slight increase in pSer345-Chk1, but not in pT68-Chk2, in siRBM3 transfected cells in the absence of induced DNA damage (Paper II). To address whether RBM3 has any impact on the activation of Chk1 and Chk2 in response to cisplatin treatment, we exposed siRBM3 transfected A2780 cells to cisplatin for 1 h, followed by examination of the phosphorylation status of Chk1 and Chk2 after 48hrs of recovery. No obvious impact of RBM3 on the phosphorylation status of Chk1 at Ser345 or Chk2 at Thr68 was observed after exposure to cisplatin (Figure 8). The observed increase in phosphorylation of Chk1 in siRBM3 transfected A2780 cells in the absence of DNA damage 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 relatively low cellular level of the phosphorylated and total proteins.

**Downregulation of RBM3 causes a delay in S-phase**

Proteins involved in DNA replication, chromatin remodeling and DNA integrity checkpoint are also important regulators of cell division, and thus, we hypothesized that RBM3 is involved in cell cycle regulation (Paper III). To elucidate the possible role

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The Role of RNA-Binding Motif 3 in Epithelial Ovarian Cancer: A Biomarker Discovery Approach
of RBM3 in the cell cycle, we examined if alterations in RBM3 expression affected the cell cycle. We found that downregulation of RBM3 in the mouse fibroblast NIH3T3 cell line caused a delay in S-phase progression in a synchronized cell population, suggesting a possible role of RBM3 in the cell cycle. The impaired G1 to S-phase transition observed in RBM3-shRNA transfected cells coincided with an observed reduction of cyclin E levels while cyclin D1 levels were not affected.

Biochemical fractionation and immunofluorescence analysis of NIH3T3 cells and the human osteosarcoma cell line U2OS demonstrated that RBM3 localizes to both cytoplasm and chromatin fractions. The level of RBM3 bound to the chromatin remained constant during the cell cycle whereas the cytoplasmic RBM3 levels fluctuated. We found a cytoplasmic accumulation of RBM3 in early G1 and G2-M phases. Elevated levels of cytoplasmic RBM3 coincided with an increase in total levels of RBM3. These data suggest that the temporary increase of RBM3 in the cytosolic fraction might reflect the cytoplasmic translation of RBM3 before its translocation to the nucleus, where it most probably exhibits its function.

In Paper II, numerous proteins involved in DNA replication were identified as differently expressed between RBM3 high versus low RBM3 expressing tumors in the performed gene expression analysis, which implies that RBM3 is a DNA regulating protein.

![Figure 8. The effect of RBM3 on activation of Chk1 and Chk2. siRBM3 transfected cells were, 24 hrs post-transfection, treated with cisplatin (50uM) for 1 h followed by 48 hrs culture in fresh drug-free media. The phosphorylation of Chk1 and Chk2 was analysed by immunoblotting with antibodies against pSer345-Chk1 and pT68-Chk2.](image)
The Role of RNA-Binding Motif 3 in Epithelial Ovarian Cancer: A Biomarker Discovery Approach

protein. To test this hypothesis, we have set up a GST-pull down assay in search for possible nuclear RBM3 interacting partners. We aim to incubate recombinant GST-RBM3 with nuclear extracts from synchronized cells and we hope to find interesting proteins and putative biomarker candidates such as: polymerase (DNA directed) delta 4 (POLD4), ORC3L, Nbs1 and Rad17, proteins that were found to be positively associated with RBM3 in the gene expression analysis.

Discussion

In this thesis, we have investigated the prognostic value of RBM3 and further uncovered the connection between RBM3 and cellular processes involved in the maintenance of DNA integrity. The identification of RBM3 associated genes by gene profiling of treatment naive EOC tumors with high versus low RBM3 mRNA expression, followed by application of the HPA as screening tool, led to the discovery of three RBM3 associated genes possessing prognostic value in EOC; MCM3, Chk1 and Chk2. Thus, this thesis is an example of a translational approach for biomarker discovery, where the HPA has served as a tool for the identification of biomarker candidates, which have then been further investigated regarding their potential clinical relevance in clinically well-annotated tumors from independent patient cohorts. In addition, in vitro studies have been performed to elucidate the biological function of the potential biomarkers, with the aim to explain the mechanisms behind the observed clinical associations.

The complexity of RBM3

RBM3 is a small glycine rich protein containing a RNA-recognition motif to which it can bind both DNA and RNA [70]. RNA binding proteins play an important role in the regulation of mRNA and are, consequently, important in the maintenance of a proper gene expression [65, 207]. The implication of RBM3 in cancer is supported by various studies, however there are discrepancies among the conclusions regarding the role of RBM3 in tumorigenesis. It becomes more and more clear that RBM3 is upregulated in a wide range of human tumors, confirmed by evidence from several studies performed by various independent groups, including ours [57, 74, 84]. It is also well established that RBM3 expression can be induced by various conditions causing cellular stress [71, 72, 74-76, 208]. However, there is discordance between the interpretations of existing experimental data and the observed favorable prognostic impact of a high RBM3 expression in human tumors.

There is now growing evidence from different experimental in vitro setups that RBM3 can provide growth advantages by mediating an increased proliferation. In colon cancer cell lines, an increased proliferation was observed as consequence of overexpression of RBM3, and an inhibitory effect on cell survival was caused by siRNA experiments targeting RBM3 in prostate cancer cell lines [84]. In line with these findings, we observed a reduced cell viability in A2780 ovarian cancer cells transfected by siRNA against RBM3. A stronger immunohistochemical staining of RBM3 has also been
demonstrated in normal intestinal epithelial cells with a high proliferation rate compared with lowly proliferative skeletal and heart muscles cells [74], indicating a positive correlation between RBM3 and proliferation during normal conditions. Taken together, these observations point towards a proliferative effect of RBM3 and RBM3 has indeed been claimed to be a proto-oncogene contributing to cell growth and the prevention of mitotic catastrophe, by Sureban and colleagues [84]. The authors further speculate that targeting RBM3 would be an efficient novel therapeutic strategy against cancer. However, this hypothesis is strongly questioned by our clinical observations, implicating a favorable outcome in breast and ovarian cancer patients with high RBM3 expression, compared to patients with none or low levels of RBM3 (Paper I) [57]. This argument is further supported by the identification of RBM3 as one of 26 proteins found to be down regulated during melanoma progression in a cDNA array [82]. It should also be emphasized that the finding that RBM3 acts as a proto-oncogene in cellular processes essential for transformation, like proliferation and apoptosis, does not necessarily contradict the favorable patient outcome associated with the presence of RBM3 in an established tumor. In EOC, the clinical observations might in part be explained by a sensitizing effect of RBM3 towards chemotherapy, which in turn could be a consequence of an increased proliferation rate in RBM3 high tumors. Notably, in prostate cancer, a downregulation of RBM3 lead to cell cycle arrest in androgen-dependent but not androgen-independent cells, the latter known to be more aggressive [75].

**RBM3 affects the DNA damage response machinery and sensitizes cells to cisplatin**

A cornerstone in the treatment of ovarian cancer is the platinum-based compound cisplatin. Cisplatin was found to have anti-tumor properties over 40 years ago and has since then had a major impact on cancer therapy, particularly in the treatment of testicular and ovarian cancer [28]. The toxicity of cisplatin is mediated by its ability to bind covalently to purine DNA bases, which causes cross-linking of DNA and activation of several signal transduction pathways involved in DNA-damage repair, cell cycle arrest and cell death [28-30]. Our *in vitro* results from *paper I* uncovered an enhancing effect of RBM3 on the cellular toxicity of cisplatin, as demonstrated in A2780 ovarian cancer cells. These findings are contradictory to observations made in the androgen dependent LNCaP prostate cancer cell line, where a down-regulation of RBM3 enhanced the toxicity of cisplatin, however this effect was not observed in the more aggressive androgen independent PC-3 cells [85]. The authors speculated that this difference in effect on chemosensitivity is dependent of the status of p53. LNCaP cells have functional p53 while PC-3 cells possesses a mutant form, and it was further demonstrated that down-regulation of RBM3 generated an inactivation of p53 and p21 and, subsequently, enhanced chemosensitivity [85]. It should however be pointed out that, in contrast to the situation in ovarian cancer, prostate cancer is not routinely treated with platinum-based chemotherapeutic agents.
RBM3 does not have a major impact on the process of apoptosis

Cisplatin is well known to induce cell cycle arrest and apoptosis [28-30]. Although A2780 cells have intact p53 and are highly sensitive to cisplatin, we could not observe a pronounced increase in apoptosis after cisplatin treatment, neither with AnnexinV/7AAD staining nor by detection of cleaved caspase-3, which was surprising (Paper I). We did, however, see a prominent G2 arrest. Although A2780 is one of the most cisplatin sensitive ovarian cancer cell lines, A2780 cells are not prone to undergo apoptosis after cisplatin treatment [209]. We could not detect any alterations in the protein levels of neither the pro-apoptotic Bax protein nor the anti-apoptotic Bcl-2 protein in RBM3 downregulated cells, supporting the theory that RBM3 promotes cisplatin sensitivity via induction of cell cycle arrest rather than apoptosis in A2780 cells. This was further strengthened in paper II, where we uncovered an association between RBM3 and cellular processes important for the protection of genomic stability, like DNA damage response, and an inverse correlation between RBM3 and Chk1, Chk2 and MCM3. Both Chk1 and Chk2 are central kinases in the DNA damage response that can drive the cell into cell cycle arrest or apoptosis, both in a p53 dependent and -independent manner [26, 142]. Exactly what determinates the nature of a DNA damage response depends on multiple of factors e.g. the form of DNA damage, the severity of the damage and, in particular, the dynamics and abundance of key mediators that direct the signal to different effector proteins [26, 152, 192]. Despite considerable advancements in the attempt to map the complex network of the DNA damage machinery, many questions still remain unresolved, e.g. the induction of cell cycle arrest or apoptosis [26, 152, 192]. It is conceivable that cisplatin induces cell cycle arrest in one cell line but apoptosis in another, not necessarily dependent on the p53 status. p53 can induce a G1 phase arrest as well as apoptosis and there are many factors involved in both the ATM/p53 and ATM/Chk2 pathways that could possibly explain the low activation of apoptosis observed in A2780 cells [26, 140].

We found it quite surprising that the cisplatin sensitive A2780 cells express Chk2 in such large amounts, considering the observation of elevated levels of Chk2 in A2780 cells as a result of RBM3 down regulation, indicating an inverse association between RBM3 and Chk2. However, in the light of the complexity of the network of proteins involved in the process of DNA damage protection, A2780 cells might possess other properties that cause elevated levels of Chk2, despite a high RBM3 expression. As observed in the gene expression profiling analysis, RBM3 affects numerous proteins and it might be that the intracellular milieu in A2780 cells favors Chk2 expression. One explanation might be that RBM3 influences Chk1 to a larger extent than Chk2, and that elevated levels of Chk2 compensate for the low Chk1 levels. However, additional studies are required to answer these questions. In the GSEA, comparing tumors with high and low RBM3 expression, apoptosis did not appear as a process significantly influenced by RBM3, which further strengthens our conclusion that RBM3 exhibits its cisplatin sensitizing effect primarily via an induction of cell cycle arrest and not
RBM3 and the activation of checkpoint proteins

In *paper I*, we demonstrate that cisplatin sensitive A2780 cells show a higher expression level of RBM3 than the cisplatin resistant A2780/Cp70 cells. In *paper II*, it is further shown that, upon cisplatin treatment, A2780 cells activate a checkpoint response by an increase in pSer345-Chk1 and pThr68-Chk2, whereas no change was observed in A2780/Cp70 cells. Notably, A2780/Cp70 cells showed a higher initial phosphorylation level of both Chk1 and Chk2 compared to A2780 cells. Another difference lies in the Chk1 levels, whereby Chk1 is strongly down regulated in A2780 cells in response to cisplatin, but not in A2780/Cp70 cells. This is interesting, given the reported association between chemotherapy resistance and defects in the capacity to downregulate Chk1 [210]. In response to stress, Chk1 is phosphorylated at Ser317 and Ser345 by ATR, which triggers the checkpoint response [143, 176, 177]. Ser345-Chk1 phosphorylation by ATR is crucial for its activity but it also marks the protein for destruction [179]. Downregulation of Chk1 is a prerequisite for re-entry into the cell cycle after an arrest. Still, it has been demonstrated that tumor cells incapable of proteolytic degradation of Chk1 become resistant to chemotherapy [210], which seems contradictory. A reasonable explanation for the observed chemotherapy resistance in Chk1 high tumors might be related to the essential function of Chk1 for stabilization of stalled replication forks [179]. Tumor cells are driven to proliferate under proliferation-promoting oncogenic signals which activate the DNA damage response already at an early stage in tumor formation [190, 191] and Chk1 activity prevents the tumor cells from stalled replication forks. If the tumor cells are exposed to additional stress induced by chemotherapy, they will respond, in an attempt to handle the introduced DNA damage, with a vigorous checkpoint activation, in an attempt to handle the introduced DNA damage, resulting in a rapid turnover of the Chk1 proteins, that will eventually diminish below a threshold for activation. This will push the stalled replication fork into a collapse due to lack of stabilization by Chk1, with the consequence of mitotic entry with lethal DNA damage, i.e. mitotic catastrophe [190, 191], which is the desirable effect of chemotherapeutic drugs. On the other hand, if the tumor cells have acquired mutations in the mechanisms promoting Chk1 degradation, the protein levels of Chk1 will remain constantly high, whereby the cell will be protected from mitotic catastrophe and tolerate higher doses of chemotherapy [190, 191]. This might explain the observed low Chk1 levels in A2780 cells with high RBM3 expression, that were even further downregulated upon cisplatin treatment, while the cisplatin resistant A2780/Cp70 cells, that are not affected by treatment, expressed higher amounts of Chk1. From these observations, together with the inverse correlation between RBM3 and the checkpoint proteins observed in the siRNA experiments, it is tempting to suggest that RBM3 is involved in the mechanisms promoting degradation or generation of low levels of cellular Chk1 in the cell. However, a down regulation of RBM3 did not counteract a decrease in apoptosis.
total Chk1 protein level after cisplatin treatment for 1 hour followed by 48 hours of recovery, which might in part be explained by the experimental set up.

These observations require verification and complementary experiments to elucidate whether RBM3 is involved in the regulation of Chk1 in response to stress. Although it is a plausible thought, there are still many remaining question marks, e.g. how RBM3 regulates the protein levels of Chk1 and Chk2 and the rationale for a cell to downregulate key proteins for the maintenance of genomic integrity. One reason for the cell to repress such an essential safe-guardian mechanism as the checkpoint machinery, which seems contradictory, might be that it serves as a protectory mechanism against improper activation of DNA damage during non-stress inducing conditions.

There is an obvious increase in protein levels of Chk1, Chk2 and MCM3 48h post-transfection with siRBM3. We were not able to detect any significant alterations at the mRNA level upon downregulation of RBM3, but a trend toward elevated levels of the transcripts was observed. It could be speculated that RBM3 inhibits translation of the mRNA of CHEK1, CHEK2 and MCM3, and most likely several other proteins involved in DNA damage and, subsequently, checkpoint response. Thus, a downregulation of RBM3 would enable translation of the released mRNA with a quick increase in the protein level but no obvious fluctuation at the mRNA level. RBM3 has indeed been reported to be involved in translation, but rather contributing to an enhanced global translation and not a suppression [74, 79, 80]. In Cohort I, tumors with low levels of RBM3 mRNA expression showed significantly higher levels of CHEK1, CHEK2 and MCM3 mRNA compared with tumors expressing high RBM3 levels. Considering the fact that RBM3 is an RNA binding protein, it could be that RBM3 binds and destabilizes the mRNA of CHEK1, CHEK2 and MCM3, which might explain their low levels in RBM3 high tumors. However, additional detailed investigations are required to draw any further conclusions about how RBM3 affects the protein levels of Chk1, Chk2 and MCM3.

Recently, CIRP, the other human RNA-binding cold-shock protein, was demonstrated to bind and stabilize ATR transcripts, thus contributing to an increase in ATR protein levels after UV exposure and, subsequently, an increase in active Chk1 [78]. This demonstrates both a direct relation between cold-shock proteins and the DNA damage response machinery. The role of RBM3 under stress-related conditions is less examined, but RBM3 is susceptible for posttranslational modifications by phosphorylation at both serine, threonine and tyrosine residues [79]. In contrast to CIRP, which promotes translation of a protein important for DNA damage response, RBM3 activity generates a decrease in the protein levels of various checkpoint proteins, which is in contrast to the enhanced global translation capacity RBM3 has been demonstrated to possess [80]. However, RNA-binding proteins have been described to both promote and repress translation and to stabilize and destabilize mRNA [63, 65]. It could well be
that RBM3 enhances the translation of proteins that trigger degradation of checkpoint proteins, which would also be of interest to investigate further.

**Nuclear RBM3 promotes S-phase entry**

Downregulation of RBM3 generated a delay in S-phase, indicating an association between RBM3 and cell cycle progression (*Paper III*). This could in part be explained by the inverse correlation between RBM3 and the checkpoint proteins Chk1 and Chk2. Downregulation of RBM3 generated an increase in the total levels of Chk1 and Chk2. Phosphorylations of Ser345-Chk1 and Thr568-Chk2 were detected even in the absence of induced stress, indicating an active checkpoint response and, consequently, a delay in cell cycle progression. Since we observed a delay and not a more robust G1 arrest, does not prove, but could indicate, that the checkpoint response is independent of p53, which is in line with what we see in A2780 cells after cisplatin treatment. Activation of p53 presumably generates a more robust G1 arrest [26]. However, it might be also possible that the observed cell cycle delay is due to a more direct affect on the DNA replication process by RBM3. In the gene expression array, a multiple of replication-associated proteins were identified to be differently expressed between tumors with high versus low RBM3 expression, among them POLD4 and ORC3, both implicated in the initiation of replication. POLD4 is a subunit of the DNA polymerase δ complex and is essential for DNA replication and repair [211, 212]. ORC3 is a component of the origin recognition complex (ORC) that binds to origins of DNA replication [213]. ORC is a dynamic complex consisting of 6 subunits (ORC1-6), and it has been proposed that this complex assembles as the cells exit mitosis and binds to chromatin in an ATP-dependent manner, serving to direct the formation of pre-replication complexes and when the cells enter S-phase the complex is degraded and disassembles [213]. The exact mechanism behind the function of the complex is not fully understood. The relationship between RBM3 and replication-associated proteins needs to be verified and investigated in more detail in order to elucidate whether RBM3 is involved in normal DNA replication or if it is a consequence of an activated checkpoint response. We are currently searching for binding partners to RBM3 in nuclear extracts from cells synchronized in S-phase and, hopefully, this will contribute to a deeper understanding of the function of RBM3.

**Do RBM3 high tumors lack an anti-cancer barrier?**

The association of RBM3 with processes involved in the maintenance of DNA integrity and the inverse correlation to MCM3 and the checkpoint proteins Chk1 and Chk2 uncovered in *paper II* additionally supports the notion that RBM3 sensitisizes cells towards DNA damage. The checkpoint proteins operate to halt cell cycle progression in the presence of DNA damage or stalled replication forks to ensure genomic stability and avoid entrance into mitosis with lethal DNA damage [26]. MCM3 is a component of the DNA replication licensing system essential for maintenance of precise chromosome duplication [103, 214]. It is well established
that Chk1, Chk2 or MCM3 are essential for genomic stability and that the loss of any of these three proteins is linked to tumorigenesis [6, 26, 113, 215-217]. The inverse correlation between RBM3 and the checkpoint proteins means that RBM3 high tumors have decreased protein levels of Chk1 and Chk2 and, accordingly, a less efficient DNA damage response. While this makes the cells more susceptible to drugs inducing severe DNA damage, it will also allow for an accumulation of genetic mutations. There are several pieces of evidence supporting a model where activation of the DNA damage signaling in pre-cancerous cells not only acts as an anti-cancer barrier inhibiting cell proliferation, but also provides an environment for selection of mutations or epigenetic silencing of checkpoint genes [190, 191]. This will in turn generate clones that are capable to progress into a more malignant phenotype, capable of uncontrolled proliferation due to the ability to bypass the cellular checkpoint machinery [190, 191]. Following this model, RBM3 high tumors might have a less active anti-cancer barrier due to the low amounts of Chk1 and Chk2, which will facilitate the accumulation of mutations and, hence, the cells are put under a lower pressure for selection of mutant clones. On the other hand, RBM3 low tumors would then have a more active anti-cancer barrier and therefore eventually be able to select for mutants with the ability to avoid an active DNA damage response. This could explain the proliferative properties of RBM3 observed in cell lines [74, 84], the higher sensitivity to cisplatin in cells expressing high amounts of RBM3 (Paper I), and the association between a high tumor-specific RBM3 expression and a significantly prolonged survival in cancer patients (Paper I) [57]. To conclude, RBM3 promotes accumulation of genetic mutations to a certain level, due to its capacity to attenuate the DNA damage response, but RBM3 high cells are less aggressive because of the lack of capability to transform into a more aggressive phenotype. The observed decrease of RBM3 along with malignant melanoma progression supports this theory [82].

The identification of novel biomarkers in EOC
In addition to uncover the prognostic and treatment predictive value of RBM3 in EOC, we have also discovered a prognostic and putative treatment predictive value of Chk1, Chk2 and MCM3. While high RBM3 expression is associated with a significantly prolonged survival, high expression of Chk1, Chk2 and MCM3 is related to an impaired survival of EOC patients. This can be explained by the earlier discussed cancer model, where a partly active DNA damage response provides an environment for selection of more aggressive tumors. Chk1 has previously been reported to correlate with tumor grade and cell proliferation in breast cancer [218] and CHEK2 mutations have been studied in the context of hereditary breast cancer [219]. High expression of MCM3 has been reported to associate with an impaired survival in malignant glioma [215], medulloblastoma [216] and malignant melanoma [217].
Major strengths and limitations
There are some aspects in thesis that need further attention and clarification. The main findings and hypotheses are based on analyses in tumors from two independent patient cohorts. Cohort I, from which the gene expression analyses are derived, consists of 285 cases of serous and endometroid carcinomas of the ovary, fallopian tube and peritoneum. Although there are biological differences between these carcinomas, they are virtually indistinguishable in clinical practice. In approximately 70% of cases, these tumors are diagnosed at an advanced stage and it is almost impossible to decide if a tumor is a primary ovarian cancer with a tubal metastasis or vice versa. The patients that had received neoadjuvant platinum based chemotherapy were excluded from the analyses. Cohort II consists of 154 prospectively collected ovarian carcinomas. In this cohort, treatment data was not available for all patients and data on the extent of residual tumor is also lacking, and therefore not included in the multivariate analysis. In this cohort, RBM3 expression was examined by employing the tissue microarray (TMA) technique and immunohistochemistry. The RBM3 antibody used in Paper I has been extensively validated, using Western blot and immunocytochemistry to verify a downregulation of RBM3 expression in si-RBM3 treated A2780 cells compared to controls. In addition, the TMAs from this cohort have been stained with the polyclonal antibody used in the paper by Jögi et al [57], with concordant results (unpublished data). A major strength in Paper I is that RBM3 has been demonstrated to be a prognostic biomarker in two independent patient cohorts, one based on mRNA expression and one on protein expression.

For the in vitro studies, investigating RBM3 and cisplatin response, two cell lines have been used, the cisplatin sensitive A2780 cells and their cisplatin resistant derivatives A2780/Cp70. Ideally, the experimental setup should be repeated in additional cell lines to verify the results obtained within this thesis work, including a p53 deficient cell line. A major strength in the siRNA experiments is that mainly all experiments have been performed using three different oligonucleotides targeting RBM3 in parallel. On the other hand, the inclusion of experiments related to the effects of RBM3 overexpression would have further strengthened the study. In Paper III we have analyzed the role of RBM3 in cell cycle progression using both mouse- and human cell lines, which is of importance when examining conserved cellular processes like cell cycle progression and replication.

Taken together, in our translational approach, we have worked our way from “bedside” to “bench” and not vice versa, the latter still being a more common approach. The fact that we have generated our hypotheses based on results from clinically well-annotated tumor tissue, and then explored the functional basis for these observations, is a major strength, since it allows for selection of the most promising biomarkers in a clinical context.
Conclusions
In this thesis we have analysed the prognostic value of the RNA-binding protein RBM3 and identified novel RBM3 associated potential prognostic markers in EOC. Furthermore, we have gained some insight into the function of RBM3 and, hence, why it is associated with a favorable clinical outcome.

We could conclude that:

- High RBM3 expression is associated with a significantly prolonged survival in EOC patients, both at the mRNA and protein levels. Therefore, RBM3 may be a useful prognostic and treatment predictive marker in EOC (Paper I).
- RBM3 sensitizes A2780 ovarian cancer cells to the platinum based compound cisplatin (Paper I).
- RBM3 expression is associated with cellular processes involved in the maintenance of DNA integrity; e.g. DNA dependent replication, DNA replication, chromatin remodeling and DNA integrity checkpoint (Paper II).
- RBM3 correlates negatively with the checkpoint proteins Chk1, Chk2 and MCM3 (Paper II).
- High protein levels of Chk1, Chk2 and MCM3 are associated with a significantly reduced survival in EOC patients (Paper II).
- RBM3 promotes entry into S-phase (Paper III).

Future perspectives
There is an urgent need to develop new diagnostic and prognostic biomarkers in addition to accurate treatment predictive biomarkers in order to obtain an effective individualized treatment regimen for ovarian cancer patients. In this thesis we have uncovered the prognostic and treatment predictive value of the RNA-binding protein RBM3 and further discovered three RBM3 associated proteins with a potential prognostic a treatment predictive value. In addition, we have revealed a link between RBM3 and cellular processes promoting the maintenance of genomic stability, like DNA damage response, replication and chromatin remodeling and demonstrated an inverse correlation between RBM3 and Chk1, Chk2 and MCM3. These findings open up for a wide range of further investigations to explore the role of RBM3 in the maintenance of genomic integrity that might have an impact on cancer therapy. It would be of interest to further investigate RBM3 in response to other stress conditions that induce different kinds of DNA lesions, like DSB, in an attempt to map the main pathways affected by RBM3. One experiment would be to examine if RBM3 co-localizes to the foci of induced DNA lesions with phosphorylated histone H2AX after induced DNA damage. Further detailed studies of the relation between RBM3 and Chk1 and...
The Present Investigation

Chk2 would give valuable information about the role of RBM3 in DNA damage response, and possible regulation of the checkpoint proteins. A great advancement in the challenge to understand the biology of RBM3 would be to identify RBM3 targets and binding partners, both proteins and possible transcripts. We have set up a GST-pull down assay in search for possible nuclear RBM3 interacting partners. We aim to incubate recombinant GST-RBM3 with nuclear extracts from synchronized cells and we hope to find interesting RBM3-interaction partners. This experiment has the potential to provide information about a possible function of RBM3 in replication during normal conditions.

Interestingly, a gene array analysis of EOC uncovered an association between BRCA1 mutations and an increased expression of a cluster of genes at Xp11.23 [13], to which RBM3 is mapped. BRCA1 did not appear as a differently expressed gene in our gene expression profiling experiment between high versus low RBM3 expressing EOC tumors. However, it would be very interesting to explore a possible relationship between RBM3 and BRCA1, since both proteins are involved in the DNA damage response and the fact that there is a link between the checkpoint proteins Chk1, Chk2 and both BRCA1 and RBM3 indicates a probability for an association between RBM3 and BRCA1 as well.

From a clinical perspective, RBM3 has the potential to provide valuable information about treatment response. EOC patients with high tumor-specific RBM3 expression will probably benefit more from cisplatin therapy than patients with RBM3 low tumors. A more detailed understanding of which pathways in the DNA damage response are affected by RBM3 would provide useful information from a therapeutic perspective, and open up for a more personalized therapeutic approach with a combination of drugs targeting different, for the cancer cell, essential pathways to obtain an optimal treatment regimen.

det bara finns ett fåtal tillförrlitliga tumörspecifika markörer tillgängliga idag och när det gäller äggstockscancer finns det ett stort behov av att identifiera nya markörer, t ex gener eller proteiner, som kan underlätta fastställandet av diagnos och prognos samt vägleda i valet av behandling.

I den här avhandlingen har vi undersökt om nivåerna av proteinet RBM3 kan ge värdefull information om prognosen vid äggstockscancer. RBM3 är ett relativt okänt litet protein som kan binda till och interagera med både DNA och RNA. RBM3 upptäcktes i en studie som identifierade proteiner vars uttryck stimulerades av stresspåverkan i form av kyla. RBM3s funktion är okänd men har i ett flertal studier förknippats med cancer då förhöjda nivåer av proteinet har detekterats i en rad olika cancerformer.


För att få en djupare inblick i RBM3s funktion i tumörceller undersökte vi i nästa delarbete vilka gener som påverkas av ett ändrat genuttryck av RBM3. Genom att jämföra genprofilerna mellan tumörer med högt RBM3 uttryck med tumörer som uppvisade betydligt lägre RBM3-nivåer, kunde vi urskilja mer än 800 gener vars uttryck samvarierade med RBM3. Tumörer med högt RBM3 uttryck uppvisade avsevärt lägre nivåer av gener som kodar för proteiner som har betydelse vid cellens försvar mot angrepp på arvsmassan, DNA. Genomisk stabilitet är enormt viktigt för cellen och hela organismens existens. En rad sjukdomar uppstår på grund av DNA-skador eller en ofullständig genomutveckling. Vårt DNA är under konstant angrepp av ämnen i vår

I det tredje delarbetet i avhandlingen undersöker vi närmare vilken roll RBM3 har vid normal celldelning. Våra resultat visar att celler som har låga nivåer av RBM3 bromsas när cellerna ska gå in replikationsfasen, det vill säga den fas där arvsmassan kopieras precis innan cellen delar sig.

Sammanfattningsvis har vi visat att höga RBM3 nivåer är kopplade till en bättre prognos av äggstockscancer och att RBM3 möjligtvis kan användas som en markör för att förutsäga effekten av behandling med platinumbaserade cellgifter. Vi har vidare påvisat en koppling mellan RBM3 och nyckelproteiner i cellens försvar mot DNA skada och sett att RBM3 möjlingen kan spela en roll i regleringen av den normala celldelningsprocessen. Den här avhandlingen är ett exempel på translationell forskning, där vi skapar våra hypoteser genom studier av mänskliga tumorer, i vilka vi försöker identifiera proteiner med intressant uttrycksmönster för att sedan undersöka om det finns en koppling mellan proteinets uttrycksnivå och överlevnad. I ett nästa steg utför vi detaljerade cellbiologiska och biokemiska studier, för att om möjligt utröna mekanismerna bakom de undersökta proteinerens betydelse för sjukdomsförloppet. Även om det fortfarande återstår en rad obesvarade frågor har vi med våra resultat kommit närmare förståelsen av biologin bakom RBM3 och därmed en möjlig förklaring till den kliniska observationen att ett tumörspecifikt uttryck av RBM3 tycks vara förknippat med en gynnsam prognos hos patienter med äggstockscancer.
Till sist vill jag tacka alla er som varit engagerade i den här resan, både direkt och indirekt, utan er hade den här avhandlingen aldrig blivit skriven.


Fredrik ”Figge” Pontén, för ditt stöd och engagemang som bihandledare och för att du introducerat mig till det intressanta HPA projektet.

Maria ”Maite” Alvarado-Kristensson som varit min bihandledare. Det har varit lika rolig som lärorikt att få ta del av din expertis. Jag uppskattar din ärlighet, din kämparanada som forskare och dina underbara glädjeyttringar: utan dig hade det varit så tyst på labbet!

TMA-teamet. För att ni är så härliga personer! Och för allt stöd ni gett mig det sista året. Elise, tack för all hjälp, inte minst med de fantastiska RBM3 färgningarna, vad skulle vi göra utan dig? Björn, ditt alltid glada humör och din positiva inställning kan inte annat än att smitta av sig. Alex, Noori, Jenny, och Sakarias för att ni bidragit med så härlig atmosfär till gruppen.

Reihaneh. Thank you for all help during the last year! It has been a pleasure to work with you. And thank you for the lovely persian food!

Donal and Darran, thank you for your hospitality, encouragement and support.

Mina medförfattare, det har varit en glädje att arbeta med er. Marianne, Sara, Henrik och Ylva på Atlas Antibodies för att ni bidragit med antikroppar och för trevliga möten.
The Role of RNA-Binding Motif 3 in Epithelial Ovarian Cancer: A Biomarker Discovery Approach

Kristin, tack för att du har stenkoll på allt administrativt som jag inte har. Christina, Siv och Elisabeth, utan er stannar labbet.


Alla på CMP! Tack för alla roliga samtalsämnen under lunch och torsdagsfrukostar och för att ni gör CMP till en trevlig arbetsplats.


Maya och Shanti för att ni är som mina extra systrar.

Jag vill även tacka min underbara mormor och vän Moa för alla intressanta samtal och ditt aldrig sinande engagemang.


Mamma och pappa, för att ni finns här för mig, och tror på mig vad jag än tar mig för. Ert stöd är alltid ovärderligt. Nu är den äntligen klar!

This work was carried out at the Department of Laboratory Medicine, Center for Molecular Pathology, Skåne University Hospital, Lund University, Malmö, Sweden.

Financial support was provided by the Knut and Alice Wallenberg Foundation, Gunnar Nilsson’s Cancer Foundation, the Swedish Cancer Society, the Crafoord Foundation and the Research funds of Malmö University Hospital.
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