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MAP Kinases Phosphorylate SPF45 and Regulate Its Alternative Splicing Function: Insights onto Phosphorylation-Dependent and -Independent Effects in Ovarian Cancer Cells

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MAP kinases phosphorylate SPF45 and regulate its alternative splicing function: insights onto phosphorylation-dependent and -independent effects in ovarian cancer cells

By

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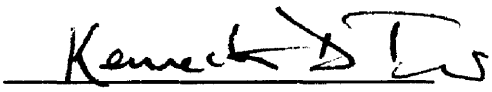

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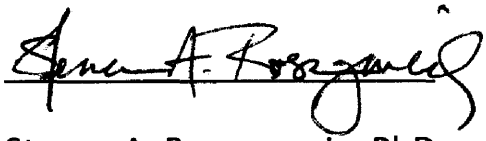

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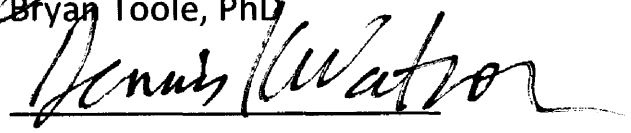

Dennis K. Watson, PhD

Dedication

To mom and dad,

Your endless love, patience, support, guidance and understanding are the reason of my success. Words fail to express my gratitude... I dedicate this work to you...

Thank you

Acknoweldgment

I would like to take this opportunity to thank my mentor, Dr Scott T Eblen, for giving me the chance to perform research in his lab and pursue my PhD under his supervision; thank you for your patience, support and dedication to my education throughout the time I spent in Graduate School, for your guidance on the path of scientific discovery and philosophy but most of all, for an everlasting friendship. To Dr Ken Tew, Dr Steven Rosenzweig, Dr Bryan Toole and Dr Dennis Watson, thank you for your willingness to serve on my committee, your continuous guidance, support and genuine interest throughout my training. To all the members of the Eblen lab, past and present, thank you for your patience, advice and kind words which made this process a pleasure. To all the members of the 6th floor Hollings Cancer Center and third floor Basic Science Building, past and present, the collegiality, friendships and happy moments will always be remembered and cherished. All the staff of the pharmacology department, past and present, thank you for handling my orders so diligently and processing them in a timely manner which made my work easier. And to all my friends and family, thank you for all the support, care and encouragement you keep on providing, I would not have been able to make it without you.

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List of Figures and Tables

List of abbreviations

The following is a simplified list of the most common abbreviations used throughout this dissertation. The list is not conclusive, however all abbreviations are described within the corresponding text.

3'ss	3' Splice Site
5'ss	5' Splice Site
Akt/PKB	Serine/Threonine Protein Kinase
ATXN1	Ataxin 1
BPS	Branch-Point Sequence
cDNA	Copy Deoxyribonucleic Acid
CLK1/2/3/4	Cdc2-Like Kinase 1/2/3/4
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EOC	Epithelial Ovarian Cancer
ErbB2/Her2/neu	V-Erb-B2 Erythroblastic Leukemia Viral Oncogene Homolog 2
ERK	Extracellular Regulated Kinase
ERK1b/1c	Extracellular Regulated Kinase Splice Variants 1b/1c
ERβ	Estrogen Receptor Beta
ESE/ESS	Exonic Splicing Enhancer/Suppressor
EST	Expressed Sequence Tag
Fas/FasL	CD95/CD95L - Death Receptor/Ligand
FGFR	Fibroblast Growth Factor Receptor
FN	Fibronectin
FN-EDIIIA/FN-EDA	Fibronectin-Extra Domain A
FN-EDIIIB/FN-EDB	Fibronectin-Extra Domain B
GAP	Gtpase Activating Protein
GEF	Guanine Exchange Factor
H2O2	Hydrogen Peroxide
hnRNP	Heterogenous Nuclear Ribonucleoprotein
HVR	Hypervariable Region
IL-1	Interleukin 1
impβ	Importin Beta
ISE/ISS	Intronic Splicing Enhancer/Suppressor
JNK	C-Jun N-Terminal Kinase

KSR	Kinase Suppressor Of Ras
MAP2K	Mitogen Activated Protein Kinase Kinase
MAP3k	Mitogen Activated Protein Kinase Kinase Kinase
MAPK	Mitogen Activated Protein Kinase
MEK	MAPK/ERK Kinase
MEKK1/2/3/4	MAPK/ERK Kinase Kinase 1/2/3/4
MKK3/6	MAPK Kinase 3/6
MKP	MAPK Phosphatase
NES	Nuclear Exclusion Signal
NLS	Nuclear Localization Signal
NMDA	N-Methyl D-Aspartate
NPC	Nuclear Pore Complex
OSE	Ovarian Surface Epithelium
p38	Protein 38 Kda Protein Kinase
PAK1	P21 Protein (Cdc42/Rac) Activated Kinase 1
PDGFR-β	Platelet-Derived Growth Factor Beta
PGK-1	Phosphoglycerate Kinase 1
PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PLCε	Phospholipase C Epsilon
PLD	Peggylated Liposomal Doxorubicin
PMA	Phorbol-12-Myristate-13-Acetate
PP2A	Protein Phosphatase 2A
pre-mRNA	Pre-Messenger Ribonucleic Acid
PTB	Polypryimidine Tract Binding Protein
PTMs	Post-Translational Modifications
Rac	Ras-Related C3 Botulinum Toxin Substrate, Rho Family, Small GTP Binding Protein
Raf	Rapidly Accelerated Fibrosarcoma, Serine/Threonine-Specific Protein Kinase
RalGDS	Ral Guanine Nucleotide Dissociation Stimulator
Ras	Rat Sarcoma Viral Oncogene Homolog, Small GTP Binding Protein
RRM	RNA Recognition Motif
RSK	Ribosomal S6 Kinase
RTK	Receptor Tyrosine Kinase
Sam68	Src-Associated In Mitosis, 68 Kda
SAP	Spliceosome Associated Protein
SCA1	Spinocerebellar Ataxia 1
SELEX	Systematic Evolution Of Ligands By Exponential Enrichmen

SF domain	SPF45 Like Domain
SF1	Splicing Factor 1
SF2/ASF	Splicing Factor2/Alternative Splicing Factor
SF3b155	Splicing Factor 3b 155 Kda
Shc	(Src Homology 2 Domain Containing) Transforming Protein 1
SMN2	Survival Of Motor Neuron 2, Centromeric
snRNP	Small Nuclear Ribonucleic Protien
Sos	Son Of Sevenless
SPF45	Splicing Factor 45 Kda
$SPF45^{AA}$	SPF45-Thr71AlaSer222Ala
$SPF45^{DD}$	SPF45-Thr71AspSer222Asp
SRC-1	(Schmidt-Ruppin A-2) Viral Oncogene Homolog (Avian)-1 , Tyrosine Kinase
SRp40	Serine/Arginine Rich Protein 40 Kda
SRp9G8	Serine/Arginine Rich Protein 9G8
SRPK	Serine/Arginine Rich Protein Kinase
STAT	Signal Transducers And Activators Of Transcription Protein
SXL	Sex Lethal Protein
TGFα	Transforming Growth Factor Alpha
TGFβ	Transforming Growth Factor Beta
TNFα	Tumor Necrosis Factor Alpha
TP53, p53	Tumor Protein 53 Kda, Tumor Suppressor Protein
U2AF/65/35	U2 Auxilliary Factor/65 Kda Subunit/35 Kda Subunit
UHM	U2AF Homology Motif
ULM	UHM-Ligand Motif
UV	Ultraviolet Radiation
VEGFR	Vascular Endothelial Growth Factor Receptor

Abstact

Alternative pre-mRNA splicing increases proteome diversity and is important in the behavior of cells in health and disease. The processes that regulate alternative pre-mRNA splicing are diverse and include regulation of splicing factors by phosphorylation. Here we report that the pre-mRNA alternative splicing factor SPF45 is a novel substrate of ERK, JNK and p38 MAP kinases in ovarian cancer cells. Using mutational analysis and phospho-specific antibodies, we demonstrate that MAP kinases phosphorylate SPF45 on Thr71 and Ser222 and that phosphorylation in cells is induced by a number of extracellular stimuli including PMA, EGF, serum, H_2O_2 and UV. Exon 6 of the death receptor Fas/CD95 has been shown to undergo alternative splicing in the presence of SPF45. Using a Fas minigene assay, we show that ERK2 and p38 inhibit SPF45 alternative splicing activity towards Fas, dependent upon these two phosphorylation sites. Other than Fas, no other pre-mRNA targets of SPF45 have been reported in mammalian cells. We generated SKOV3 cells stably-overexpressing wild-type SPF45 or a phosphorylation site mutant and performed an exon and gene array analysis to identify novel SPF45 splicing targets and genes whose expression is changed downstream of SPF45 splicing activity, respectively. From this analysis, 139 potential splicing targets and over 150 genes with altered expression were identified. We focus on four genes for validation with emphasis on ErbB2 and fibronectin. We show that SPF45 downregulates cellular proliferation in SKOV3 cells in a phosphorylation-dependent manner. Furthermore, we demonstrate that SPF45 regulates fibronectin alternative splicing, enhances inclusion of

FN-EDIIIA region, and affects cellular adhesion to fibronectin matrix. We also assess SPF45 binding to SF1 and SF3b155, essential components of the spliceosome, as well as the impact of Thr71 and Ser222 mutations on this interaction. Finally, we determine the effect of SPF45 overexpression in SKOV3 cells on their drug resistance profile. This study provides a link between MAP kinase signaling and splicing factors and identifies the role of this interaction in regulating molecular processes in ovarian cancer cells. Additionally, it provides the basis to investigate the role of SPF45 in ovarian cancer and to produce successful therapeutic interventions targeting SPF45-mediated effects.

Chapter 1: Introduction

Ovarian Cancer

The most recent statistics indicate that ovarian cancer is the second most common yet deadliest gynecological malignancy and ranks as the fourth leading cause of cancer death among women in the western world (Jemal 2009) surpassing mortality from both endometrial and cervical cancer combined (Ozols 2004). The etiology and pathogenesis of the disease are still poorly understood compared to other forms of cancer, primarily due to a lack of a progression model that can accurately explain the course of the disease (Shih Ie 2004). Ovarian cancer is usually designated as epithelial ovarian cancer (EOC) since 90% of ovarian tumors are believed to originate from the ovarian surface epithelium (OSE) (Auersperg 2001), with granulosa cells accounting for most of the rest, and a rare contribution from stromal or germinal cells (Auersperg 2001). The 5-year survival rate for ovarian cancer patients can be extremely good if detected early (~ 90% FIGO stage IA, IB), however it declines raidly with increased stage (32.5% FIGO IIIC, 18.6% FIGO IV) when unfortunately most patients (> 55%) are diagnosed (Heintz 2006).

Risk factors

The risk factors for developing ovarian cancer can be divided into three main categories: hormonal, genetic and environmental (Chen 2010).

Hormonal factors – such as early menarche or late menopause, nulligravidity, infertility, endometriosis (mainly endometrioid and clear cell ovarian cancer) and postmenopausal estrogen therapy.

Genetic factors – such as family history of ovarian cancer, BRCA1/2 mutations and Lynch syndrome.

Environmental factors – such as talc powder, cigarette smoking (mainly mucinous ovarian cancer) and obesity.

In contrast to the aforementioned risk factors, multiple pregnancies, oral contraceptives, tubal ligation, hysterectomy and oophrectomy, and breastfeeding are considered protective factors from developing ovarian cancer. Based on these observations and other indications, two theories have been proposed to explain the pathogenesis of ovarian cancer and are discussed later in this chapter.

Clinical manifestations, physical exam and diagnostic evaluation

Epithelial ovarian cancer is primarily a disease of menopausal and post-menopausal women. It typically manifests between the ages of 40 and 65, unlike the non-epithelial varieties which tend to be more common in younger population. One of the main hurdles in detecting ovarian cancer is the dull and vague presentation of symptoms that often leads to delayed discovery, and unfortunately at an advanced stage. The most common symptoms include: abdominal distention, nausea and anorexia. It is suggested that a frequent and more severe presentation of these symptoms should prompt ovarian cancer in the differential diagnosis (Chen 2010). Equally, an adnexal mass palpated during pelvic or rectovaginal examination should also

prompt further workup to rule out ovarian cancer (Chen 2010). Ultrasound sonography is the tool of choice for diagnostic evaluation of malignant growth versus ovarian cysts, the former of which requires further surgical intervention.

Etiology of epithelial ovarian cancer - Proposed hypotheses

Two main theories have been proposed and accepted to explain the origin of EOC. The first theory, proposed by Fathalla (Fathalla 1971), also known as "the incessant ovulation" theory, is based on observations of development of adenocarcinoma of the ovaries of domestic fowls that are maintained at a continual ovulation rate without seasonal rest. Epidemiological studies point to strong relationship between the development of ovarian cancer and nulliparity compared with a lower risk for multiple pregnancies especially a younger age of first pregnancy; equally, there is higher incidence of ovarian cancer associated with fertility treatment regimens that induce hyperovulation compared to a protective effect associated with oral contraceptives (Cramer 1983, Greene 1984). Due to an increased rate of wound healing and repair of the surface epithelium, the hypothesis postulates that adenocarcinoma of the OSE is induced by the formation of foci of genetic mutations known as "inclusion cysts" providing a fertile ground for tumor initiation and growth at the sites of ovulation, which is greatly increased by a higher rate of ovulation similar to the cases of nulliparity and fertility treatment. The hypothesis is also supported by observations of malignant progression of rat OSE that are extensively subcultured (Godwin 1992, Testa 1994) and an increase in inclusion cysts and changes of ovarian surface epithelium in women with higher risks of ovarian cancer (Feeley 2001, Schlosshauer 2003).

The second hypothesis, developed by Cramer and Welch (Cramer 1983), is known as "the gonadotropin theory". The process of ovulation is under strict hormonal regulation by pituitary-secreted gonadotropins, which by themselves have no tumorigenic effect on ovarian epithelial cells grown in culture (Ozols 2004), although they cause a disruption of the ovarian epithelial basement membrane altering tissue regeneration and cellular signaling (Roland 2003). Additionally, ovulation constitutes an inflammatory-like process that culminates in cell rupture (Espey 1994). Based on these observations and the fact that inflammation is one of the hallmarks of cancer (Hanahan 2000) that increase mutagenic alterations, persistent gonadotropins secretions in the body even after menopause, along with any other potential source of inflammation are thought to be carcinogenic and lead to the development of ovarian cancer.

Interestingly, a third hypothesis/theory has emerged in the last decade implicating the fallopian tube as a potential source for serous ovarian adenocarcinoma. Studies by Piek et al identified tubal intraepithelial carcinoma (TIC) in fimbriae from patients who underwent elective bilateral salpingo-oophorectomy due to high risk of ovarian cancer (Piek 2001). Furthermore, a study by Carcangiu et al identified hyperplastic changes in prophylactically resected fallopian tubes of BRCA1 mutation carriers (Carcangiu 2004). Furthermore, TICs are identified in more than 50% of cases with ovarian cancer, beside shared mutations in TP53 of TICs and adjacent bulky ovarian masses (Kindelberger 2007).

Histopathology

As mentioned earlier, the majority of ovarian cancer cases are of epithelial origins, therefore the discussion will be limited to the different histopathological types of EOC. EOCs are divided into the following subtypes: serous, mucinous, endometrioid, clear cell, transitional cell (Brenner type), squamous and mixed epithelial based on their resemblance to the epithelia neighboring the ovaries (Seidman 2002, Scully 1999) with each of these subtypes further divided into benign, borderline and malignant. The most important findings of the different EOC histological types are summarized in table 1.1 below.

Histology	Characteristics
Serous (benign, borderline and malignant)	Endosalpingeal. 75% high-grade, 25% low-grade (Ben-Baruch, 1996). Low-grade less responsive to CRx (Schmeler 2008, Gershenson 2009)
Mucinous (benign, borderline and malignant)	Endocervival. Forms largest ovarian tumors with a median diameter of 18 to 20 cm (Seidman 2002)
Endometrioid (benign, borderline and malignant) **Epithelial-stromal and stromal**	Endometrial. Foci of endometriosis: precursor lesions (Heaps 1990, McMeekin 1995). Better survival than serous (Storey 2008).
Clear cell (benign, borderline and malignant)	Mullerian. (Also called mesonephroid). May arise from endometriosis (Scully 1998). Lower response rate to platinum- and taxane-based CRx (Behbakht 1998, Goff 1996)
Transitional cell (benign, borderline and malignant) **Non-Brenner transitional cell**	Transitional. Benign – small solid masses within stroma. Intermediate – multicystic, low malignant potential. Both unilateral. Malignant – atypical features and invasive characteristics.
Squamous cell tumors	Squamous
Mixed epithelial (benign, borderline and malignant)	Mixed. Two or more distinct histologic types. Presence of serous carcinoma or sarcoma associated with poorer prognosis (Brown 2004)
Undifferentiated	Anaplastic
Unclassfied	Mesothelioma, etc

Table 1.1 EOC types and significant findings

Genetics of Ovarian Cancer/Dualistic model of ovarian carcinogenesis

According to Robert Kurman, director of the division of gynecologic pathology at Johns Hopkins, EOC tumors are divided into types I and II based on the histological presentation and molecular aberrations found (Shih 2004). Type I tumors tend to follow an indolent course and develop gradually from precursor lesions, developing from cystadenomas and adenofibromas, progressing to borderline malignancies then finally becoming low grade tumors. They are also associated with several signaling pathways and genetic mutations most noticeable of which are K-Ras and B-Raf mutations. In a way, type I tumors are reminiscent of the progression model of colorectal adenocarcinoma; unfortunately, they represent a minority of ovarian cancer cases (Shih 2004). Type II tumors, on the other hand, are more common and are associated with an aggressive and fast course, which prevents the detection of precursor lesions if any. At times, they are thought to develop *de novo* (Shih 2004). Unlike type I tumors, there are no noticeable changes in signaling pathways, but *TP53* mutations are observed in at least 80% of cases and seem to present a unifying feature of type II tumors. The following two tables (tables 1.2 and 1.3) summarize the main characteristics of these two types.

Type I tumors	Common molecular genetic alterations
Low-grade serous carcinoma (invasive MPSC)	***BRAF* or *KRAS* mutations**
Mucinous carcinoma	***KRAS* mutations**
Endometrioid carcinoma	**β-catenin gene mutations** **Microsatellite instability** LOH or mutations of PTEN *KRAS* mutations
Clear cell carcinoma	**TGF-β RII mutation** *KRAS* mutations Microsatellite instability

Table 1.2 Type I EOCs and significant molecular changes. *Adapted from Shih and Kurman*. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 2004.

Type II tumors	Common molecular genetic alterations
High-grade serous carcinoma	***p53* mutations** *HER2/neu* gene overexpression *AKT2* gene overexpression Inactivation of *p16* gene
Malignant mixed mesodermal tumor (carcinosarcomas)	***p53* mutations**

Table 1.3 Type II EOCs and significant molecular changes. *Adapted from Shih and Kurman*. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 2004.

Chemotherapy for epithelial ovarian cancer

The standard of care for patients newly diagnosed with EOC depends on the stage of the disease upon presentation. 75% of patients are diagnosed at an advanced stage, usually stage III (dissemination into peritoneal cavity or lymph node involvement) or IV (disseminated to more distant tissues). For these patients, surgical debulking followed by chemotherapy (taxanes and platinum compounds) remains the method of choice (Young 1983). For the remaining 25% of patients (stages I and II, I being restricted to the ovary while II is in the pelvic cavity), surgery alone is the main approach (Young 1990, Heintz 2006), with further chemotherapy indicated in high-risk stages I or II in order to avoid relapse. Optimal debulking (residual tumors < 1cm) has been associated with improved relapse rates (Herzog 2010) however the majority of these patients develop recurrent refractory disease from which they ultimately die. In the following I will discuss the two main anti-neoplastic agents employed for ovarian cancer and then approach recurrent ovarian cancer and the different modalities used.

Platinum compounds

Before the introduction of platinum compounds, cyclophosphamide and doxorubicin were the drugs of choice for cancer patients. Serendipitously discovered to have anti-growth effects (Rosenberg 1973, 1985), cisplatin represents the prototype of platinum compounds. Cisplatin is composed of a platinum atom complexed to two ammonia and two chloride atoms in a square planar geometry. Cisplatin inhibits tumor cells growth and it has been postulated that the central platinum atom binds the DNA forming diaminoplatinum adducts causing cross-links and disrupting the DNA structure

(Huang 1994, McGuire 1998). Addition of cisplatin to the established chemo regimens resulted in a substantial improvement in response rates (Omura 1986, Thigpen 1994). Due to its undesirable side effects such as neurotoxicity, nephrotoxicity, ototoxicity and gastrointestinal toxicity (Ozols 1984), chemical modifications of the cisplatin led to the development of several other platinum compounds including carboplatin and oxaliplatin. Carboplatin has equal efficacy to the parent compound (ICON2, 1998) with lesser associated side effects, except for an increase in myelosuppression that can be dose-limiting (Ozols 1989). Oxaliplatin is active in ovarian cancer and overcomes cisplatin resistant tumors *in vitro* (Vaisman 1998). The main side effect of oxaliplatin is the development of peripheral neuropathy that is cumulative and dose-limiting (Chollet 1996).

Taxanes

Paclitaxel represents the parent molecule in this family, and was first isolated from the bark of the pacific yew tree *Taxus brefivolia* (Wani 1971). Paclitaxel works by promoting microtubule formation, inhibiting the disassembly of β-tubulin subunits, leading to aberrant microtubules-derived structures and arrested mitosis (Rowinsky 1993). Chemically, it has a complex molecular structure and is currently obtained as a semi-synthetic product from 10-desacetylbaccatin precursor extracted from the yew tree leaves. Paclitaxel displays preference to malignant tumors of epithelial origin and occupies a central role in the treatment of cisplatin-refractory ovarian cancer (Rowinsky 1995). Modification of two side chains on paclitaxel led to the development of

docetaxel, with enhanced clinical response in the therapies of ovarian and breast cancer.

Recurrent epithelial ovarian cancer

Currently, 70-80 % of patients with ovarian cancer are detected at an advanced stage, usually III or IV, with a recommended management of cytoreductive surgery followed by combination chemotherapy of platinum compounds and taxanes. Unlike other types of cancer, where diagnosis at an advanced stage deters surgery, the combination of surgery and chemotherapy has shown an advantage over chemotherapy alone, leading to remission in around 80% of cases (NIH Consensus Conference 2005). Unfortunately, relapse rates are as high as 90%, and cancer resurges with the added disadvantage of drug resistance. The decision for managing these patients depends on their response to the first-line treatment (Thigpen 1993), which leads to categorizing patients into platinum-sensitive and platinum-resistant. While platinum-sensitive patients usually have a very favorable response to platinum in their first round of chemotherapy, platinum-resistant patients have the following characteristics: i. platinum-refractory phase, where patients progress while on platinum therapy ii. at best these patients have a stable response while on their first round of chemotherapy and iii. they tend to relapse less than six months following the completion of the first round of therapy (Herzog 2010). Unfortunately, the response of this category to second-line platinum therapy is dismal and these patients are usually treated with non-cross resistant anti-neoplastic agents. Additionally, since a cure is almost unattainable, and to

preserve a decent quality of life for these patients, palliative therapy is more common than an aggressive regimen that has no obvious benefit.

In the platinum-sensitive category, a higher treatment-free interval is associated with a better outcome and helps in the decision of the second-line regimen. A phase II study showed that addition of platinum compound (cisplatin) to cyclophosphamide and doxorubicin regimen has a better response than the single agent paclitaxel in treatment of recurrent EOC (Cantu 2002) and a recent randomized phase III trial showed an advantage of combination of carboplatin and pegylated liposomal doxorubicin (PLD) to carboplatin alone (59 vs. 28 percent) in recurrent ovarian cancer treatment (Markman 2010). Additionally, it was found in the same study that the combination of carboplatin with PLD is associated with lesser alopecia and chronic neurotoxicity as well as decreased hypersensitivity due to fewer infusion reactions compared to the combination of paclitaxel and carboplatin. Altogether, promising outcomes are expected in combination therapy as a second-line treatment, demonstrating the efficacy of inclusion of platinum compounds in the regimens. In the following, a brief overview of the chemotherapeutic agents under investigation in the second-line management of EOC, especially non-platinum non-taxane cross-resistant, is provided.

Etoposide belongs to the family of podophyllotoxins that inhibit topoisomerase II causing DNA strands breaks. Oral etoposide offers an advantage in regimen scheduling compared to intravenous administration and causes a 27% response rate in platinum-resistant patients (Slayton 1979). Toxicity includes myelosuppression (neutropenia) and gastrointestinal symptoms (nausea and vomiting) (Rose 1998). Because of its highest

response rate compared to other antineoplastics, it is currently employed as the first-line of therapy of recurrent EOC after failure of platinum and taxane therapies.

Doxorubicin is an anthracycline antibiotic obtained from *Streptomyces peucetius*. The quinone and hydroquinone moieties allow for electron transfers promoting formation of free radicals. Several mechanisms have been proposed to explain doxorubicin actions including: DNA intercalation, topoisomerase II inhibition and formation of free radicals, all of which lead to destruction of DNA integrity. A pegylated liposomal formulation of doxorubicin is employed in the treatment of EOC, and a 17% response rate is observed with PLD monotherapy in platinum- and taxane-resistant EOC (Lorusso 2004, Thigpen 2005). Major toxicities are stomatitis and "hand-and-foot" syndrome.

Topotecan is a camptothecin analog that acts by inhibiting topoisomerase I and is active against both platinum-sensitive and -resistant cancer. Myelosuppression is a dose-limiting toxicity (neutropenia). Compared to PLD, topotecan has a similar response rate (Gordon 2001); however the myelosuppression induced by topotecan favors PLD.

Gemcitabine is a fluorinated analog of cytidine that acts an inhibitor of DNA synthesis during the S phase. Upon conversion to di- and tri-phosphate metabolites, it prevents the incorporation of dNTPs into the nascent DNA. The tri-phosphate metabolite is also incorporated into the DNA causing chain termination. In treatment of recurrent EOC, gemcitabine is active against both platinum- and taxane-resistant disease (Markman 2003, Rose 2003). Compared to PLD, no difference in response rate is

observed (Mutch 2007); however due to lesser cytotoxicity, it represents a tolerable alternative to PLD in women with platinum-resistant EOC.

Vinorelbine is a semi-synthetic analog of vinblastine, a vinca alkaloid. Vinca alkaloids are mitotic spindle inhibitors and act by binding tubulin and preventing polymerization. Response rate to vinorelbine in EOC-patients refractory to platinums has been variable with no obvious advantage (Bajetta 1996, Burger 1999 and Rothenberg 2004).

Ifosfamide is a nitrogen mustard prodrug that is converted enzymatically in the liver to its active electrophilic metabolite which attacks the N7 nitrogen of guanine, forming covalent bonds resulting in DNA cross-linking. In treatment of recurrent platinum-resistant EOC, ifosfamide is moderately active (Baur 2006). Toxicity includes hematologic, renal and central nervous system such as disorientation, hallucinations, somnolence and agitation (Herzog 2010).

Pemetrexed is an antifolate prodrug metabolized to its active polyglutamate forms which inhibit THF4-dependent enzymes. Pemetrexed monotherapy showed a 21% response rate in platinum-resistant EOC despite the severe toxicity that could be associated with its administration (Vergote 2009) and folic acid and vitamin B12 are required supplements during treatment.

Anti-hormone therapy, similar to that used with other hormone-dependent cancers like breast and prostate, has been tested in ovarian cancer using tamoxifen - a selective estrogen receptor modulator - , and letrozole and anastrazole- aromatase inhibitors. Several trials and one meta-analysis showed that the response to hormone

therapy in ovarian cancer is, at best, modest (Williams 2001, Smyth 2007 and Argenta 2009)

Bevacizumab is a humanized vascular endothelial growth factor (VEGF) - monoclonal antibody. Several phase II trials showed improved response rates in combination with other chemotherapeutic agents (Burger 2007, Cannistra 2007, Chura 2007, Micha 2007, Spannuth 2008), however, the high potential for GI perforation keeps it as a last-line of treatment (3rd, 4th or even 5th-line of treatment).

Intraperitoneal chemotherapy can provide, theoretically, a several fold increase of drug concentration at the site of a tumor with the feasibility of overriding systemic toxicity. Its role in ovarian cancer therapy remains controversial as it hasn't produced an advantage over intravenous therapy (Markman 1991) and has been limited to microscopic rather than macroscopic disease (Feun 1998, Berek 1999). Further clinical trials are needed in order to establish the true role of IP therapy.

Ovarian Cancer and Intracellular Signaling

As in many cancers, several signaling cascades are altered in ovarian cancer leading to the different pathological phenotypes expressed by these cells. ERK/MAP kinase and PI3K/Akt pathways have both received special attention in ovarian cancer for their involvement in survival, development, differentiation and migration, among other functions. In fact, Ras or B-Raf activating mutation occur in two thirds of low grade ovarian tumors and 16% of high grade tumors (Singer 2003 a/b, Ho 2003). Additionally, activation of ERK/MAP kinase has been observed in 80% of low grade ovarian tumors

and 40% of high grade tumors (Hsu 2004) indicating the existence of mechanisms, different than upstream Ras or Raf mutational activation, responsible for elevated signaling through this pathway and pointing to its importance in ovarian tumors. In this dissertation I will focus on the MAP kinase pathway and provide a summary of the major components of this cascade, as well as the therapeutic strategies targeting this pathway.

MAP Kinase pathway

The discovery of the MAP kinase pathway (Fig 1.1) in the lab of Tom Sturgill (Rossomando 1989) about 20 years ago, marked a breakthrough in the field of signal transduction. The MAP kinase pathway was initially described as a linear cascade of sequentially activated protein kinases with "little or no cross-reactivity" and the namesake MAP kinase as a "unifying explanation of most, if not all, nonnuclear oncogenes" (Seger 1995). Our view of the pathway has changed and evolved over the last twenty years with increasing complexity and cross-talk between the different signaling cascades; however, the linear representation of the pathway remains the best start to examine and understand its different details. In its simplest topology, the MAP kinase pathway is a sequentially activated pathway of three conserved protein kinases that convey an extracellular signal in the form of phosphorylation events (Tanoue 2003). Four pathways have been well described in mammalian cells: ERK1/ERK2, p38α/β/γ/δ, JNK1/2/3 and ERK5 MAP kinase pathways. ERK pathways are preferentially activated by growth factors, phorbol esters and adhesion, while JNK and p38 MAP kinases are preferentially activated by cytokines and stress. In response to these extracellular cues, receptor tyrosine kinases, G-protein coupled receptors and integrin receptors recruit a

small GTP-binding protein to the cell membrane (Ras superfamily) that recruit a MAP3K, the first kinase in the cascade. Activated MAP3K leads to the sequential phosphorylation, and thereby activation, of MAP2K and finally the namesake MAPK that phosphorylates a plethora of targets across the cell, from the cell membrane to the nucleus. In the following, we will focus on the ERK1/2 MAP kinase pathway and then briefly discuss JNK and p38 MAP kinase pathways.

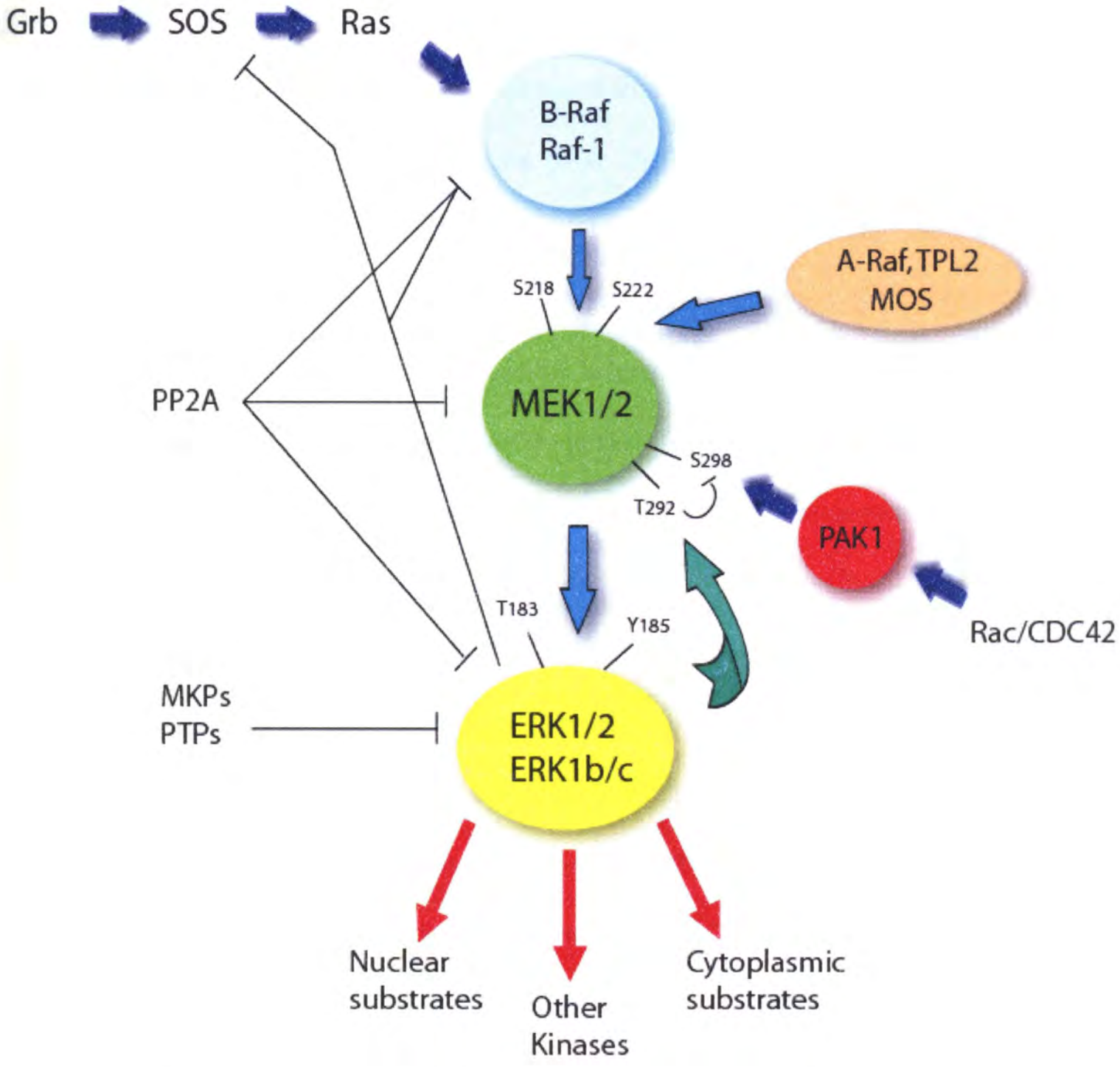

Fig 1.1 Ras-MAP kinase pathway A simplified and linear overview of the ERK/MAP kinase pathway topology.

Ras

Ras belongs to a family of small G-proteins that alternate between an inactive GDP-bound and an active GTP-bound state (Shih 1980) due to an intrinsic GTPase activity. Transmembrame receptor activation by extracellular stimuli induces a conformational rearrangement that recruits the molecule adapters Grb2, Shc and guanine-nucleotide exchange factor SOS which in turn binds and recruits Ras to be activated at the cell membrane (Olivier 1993, Rozakis-Adcock 1993, Buday 1993 and Egan 1993). An intricate interplay between guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) is believed to have a major role in activating Ras (reviewed in Konstantinopoulos 2007). Four main isoforms of Ras have been described: K-Ras4A, K-Ras4B, H-Ras and N-Ras. Except for the C-terminal 23-24 amino acids, known as the hypervariable region (HVR), all isoforms share a near identical homology (Dhar 1982, Tsuchida 1982, Shimizu 1983). Ras proteins are subject to lipid modifications in their C-termini (prenylation and palmitoyilation) that target the proteins for cell membrane anchoring and subsequent activation (Sefton 1982, Casey 1989, Hancock 1989). It is also believed that lipid modifications of Ras target it for microlocalization on the cell membrane (Boyartchuk 1997, Prior 2001).

Among the first evidences into the role of Ras in cancer is the finding of its ability to mediate cellular transformation in NIH 3T3 cells (Ellis 1981). In 1982, Channing Der showed an elevated expression levels of K-Ras and H-Ras in human bladder and lung carcinoma cell line and identified their role in transforming cells (Der 1982) while Luis

Parada, then in the lab of Bob Weinberg, demonstrated H-Ras as the oncogene in EJ human bladder carcinoma (Parada 1982). Further studies demonstrated Ras to be activated in about 30% of all human cancers, with the highest activation observed in pancreatic cancer (90%) – arguably the worst kind of cancers –, in colon and thyroid (50%), in lung cancers (30%) (Malumbres 2003). Elevated Ras activation in cancer can be attributed to two main processes: i. activating mutations and ii. continuous Ras activation via upstream stimulation. Concerning the former, several Ras mutations have been described in a variety of diseases; however mutant K-Ras genes encoding proteins harboring the single amino acid mutation at G12 or Q61 (or their homologs on the other Ras isoforms) are reserved for cancer cells (Taporowsky 1983, Shimizu 1983 and Fasano 1984). Ras G12/Q61 are GAP-insensitive therefore they maintain a GTP-bound state and remain activated in a stimulus-independent manner (Adari 1988). Alternatively, Ras is activated by aberrant upstream stimulation. For instance, several receptor tyrosine kinases (RTKs) such as EGFR and ErbB2 are either overexpressed or mutationally activated in several human cancers leading to subsequent Ras-Raf-MEK-ERK activation (Lynch 2004, Stephens 2004 and Grandis 2004). Additionally, continuous Ras activation leads to transcriptional activation of transforming growth factor α (TGF α), heparin-binding-EGF and amphiregulin (McCarthy 1995, Gangarosa 1997 and Schulze 2001) which are natural EGFR ligands leading to a positive feedback loop activation of Ras/ERK pathway .

While the Raf-MEK-ERK axis is the main effector target of Ras activation, other pathways can be induced by Ras activation. In particular, PI3K/Akt, Tiam1/Rac, RalGDS

and PLCε are additional pathways activated by oncogenic Ras and have a strong impact on tumorigenesis (reviewed in Repasky 2004, Shaw 2006). Accordingly, targeting the Ras/ERK pathway for inhibition might not suffice for a complete abrogation of Ras oncogenic effects. Alternatively, developing specific inhibitors for ERK targets might represent a more efficient strategy in inhibiting Ras/ERK-mediated effects in cancer. Further discussion on different inhibitors of the Ras/ERK cascade is provided later in this chapter.

Raf

The regulation of the MAP kinase pathway at the Raf level is very complex and remains under extensive investigation. Raf is a serine/threonine (S/T) kinase that exists under 3 main isoforms: A-Raf, B-Raf and Raf-1 (or C-Raf). Activation of Ras at the cell membrane initiates a complex series of phosphorylations and dephosphorylations of Raf where it constitutes a relay for several pathways like PI3K/Akt, Src, PKA, PKC and PP2A (reviewed in McCubrey 2006), self-dimerization or heterodimerization with the Raf-like protein kinase-suppressor of Ras (KSR) (Xing 1997), and association/disassociation with scaffolding proteins like HSP90 and RKIP (Blagosklonny 2002, Corbit 2003). Of the three isoforms, B-Raf is mutated most in cancer cells; more than 30 mutations of B-Raf, clustered within the kinase's activation loop and P-loop, have been reported (Wan 2004). Interestingly, B-Raf(V599) is the most common mutant isoform and its basal kinase activity exceeds 400-folds that of the wild type kinase (Wan 2004) leading to a sustained ERK/MAP kinase pathway activation independent of Ras and Src function (Marais 1997, Brose 2002 and Davies 2002). Also, Src-dependent activating residues on

Raf-1 and A-Raf are replaced by the negatively charged aspartic acid residues, mimicking a continuously phosphorylated enzyme and giving B-Raf a higher basal activity to activate MEK/ERK than A-Raf or RAf-1 (Marais 1997). B-Raf mutations are reported in up to 70% of melanomas, 50% in thyroid cancer, 22% in colon cancer and 30% in ovarian cancer (Davies 2002, Garnett 2004 and Libra 2005). It is interesting to notice that, in ovarian cancer, K-Ras and B-Raf mutations are mutually exclusive (Singer 2003) as either mutation is sufficient for Raf-MEK-ERK activation. The exact role of B-Raf in tumorigenesis remains to be determined as introducing B-Raf mutants into cells does not initiate neoplastic transformation and hence a maintenance role for B-Raf is predicted in these cancers (Rajagopalan 2002, Yuen 2002).

MEK

MAP/ERK kinase (MEK1/2) is the direct activator of ERK/MAP kinase. It is a dual specificity tyrosine and serine/threonine (Y/S/T) kinase (Alessi 1994) that is activated by Raf upon phosphorylation on S218 and S222 (or Ser217 and Ser221) (Alessi 1994). MEK is also phosphorylated on S298 by PAK1 in order for Raf to phosphorylate MEK in response to integrin adhesion (Coles 2002, Slack-Davis 2003). MEK is inhibited by a negative feedback phosphorylation on T292 by ERK (Brunet 1994b, Eblen 2004). In the ERK/MAP kinase pathway, MEK targets a unique substrate, namely ERK1/2 (Crews 1992). This feature in the ERK pathway allows for specific inhibition of ERK by pharmacologically targeting its upstream activating kinase MEK1/2. Interestingly, unlike Ras and Raf, there are no known activating mutations of MEK in cancer. However, mutation of Ser218/222 in MEK1 to Asp or Glu generates a constitutively active MEK1

that is oncogenic in fibroblasts (Brunet 1994a). Recently, mutations on F35S and Y130 in MEK1 and F57C in MEK2 have been identified as part of the cardio-facio-cutaneous syndrome (Rodriguez-Viciana 2006), but have no implications on cancer. Also, a recent activating mutation of MEK1 (F129L) has been identified and is proposed to impart an acquired resistance to MEK inhibitors (Wang 2011).

In resting cells, MEKs are known to localize to the cytoplasm due to a nuclear export signal (NES) on their N-termini (Fukuda 1996, Jaaro 1997). Additionally, interactions with scaffolding proteins and cytoplasmic anchors such as MP1, KSR1, Sef1 and paxillin confine MEKs to endosomes, the plasma membrane, outer Golgi membrane and cytoskeleton, respectively (Shaeffer 1998, Kornfeld 1995, Therrien 1995, Torii 2004 and Ishibe 2003). More recently, MEKs were shown to localize to the mitochondrial membranes and lumen (Poderoso 2008), however the significance of this localization remains poorly understood. Although mainly localized to the cytoplasm, MEKs are subject to continuous and rapid shuttling in and out of the nucleus in response to stimulation (Jaaro 1997, Tolwinski 1999 and Yao 2001). In the nucleus, MEKs interact with inactivated ERKs through their D and CD domains (described below) and are subsequently exported via the NES/exportin system into the cytoplasm (Adachi 2000). In that regard, MEKs explain the transport process for ERK out of the nucleus and provide a cytoplasmic anchor for inactive ERKs.

ERK

ERK represents the last tier in the core MAP kinase pathway. Two isoforms of ERK exist, ERK1 (p44 MAPK; 44 KDa) and ERK2 (p42 MAPK; 42 KDa) with 83% amino acid

homology (Owaki 1992). Additionally, two additional splice variants of ERK1 have been described. The first, ERK1b, is a 46 KDa protein with a 26 amino acid insertion between residues 340 and 341 of ERK1 and expresses similar kinetics of activation to the main ERK isoforms, however with a tissue specific distribution and higher response to osmotic stress (Boulton 1991, Yung 2001). The second splice variant is ERK1c, a 42 KDa protein with slower kinetics than ERK1/2, and displays high density in the Golgi apparatus where it is implicated in Golgi fragmentation (Aebersold 2004, Jesch 2001).

ERK1/2 are activated by phosphorylation of the TEY motif (202/204 or 185/187) by the dual specificity kinase MEK (Boulton 1991). As an enzyme, ERK is a serine/threonine kinase and phosphorylates a plethora of substrates distributed around the cell, from the cell membrane (CD120a, calnexin), to cytoskeletal proteins and kinases (neurofilaments, paxillin, RSK, MSK), all the way to transcription factors in the nucleus (SRC-1, Elk1, MEF2, c-Fos, c-Myc, STAT) (reviewed in Yoon 2006). To date, more than 160 substrates of ERK have been reported, mediating its diverse functions in cellular proliferation, development and migration. Obviously, such a diverse response from ERK activation requires a tight control of substrate phosphorylation in order to maintain a high fidelity signal. Regulation through kinases scaffolds, protein phosphatases and localization represent the major routes to reach this end.

ERK1/2 are predominantly located in the cytoplasm of quiescent cells and translocate to the nucleus upon activation. In the cytoplasm of resting cells, around 50% of ERKs are bound to microtubules (Reska 1995). ERK1/2 also localize to focal adhesions and adherens junctions where they are necessary for paxillin phosphorylation during cell

migration (Ishibe 2003). Three mechanisms have been elucidated describing ERK1/2 nuclear translocation through the nuclear pores: i. passive diffusion of a monomer, ii. active transport of an ERK1/2 dimer (both phosphorylated and unphosphorylated) and iii. nuclear pore complex (NPC)-mediated translocation via active, cytosol- and ATP-independent mechanisms (Khokhlachev 1998, Adachi 2000, Matsubyashi 2001 and Whitehurst 2002). As it doesn't have either a nuclear localization (NLS) or export signals (NES) of its own, ERK is anchored in the cytoplasm by binding the N-terminus of MEK1/2 (Fukuda 1996) which is disrupted upon ERK1/2 phosphorylation, inducing ERK1/2 translocation to the nucleus (Fukuda 1997). ERK1/2 is dephosphorylated in the nucleus by MAP kinase phosphatases (MKPs), which causes its association with MEK1/2 for shuttling back to the cytosol (Tanoue 2000).

When localized to the nucleus, ERKs are kept under tight spatiotemporal control by several molecules that act as scaffolds, which act to anchor kinases within close proximity in certain areas of the cell in a way to achieve localized response in a stimulus-specific manner and integrating different signals within controlled microdomains (Elion 2001, Kolch 2005). Several cytoplasmic scaffolds have been identified, including KSR, MP1, β-arrestins and Sef. KSR interacts with Raf-1, MEK1/2 and ERK1/2 and is translocated with MEK1/2 to the plasma membrane, bringing the Raf-MEK-ERK in close proximity for increased signaling efficiency (Sundaram 1996, Therrien 1996). MP1 binds to MEK1/2 and ERK1/2 in endosomes, enhancing their interaction and ERK1/2 signaling in response to EGF (Schaeffer 1998, Teis 2002). B-arrestins improve Raf-MEK-ERK signaling in endosomes downstream of G protein coupled receptors (Luttrell 2001) and

Sef colocalizes with active ERK1/2 and MEK1/2 on Golgi membranes, preventing signaling to the nucleus (Torii 2004). Altogether, these molecules provide examples of regulation and control of ERK signaling through ERK localization.

Phosphatases also control ERK signaling and play an essential role in positive and negative regulation of ERK signal propagation. Similar to MAP kinases, phosphatases are also governed by localization-mechanisms within the cell, hence they convey their effects in a stimulus- and spatial distribution-dependent manner. Protein phosphatase 2A PP2A binds KSR and dephosphorylates it along with Raf-1, removing an inhibitory phosphorylation on the latter and allowing Ras-Raf activation in response to growth factor stimulation (Dougherty 2005). The nuclear phosphatase DUSP5 is a specific ERK-phosphatase and behaves as an inhibitor of the kinase but also as a nuclear anchor (Mandl 2005). Phosphatases are also regulated by phosphorylations in the vicinity of the ERK interaction region. Hematopoietic protein tyrosine phosphatase (He-PTP) dephosphorylates ERK1/2 only when an inhibitory phosphorylation on Ser23 in the D-domain is removed (Gjorloff-Wingren 2000). STEP, a striatal neuron specific phosphatase, dephosphorylates ERK1/2 in response to NMDA receptor activation, which activates calcineurin to dephosphorylate and subsequently activate STEP (Paul 2003). Phosphatases are diverse and represent a crucial level of regulation and maintenance of a high fidelity signal transducing extracellular cues, their discussion is however beyond the scope of this work.

Docking interactions: D, DEF and CD motifs

Since all MAP kinases share a common substrate consensus sequence, separate motifs within the different proteins, both enzymes and substrates, are necessary to achieve a higher fidelity of binding and phosphorylation. Four motifs have been described: D, DEF, CD and ED motifs. The D-domain consists of a cluster of basic (positively charged) residues in the vicinity of an LXL motif, either upstream or downstream of the phosphorylation site (Yang 1998). D-domains are thought to enhance substrates recognition and to improve signaling specificity. They have been described in MAP kinase regulatory proteins, such as MAPKKs (MEK1/2), phosphatases (STEP, MKPs) and scaffolding proteins (KSR) and are believed to enhance the propagation and regulation of the signal specificity and fidelity similar to MAP kinase substrates (reviewed in Enslen 2001). The DEF motif (Docking site for ERK and FXFP) forms another class of interacting domains: the (F/Y)X(F/Y)P sequence is found in several transcription factor substrates of ERK1/2 across different species and is believed to be an ERK specific sequence (Jacobs 1999). The FXFP motif tends to be C-terminal to the phosphoacceptor site, and it is not mutually exclusive from the D-domain; in fact, together (such as in Elk1 and KSR) they strengthen substrate recognition and binding (Jacobs 1999, Fantz 2001, Murphy 2002). While the DEF interacting module on ERK has not been identified yet, hydrophobic sequence in and downstream of the common docking (CD) motif has been shown to mediate ERK, p38 and JNK interactions with the D-domains (Rubinfeld 1999, Tanoue 2000). It is separate from the kinase active site but lies in its vicinity, and consists of a cluster of acidic residues (negatively charged)

surrounded by hydrophobic residues (Rubinfeld 1999 and Tanoue 2000) mediating electrostatic and hydrophobic interactions with the D-domain residues, respectively. Fig 1.2 below depicts ERK domains discussed above.

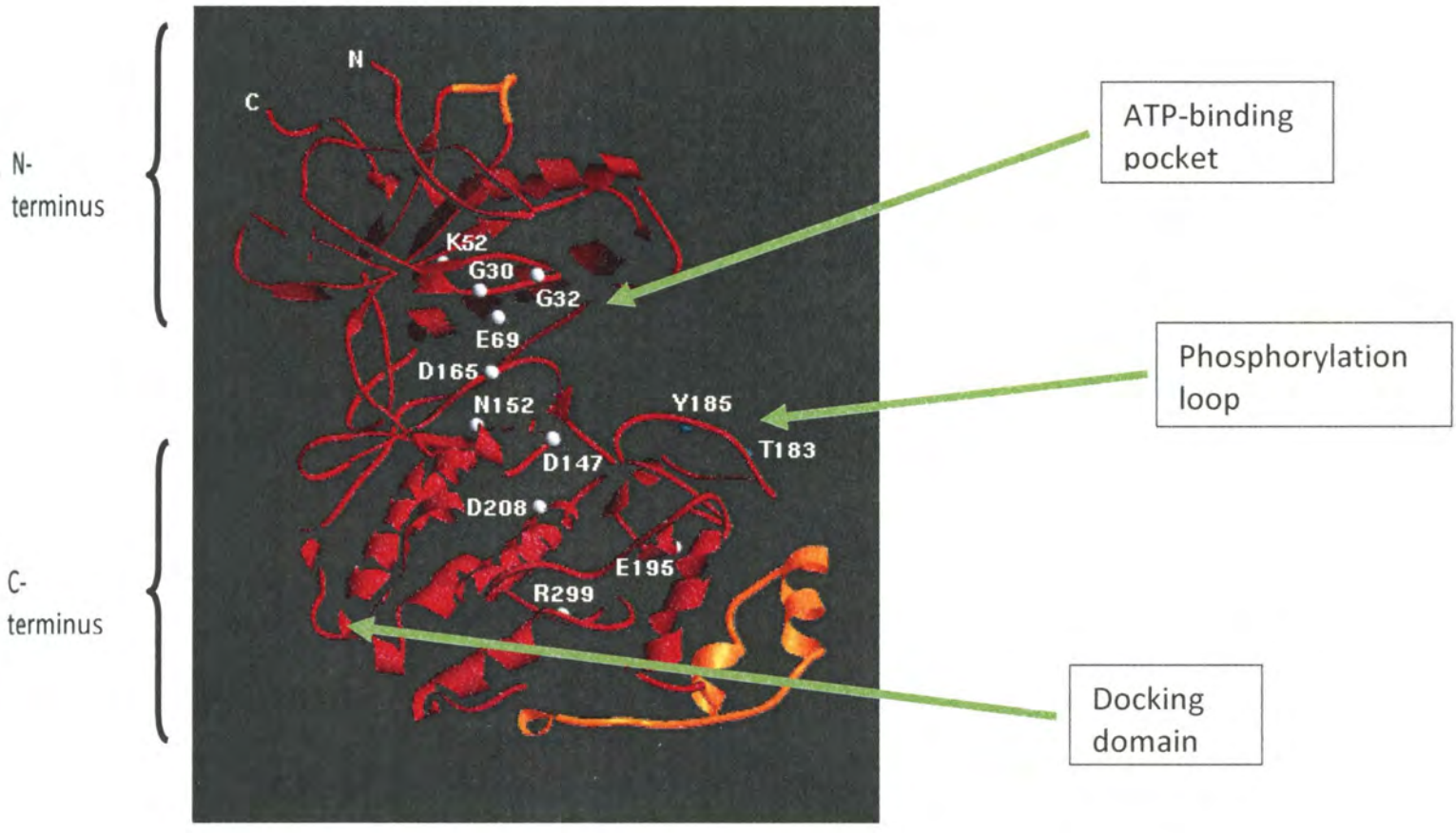

Fig 1.2 Crystal structure of inactive ERK2 bound to ATP, above, was resolved in the lab of Elizabeth Goldsmith (Zhang 1994).

P38 MAP kinase module

Similar to the ERK/MAP kinase pathway, the p38 pathway is a three-tiered cascade (Han 1994, Lee 1994) consisting of several MAP3Ks including MEKK1/2/3/4, MLK2/3, ASK1 and TAK1 that are activated in response to environmental stresses such as UV irradiation, osmotic pressure, oxidative stress, ischemia and inflammation along with its cytokines such as IL-1 and TNFα (reviewed in Roux 2004). Consequently, MAP3Ks phosphorylate the dual specificity kinases MAP2Ks, MKK3 and MKK6, to finally activate the four isoforms of p38α/β/γ/δ MAP kinase (Han 1993, 1994, Freshney 1994, Rouse 1994, Lechner 1996). P38 is a serine/threonine kinase and is activated by phosphorylation on the TGY motif in its activation loop (Han 1995, Wang 1997). Structurally, p38 shares around 50% homology with ERK, however, it differs in the length of its activation loop suggesting a potential explanation for the discrepancy of substrate specificity (Wilson 1996). Similar to ERK, p38 substrates are found around the cell (Raingeaud 1995, Ben-Levy 1998). P38 is strongly involved in immune and inflammatory processes and is activated in macrophages and neutrophils (Ono 2000). Accordingly, it is not surprising that several p38 inhibitors in development are being targeted towards autoimmune and inflammatory diseases. P38 inhibitors are discussed later in this chapter.

JNK module

In the same pattern as ERK and p38, the JNK subfamily is a three-tiered cascade that shares similar activating stimuli with p38 MAP kinases. Environmental stresses and inflammation are the main activators of the pathway which is commonly turned on with

p38 by phosphorylating MEKK1/2/3/4, MLK2/3, ASK1 and TAK1 but also separately by activating TAO-1/2 and ASK2 (reviewed in Bogoyevitch 2010). At the MAP2K level, MKK4 and MKK7 mediate the upstream signal to phosphorylate JNK1/2/3 on the TXY motif where X is a proline. MKK4 also phosphorylates p38 in some cell types, suggesting a role for MKK4 in integrating signals from two pathways activated by similar cues (Derijard 1995). Although it is localized throughout the cell, JNK is involved in the phosphorylation and activation of transcription factors such c-jun, ATF-3, STAT-3 and HSF-1. Despite the fact that JNK shares a similar implication in inflammation as p38, more inhibitors of the latter managed to reach clinical trials (Roberts 2007).

Pharmacological inhibition of MAP kinases

Due to their centrality to oncogenic and mitogenic extracellular signals, the MAP kinase pathways have been under extensive investigation for pharmacological inhibition, with Ras/Raf/MEK/ERK and p38 MAP kinase cascade being the most alluring targets for intervention. The majority of inhibitors are discovered by *in vitro* screens of protein kinases or in cells assays, and have focused on exploiting the ATP-binding domain of protein kinases, to generate ATP-mimetic molecules that work by competitive ATP inhibition. A major drawback of this approach is the non-specific inhibition of other kinases since most of these proteins share a similar topology like the one described above for ERK. Pharmacological inhibitors have been synthesized to inhibit the pathway at each tier of the canonical sequence Ras→MAP3K→MAP2K→MAPK.

Inhibitors of Ras

Ras mutations are common in several cancers, including pancreatic, colon, lung, gall bladder and thyroid cancer (Rowinsky 1999) and altogether account for 30% mutations in human cancer (Bos 1989). Ras inhibitors have focused on a common property to all Ras isoforms – the CAAX box (Giehl 2005, Roberts 2007). The CAAX box is a common motif in the hypervariable region of Ras proteins that is subject to lipid modification responsible for anchoring the proteins to the cell membrane. Prenylation (addition of farnesyl group) is the most common modification to the C-terminus of Ras. Targeting farnesyltransferase for inhibition, the enzyme that catalyzes this modification, is the main approach followed to inhibit Ras anchoring to the cell membrane; however, the discovery that K-Ras is the most commonly mutated isoform in cancer decreased the interest in FTIs because K-Ras undergo geranyl geranylation, another lipid modification that compensates for the loss of farnesylation (Adjei 2001, Rajagopalan 2002).

Inhibitors of Raf

Raf, a serine/threonine kinase that occupies the middle tier of the canonical ERK/MAPK pathway, is under extensive regulation by no less than 13 regulatory phosphorylations (Steelman 2004), which makes it an important crossroad for several signaling pathways. Raf is heavily involved in cancer, and is reported to be mutated (mainly B-Raf) in approximately 30% of solid cancers (Bos 1989) making it a valid to target Raf for inhibition. The first successful inhibitor of Raf to reach clinical trials is BAY439006, also known as Sorafenib, a small molecular weight inhibitor shown by crystallographic data to bind an overlapping region of the ATP-binding domain on Raf

and interestingly, the mechanism of inhibition is non-ATP competition (Wan 2004). However, like most kinase inhibitors targeting the ATP-binding domain, Sorafenib shows a promiscuous behavior towards other kinases, mainly receptor tyrosine kinases like VEGFR-2/3, PDGFR-β, c-KIT and FGFR-1 (Wilhelm 2004). Sorafenib is currently exploited in several clinical trials for its RTK inhibitory effects, however, the crystallographic resolution of B-Raf led to the development of more specific inhibitors, such as PLX4032, which is currently showing promising Raf inhibition in Phase I melanoma clinical trials (Wellbrock 2010).

Inhibitors of MEK

Other than an enhanced activity in several types of cancer, MEK has not been shown to be overexpressed or mutated in cancer (Hoshino 1999). Arguably, targeting the ERK/MAP kinase pathway at the MEK level is perhaps the most effective route to achieve strong ERK inhibition as MEK and ERK represent a unique feature among protein kinases: one enzyme-one substrate duo (Seger 1992). PD98059 and U0126 are two small molecule inhibitors of MEK1/2 identified by *in vitro* and cell-based screen for inhibitors of ERK activation, respectively (Dudley 1995, Favata 1998). Both inactivate MEK by allosteric interactions, non-competitive with ATP-binding (Davies 2000). Another family of MEK inhibitors is identified from microbial extracts. This discovery led to the development of two small molecules, Ro092210 (Williams 1997) and L783277 (Zhao 1999) that contain an active ketone moiety shown to inhibit MEK1/2 competitively with ATP. Currently, several MEK inhibitors are in clinical development including AZD8330, GSK1120212, and Ro5126766. Recently, AZD6244 (Selumetinib), a non-ATP competitive

MEK inhibitor has shown promising results in phase II clinical trials of colorectal, hepatocellular, non-small-cell lung, and melanoma tumors (Davies 2007, Hersey 2009, Hainseworth 2010 and Bennouna 2010).

Inhibitors of JNK and p38 MAP kinase

P38 has received the most extensive attention among the MAP kinases from the pharmaceutical industry due to its involvement in inflammatory diseases and several inhibitors have entered clinical trials for rheumatoid arthritis and psoriasis (Lee 2000). In fact, p38 inhibitors have shown promising efficacy in animal models as well as clinical trials of arthritis and inflammatory lung disease such as: rheumatoid arthritis (e.g. VX-745, Scio-469, Ro-320-1195), psoriasis (e.g. HEP 689), COPD (e.g. GSK-681323) and Crohn's disease (e.g. GSK-681323, RWJ-67657). Equally, several JNK inhibitors are also developed and their involvement in the therapy of inflammatory diseases exceeds their use in cancer. Examples of JNK inhibitors currently in clinical trials include CC-401, CNI-1493 and CEP-1347. The list of kinase inhibitors is extensive and is beyond the topic of this dissertation; the reader is directed to the following reviews for further information concerning these pharmacological agents (Adcock 2006, English 2002).

Pre-mRNA Splicing and Alternative Splicing

Eukaryotic genes almost entirely consist of interrupted coding nucleotide sequences called "exons" and non-coding sequences called "introns". Pre-mRNA splicing is the molecular process that leads to intron removal and subsequently, ligation of exons to form the mature messenger RNA (mRNA) which is transported to the

cytoplasm for translation and protein synthesis. The general mechanism of intron removal and exon ligation has been well characterized in its broadest terms. It consists of a sequential formation of a multiprotein-nucleic acid complex known as the spliceosome followed by a stepwise removal of the two intronic borders. Nearly all introns are flanked by a GU dinulceotide at the 5' splice site (ss) and an AG dinucleotide at the 3' splice site (Grabowski 1984, Krainer 1984, Ruskin 1984). The 3' ss is preceded by a polypyrimidine tract that separates it from an important adenine known as the branching point in the branching point sequence (BPS) essential for the first catalytic step (Padgett 1984).

An efficient and precise splicing reaction requires the exact recognition of the borders of each intron/exon, a process accomplished by the spliceosome. The latter is a large complex, consisting of 5 small nuclear ribonucleoproteins (snRNPS, U1, U2, U4/U6 and U5) and more than 150 associated auxiliary proteins that coordinate the efficient binding and recognition of the splice sites, as well as catalyzing the trans-esterification reactions. As the spliceosome assembles, U1 binds to the 5' splice site while splicing factor 1, SF1 (also known as mammalian branch point binding protein mBBP) binds the adenine in the branch-point sequence (BPS), forming the E' complex (Michaud 1991). E' complex is converted to the E complex by binding of the U2snRNP auxiliary factor (U2AF) to the polypyrimidine tract and the 3' splice site via the 65KDa and 35KDa subunits, respectively (Séraphin 1989, Michaud 1993). U2AF facilitates U2snRNP recruitment to the adenine in the BPS replacing SF1 in the first ATP-dependent step of the spliceosome formation (Frendewey 1987, Konarska 1987, Jamison 1992) forming

complex A and inducing a conformational rearrangement facilitating the recruitment of the tri-snRNP U5/U4/U6 (Konarska 1987, Cheng 1987). U5 binds the upstream exon at the 5' splice site while U6/U4 binds U2 forming complex B1, which is rapidly replaced by complex B2 upon U1 release and U5 and U6 binding the 5' intronic splice site (Cheng 1987, Lamond 1988). Extensive remodeling and rearrangements lead to the formation of the catalytic C complex. At this point, U4 is released, the spliceosome is fully mature and intron splicing occurs via a stepwise two trans-esterification reactions. The phosphodiester bond at the 5' splice site is cleaved by a nucleophilic attack of the 2'-OH of the adenine (A) at the BPS resulting in two intermediates, the upstream exon with a free 3'-OH on a guanosine and a lariat structure consisting of the downstream exon and the intron coiled on itself by 2', 5'-phosphodiester bond. The free 3'-OH on the upstream exon subsequently liberates the lariat intron by another nucleophilic attack in a 3',5'-phosphodiester bond on the 3' splice site leading to the release of the lariat intron and ligation of the upstream and downstream exons. This process continues on all intronic sequences until the mature mRNA is formed and ready for translation. The different steps in spliceosome assembly are depicted in Fig 1.3 and the two nucleophilic transeterification reactions are depicted in Fig 1.4.

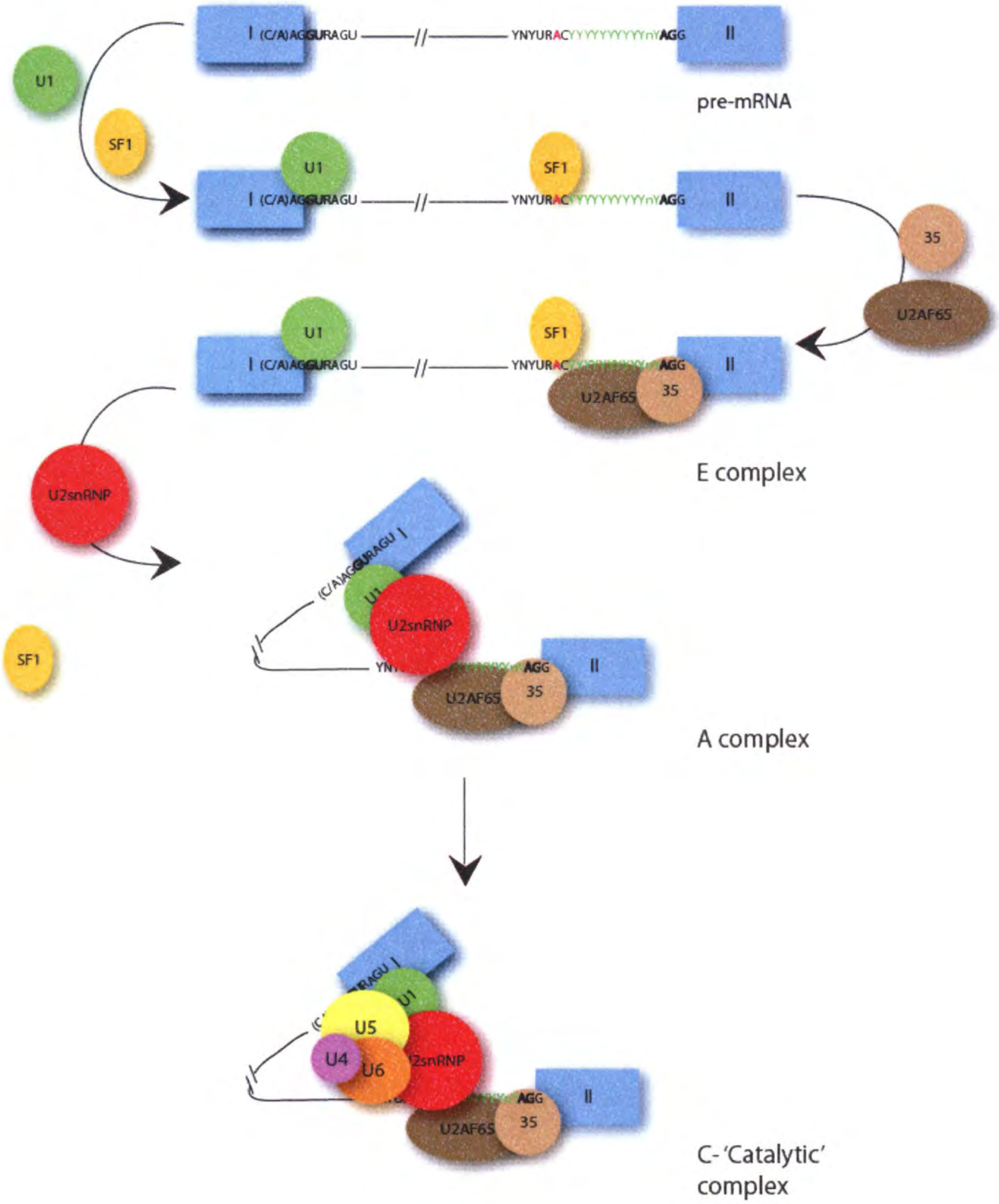

Fig 1.3 Spliceosome assembly Pre-mRNA alternative splicing is a multi-step mega-reaction that includes several intermediate complexes involving a large number of proteins/nucleoproteins.

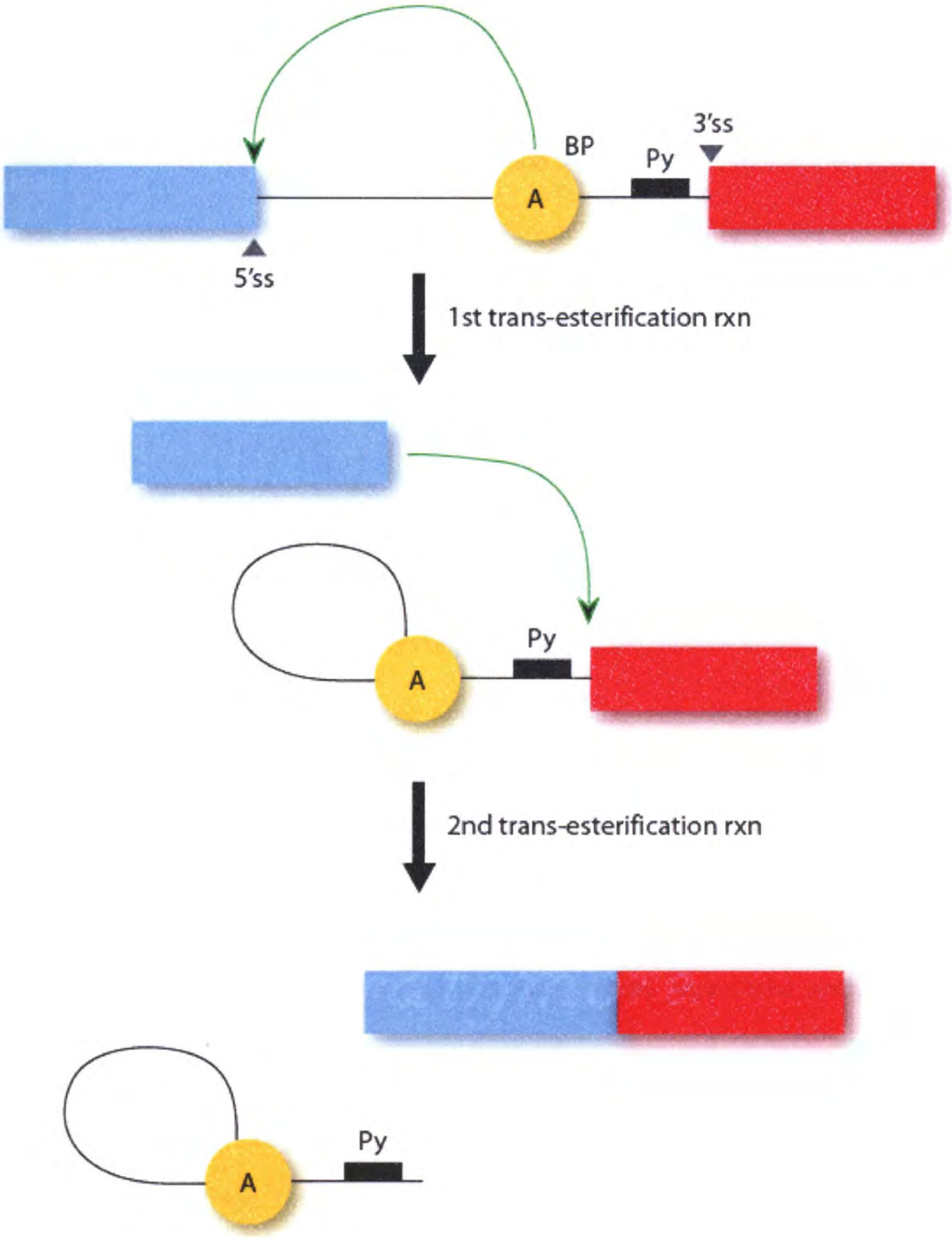

Fig 1.4 Double transeterification reactions and intron excision In the 1st reaction, the 2'-OH group of the BPS Adenine cleaves the phosphodiester bond at the 5' ss. In the 2nd reaction, the 3'-OH on the free upstream exon cleaves the lariat intron and joins the downstream exons at the 3'ss by a 2nd nucleophilic attack.

Alternative splicing

Alternative splicing is the process of generating two or more transcripts of the same gene from a common pre-mRNA sequence. In eukaryotes, alternative splicing is a common mechanism for generating a wide array of protein products involved in diverse cellular functions that range from sex determination to growth, differentiation and even apoptosis. It has been estimated in the recent literature, based on bioinformatics analysis of expressed sequence tags (ESTs) and cDNA datasets, that 95-100% of pre-mRNAs undergo alternative splicing yielding multiple mRNAs (Wang 2008, Pan 2008). Alternative splicing gained substantial interest after the discovery that mutations in no less than 15%, and up to 50% of human genetic diseases involve changes in alternative splicing, either in the consensus sequence or in weak regulatory regions within the introns and/or exons, and dictate the fate of the final isoform produced (Matlin 2005). Similar to constitutive splicing, the process is carried out by the spliceosome; however, differences between weak and strong sequences on the pre-mRNA, called *cis*-factors, and the relative concentrations of several spliceosome associated proteins (SAPs), known as *trans*-factors, play a crucial role in determining the outcome of splicing. Additionally, the interplay between the *cis* and *trans* factors is subject to post-translational modifications, which increases the complexity of the system. Ultimately, introns and exons forming the nascent pre-mRNA are cut and ligated in a multitude of combinations generating several transcripts that are translated by increased proteomic diversity. Several patterns of alternative splicing exist, with the most common modules

being alternative 5' or 3' splice-site selection, mutually exclusive exons, cassette-exon inclusion or skipping, and intron retention (Nilsen 2010) and are depicted in Fig 1.5.

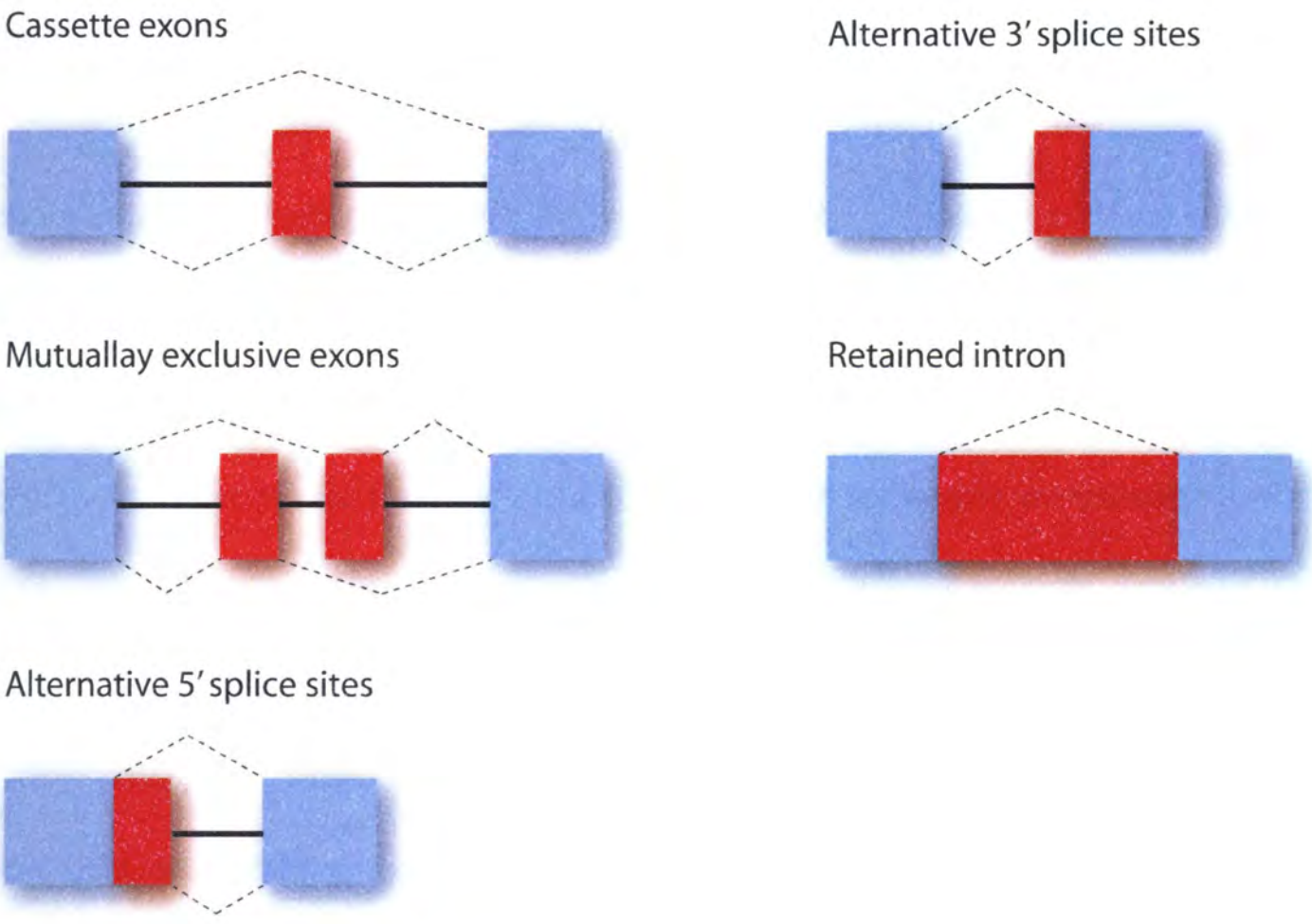

Fig 1.5 Splicing patterns in mammalian cells Different combinations of splicing and ligation can potentially generate a countless amount of transcripts. Of the multiple patterns of alternative splicing, cassette exons splicing pattern is the most identified and most studied form of alternative splicing.

Regulation of pre-mRNA splicing

A major requirement for an efficient splicing reaction is the ability of the spliceosome to differentiate exons from introns, a process known as exon definition, which is mainly dictated by the presence of three short sequence *cis* elements: the 5' splice site, the 3' splice site and the branch point. Although intronic sequences are spliced-out and removed from the pre-mRNA, their relative length compared to average exons (50-250 nt) supports the hypothesis that splicing occurs by exon definition first, subsequently converted to intron definition with cross-bridging between U1 and U2 snRNPs on opposite sides of an exon. As such, controlling splice sites selection, either by facilitating or inhibiting their recognition, is an important step in the regulation of splicing. In yeast, the consensus of splice sites selection is strictly followed while higher eukaryotes represent a more challenging case and divergence from the consensus is observed (Burset 2000, 2001). To improve exon control and recognition, additional elements known as exonic or intronic enhancers (ESE or ISE) and exonic or intronic silencers (ESS or ISS), depending on their location and impact on splicing, are present in the pre-mRNA. Two major classes of splicing factors, arginine-serine rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs), bind the regulatory elements mentioned above to modulate splicing.

SR proteins

The serine and arginine rich proteins (SR proteins) are an essential family of constitutive splicing factors with abilities to bind RNA specific sequences and to modulate protein-protein interactions. SR proteins are recognized for their role as

molecular adapters between the pre-mRNA and the spliceosome. SR proteins are highly conserved and consist of one or two N-terminal RNA recognition motifs (RRMs) and a C-terminal domain rich in repeating sequences of arginine and serine residues (RS domain). RRM domains are approximately 80 amino acid in length (Nagai 1990), and function in RNA binding (Nagai 1995). SELEX protocols (systematic evolution of ligands through exponential enrichment; Tuerk 1990) identified purine-rich exonic enhancer elements as preferred binding sites for individual SR proteins (Tacke 1999). The C-terminal RS domains are highly conserved among the SR proteins (Zahler 1992, Birney 1993) and function in cellular localization through protein-protein interactions with other RS-containing proteins (Caceres 1997, Kataoka 1999, Lai 2000, 2001). Additionally, RS domains are necessary for mediating protein-protein interactions with the spliceosomal machinery (Wu 1993, Kohtz 1994, Graveley 1998). As mentioned above, higher eukaryotes tend to diverge from the consensus exon definition, and SR proteins have been determined as crucial factors in identification of enhancer sequences within the exons or introns and facilitation of the interactions between the upstream 3' and the downstream 5' splice sites surrounding an exon (Berget 1995). SR proteins are also involved in intron bridging, the process of converting the exon definition into intron definition, which leads ultimately to intron removal and ligation of exons (Wu 1993, Abovich 1994, 1997 and Berglund 1997). It is noteworthy to mention that most SR proteins were discovered by their accidental reactivity to a monoclonal antibody, mAb104, mounted against active sites of RNA pol II on loops of the lampbrush chromosomes and germinal vesicles (Roth 1990). Altogether, the combination of the

RRM and RS domains functions support the role of SR proteins as facilitators of splicing: through binding ESEs and mediating critical protein-protein interactions, SR proteins strengthen weak splice sites, dictating the outcome of pre-mRNA splicing.

HnRNPs

Another group of essential modulators of the splicing process are the heterogeneous nuclear ribonucleoproteins (hnRNPs). HnRNPs contain one or two RNA recognition motifs (RRMs) that mediate their binding to the pre-mRNA. The process of specific hnRNPs binding to pre-mRNA is not fully understood and still under debate; whereas some hnRNPs bind in a sequence-specific fashion, many others bind randomly. HnRNPs are involved in several layers of gene expression and RNA processing including transcription, polyadenylation, stabilizing mRNA structure, shuttling of mRNA to the cytoplasm as well as pre-mRNA splicing. A C-terminal glycine G-rich motif, involved in protein-protein interactions within the spliceosome, is commonly found in several hnRNPS, although not universally. In contrast to SR proteins which function in enhancing splicing, hnRNPs act as splicing repressors by binding to silencing sequences within the introns and exons (ISS and ESS). More than 25 hnRNPs have been identified to date (Dreyfuss 1993), of which SXL, PTB (also known as hnRNP I) and hnRNP A/B family have shown to have the strongest involvement in modulating splicing. Two hypotheses are advanced to explain the inhibitory effect of hnRNPs in both of which they sterically encumber the interaction between positive enhancers or splicing factors to the pre-mRNA. In the simplest form, repressor proteins directly antagonize enhancers by binding either close to a splice site or to ESE/ISE. PTB for instance, competitively binds

the polypyrimidine tract blocking U2AF (Singh 1995, Spellman 2006). Similarly, SXL binds the polypyrimidine tract of an upstream cryptic 3' splice site preventing U2AF binding, thereby activating a downstream 3' splice site (Lin 1995, Lallena 2002). Another example of direct steric hindrance comes from FOX1 and FOX2, splicing factors expressed specifically in the heart, brain and skeletal muscles, which bind an ISS preventing SF1 binding to the branch point of the calcitonin-related polypeptide-α, *CALCA*, hence inhibiting E' complex formation (Zhou 2008). On the other hand, simple steric hindrance fails to explain the inhibitory effect of hnRNP A1 on its own exon 7B inclusion. The exonic sequence suppressor (ESS) is > 100 nt far from the enhancers. Oligomerization of A1 through the C-terminal G-rich domain extends the inhibitory effect of A1 towards the enhancer sequence and probably mediates a conformational rearrangement leading to the A1 observed effect (Del Gatto-Konczak 1999); a similar hnRNP A1 effect has been shown for exon 7 exclusion of *SMN2* (Kashima 2007).

Combinatorial control

SR proteins and hnRNPs interactions with the pre-mRNA and the spliceosome provide an attractive and somewhat simplistic view of the process of splicing regulation; however, a combinatorial effect of these RNA-associated proteins is more likely to represent a closer look into the real mechanisms of regulation. Several ways help accomplish that combination. The simplest and most studied form is the effect of relative abundance of enhancers and inhibitors. HIV1 *tat* exon 3 inclusion is influenced by the amount of the inhibitor hnRNP A1 and enhancer SF2/ASF – an SR protein – through interplay of their binding to their respective sequences (Zhu 2001). SF2/ASF and

hnRNP A1 provide similar opposing effects on β-tropomyosin exon 6B inclusion (Expert-Bezancon 2004). The picture becomes more complicated when proteins within the same family display antagonistic influences. There are many examples of functional antagonism within SR proteins, as well as hnRNPs. For instance, SRp20 self-regulated splicing is antagonized by SF2/ASF (Jumaa 1997), SRrp86 is antagonized by SRp9G8 (Li 2003) and SRrp86 itself has antagonistic effects: it enhances SRp20 while repressing SC35, SF2/ASF and SRp55 (Barnard 2000). Functional antagonism is also observed among hnRNPs: PTB and CELF (CUG-BP and ETR3-like factors) have opposite effects on α-actinin (Suzuki 2002, Gromak 2003) and cardiac troponin-T (Charlet-B 2002).

Regulation of splicing factors activities by phosphorylation/signaling

Adding an extra layer of complexity, post-translational modifications of splicing factors are also complicit in modulating their activities and hence affecting both constitutive and alternative splicing. Phosphorylation, glycosylation and methylation are documented post-translational modifications (PTMs) on splicing factors (Soulard 1993, Rho 2007, Stamm 2008, Sinha 2010) and are implicated in regulating their functions. In this subsection, I will focus on the phosphorylation PTMs as they are most pertaining to this dissertation and the reader is directed to the references above for more information regarding the other forms of PTMs. As discussed earlier under this section, proteins and nucleic acids within and around the spliceosome are governed by weak hydrophobic and electrostatic protein-protein, protein-RNA and RNA-RNA interactions, which lead in a sort of "organized chaos" to a highly specific pattern of splicing. It is believed that this type of weak interactions is necessary to allow smooth and efficient

transitions in a highly complex and dynamic system. In that context, phosphorylation of splicing factors plays an important role in "bending and twisting" these interactions to modify the splicing outcome. Traditionally, phosphorylation modifications are studied on the two major classes of splicing protein regulators, namely the SR proteins and hnRNPs. In what follows, I will address the roles that phosphorylation is identified to play in splicing, giving examples in each case. With respect to the role of the MAP kinase pathway in signaling to splicing factors, Sam68, remains the sole ERK/MAP kinase direct substrate implicated in splicing so far (Matter 2002). One of the earliest evidences of the involvement of protein phosphorylation in splicing is the fortuitous identification of SR proteins. Indeed, SR proteins were identified based on their reactivity to the monoclonal antibody mAB104 which reacts with a phosphate-containing peptide sequence (Roth 1990, 1991; Zahler 1992). Emerging evidence of involvement of protein kinases and phosphatases in splicing was accumulating (Mermoud 1992, 1994; Tazi 1992, 1993; Woppmann 1993; Gui 1994; Colwill 1996a/b; Rossi 1996). Interestingly, replacement of ATP with ATP(γS) stalls the catalytic step of the spliceosome, but not its assembly (Mermoud 1992, 1994; Tazi 1992) and a continuous and dynamic cycle of phosphorylation/dephosphorylation is proposed to explain these observations (Tazi 1993). The main impact of phosphorylation on splicing factors is its ability to alter their interactions within the spliceosomal complex, leading to changes in the mRNP formation, localization, or both; examples are provided below.

Changes in pre-mRNA/protein complex formation

Protein splicing factors interactions with the RNA are dominated by weak hydrophobic and electrostatic interactions. Studies of the SR proteins RS domain interactions are among the best characterized; they are responsible for determining homophylic and heterophylic SR proteins binding. For instance, SF2/ASF phosphorylation enhances its binding to U1-70K (U1 snRNP 70K subunit) but has no effect on its binding $U2AF^{35}$ (Xiao 1997) while it has a negative effect on its binding the RNA export factor TAP/NXF1 (Huang 2004), SRp40, hTra2 and itself (Xiao 1997, 1998). Not limited to protein-protein interactions, phosphorylation alters SR proteins-RNA interactions as well, where phosphorylated RS domains prevent the non-specific RNA binding observed under unphosphorylated forms (Xiao 1998) showing that it increases specificity of splicing. Phosphorylation events in the process of splicing seem to play a regulatory switch, decreasing random interactions and improving specificity.

HnRNP proteins are also subject to post-translational phosphorylation. SF1 is a member of the hnRNP K family and is phosphorylated by cGMP-dependent protein kinase-I (PGK-I) at Ser20 blocking its interaction with $U2AF^{65}$, thereby pre-spliceosome assembly (Wang 1999). HnRNP C is phosphorylated by casein kinase II-type activity (Fung 1997), which decreases its RNA-binding capacity; however, it increases its target-specific binding. TIA-1, an apoptosis-activated splicing factor, is also phosphorylated *in vivo* by FAST (Fas-Activated Ser/Thr kinase) in response to Fas ligand binding (Tian 1995). It is interesting to note that in most cases, an adequate amount of phosphorylation is necessary and sufficient for optimal functioning of these splicing

factors, as hypo-phosphorylated and hyper-phosphorylated states tend to inhibit splicing (Prasad 1999).

Changes in cellular localization

An efficient and precise splicing requires specific contribution from each member of the spliceosome, therefore spatial distribution of splicing factors is a major determinant of the reaction's outcome. Phosphorylation of SR proteins and hnRNPs modulates their spatial distribution both between the nucleus and cytoplasm and within the nucleus itself, rendering them more or less available to the splicing machinery. Members of the importin β (imp β) and transportin (TRN) family, TRN-SR1 and TRN-SR2 have been shown to interact with SR proteins for nuclear import both in humans and *Drosophila* (Kataoka 1999, Lai 2000, 2001) and this interaction is dependent on phosphorylated RS domains (Lai 2000, 2001). Dephosphorylation of SRp9G8 and SF2/ASF decreases their binding to the nuclear export factor TAP/NFX-1 leading to cytosolic accumulation (Huang 2004), while phosphorylation of hnRNP A1 by Mnk1/2 (Guil 2006) in a $MKK_{3/6}$/p38-dependent fashion reduces hnRNP A1 binding to transportin-1 under osmotic stress (Allemand 2005) and confines it to the cytosol (van der Houven van Oordt 2000). Spatial rearrangement is also observed in the nucleus where splicing factors (SR proteins, YB-1), in response to CLK1/2/3/4 and phosphatases activities, leave their stronghold "nuclear speckles" following phosphorylation, causing their nuclear redistribution and change in splice site selection (Hartmann 2001, Allemand 2007).

Splicing Factor 45 KDa - SPF45

The pre-mRNA alternative splicing factor 45 KDa, SPF45, was discovered by two groups working independently. Neubauer et al. (Neubauer 1998) used an ingenious technique to identify components of the spliceosome multi-protein complex. Following 2D-gel separation of the components of the fully assembled spliceosome, spots were excised, analyzed by mass spec and then coupled to EST-databases. A spot with an apparent molecular weight of 45 KDa was named SPF45, for splicing factor 45 KDa. SPF45 is highly homologous to the DNA damage repair protein DRT111 in *A.thaliana*. Another group was working on identifying novel genes expressed in drug-resistance cancer cells; using a suppressive-subtractive polymerase chain reaction, Sampath et al. identified SPF45 as a gene expressed in the cyclophosphamide-resistant EMT-6 mouse mammary cancer cells (Sampath 2003).

SPF45 consists of 401 amino acid residues. It has an N-terminal domain that is projected to be largely unstructured (Corsini 2007), a ~40-residues G-patch motif spanning between residues 235 and 281 (Aravind 1999), and a C-terminal domain harboring the RNA Recognition Motif (RRM). The G-patch domain has 6 highly conserved G nucleotides and is predicted to have an unstructured secondary structure with possibly two α helices. It also contains repetitive RS and RGG sequences making it a candidate for non-specific protein-RNA interactions (Aravind 1999). Directly upstream to the G-patch is a ~10 residue domain named SPF45-like domain (or SF motif) and is common to several proteins with DNA repair function in *T.gondii*, *C.elegans*, *D.melanogaster* and *A.thaliana* (Frenal 2006). The SF domain assumes a helical

conformation and is involved in protein oligomerization (Frenal 2006). It has been shown that SPF45 interacts with SXL (*sex lethal*) in *Drosophila* through their N-termini (Lallena 2002) while it interacts with constitutive components of the spliceosome through the RRM domain (Corsini 2007). This latter is highly homologous to a group of protein-binding RRM domains in nuclear proteins such as PUF60, KIS kinase, HCC1 and U2AF65 and are thus named UHM, for U2AF-homology motifs (Corsini 2007). The UHMs of these proteins interact with tryptophan-containing linear peptide motifs (Kielkopf 2001, Selenko 2003) and are subsequently named UHM-ligand motifs ULMs. It is through the UHM-ULM type of interactions that SPF45-UHM binds the N-terminal ULMs of the constitutive spliceosomal proteins U2AF65, SF1 and SF3b155 (Corsini 2007) and in fact, the C-terminus of SPF45 is capable of mediating the alternative splicing of pre-mRNA without the need of the G-patch or the N-terminus.

SPF45 is a ubiquitously expressed protein at low levels in normal tissues. Its highest expression is seen in breast, bladder, colon, kidneys and ovaries with main distribution to the ductal epithelia (Sampath 2003). SPF45 is a nuclear protein that localizes to the nuclear speckles along with other proteins from the spliceosome like U1snRNP and SR proteins (Neubauer 1998, Sampath 2003). In cancer cells, it has the highest expression levels in tumors of epithtelial origin such as bladder, breast, colon, lung, ovarian, pancreatic and prostate carcinomas (Sampath 2003).

We currently have very limited understanding of the potential targets for SPF45-mediated pre-mRNA alternative splicing. So far, only 3 genes are reported to be affected by SPF45 expression. In *D.melanogaster*, SPF45 is responsible for the alternative splicing

of the *sxl* gene (Lallena 2002). SPF45 activates a cryptic AG dinucleotide upstream of the normal 3'AG-dinucleotide responsible for exon 2 definition, leading to expression of exon 2 which contains a premature early stop codon and hence preventing its expression. However, in the presence of SXL, it binds SPF45 through their N-termini preventing the activation of the cryptic AG and leading to exon 2 skipping. The *sex-lethal* protein, SXL, is responsible for female secondary traits maturation (Skripsky 1982). SXL is responsible for its own auto-regulation, being expressed only in female but not male flies. Similarly to SXL, SPF45 is involved in the development of β-thalassemia; a single point mutation of a guanine to adenine in the first intron of the β-globin gene generates a cryptic AG dinucleotide recognized by SPF45 for activation, leading to expression of the β^{110} mutation responsible for the thalassemic condition (Lallena 2002). Another target for SPF45-mediated alternative splicing is the death receptor FAS (Corsini 2007). SPF45 overexpression causes skipping of *fas* exon 6 which encodes the single pass trans-membrane domain that anchors the receptor to the cell membrane (Cascino 1994). Although exon 6 skipping does not inhibit protein expression, the product fails to anchor into the cell membrane and is released as a soluble isoform extracellularly. The soluble FAS acts as a decoy; it binds the FAS ligand (FASL), however it fails to initiate the apoptotic cascade (Cheng 1994). The FASL signal is then transformed from pro-apoptotic to anti-apoptotic by the deletion of the trans-membrane domain.

Sampath et al. showed SPF45 to be strongly over-expressed in tumors of epithelial origins (Sampath 2003). More importantly, when they forced SPF45 expression in HeLa cells, they induced a drug resistance phenotype, as the cells became

resistant to doxorubicin and vincristine. A later study by Perry et al. (Perry 2005) showed that SPF45 overexpression in the ovarian cancer cell line A2780 –traditionally used for measuring drug sensitivities – caused a multidrug resistant phenotype. SPF45 confered drug resistance to the following chemotherapeutic agents: cisplatin, doxorubicin, mitoxantrone, etoposide and vincristine. The mechanism of drug resistance is not identified, however it was speculated to involve, at least partially, interaction between SPF45 and ERβ, since co-treatment of the cells with the selective estrogen receptor modulator, tamoxifen, partially reversed the drug resistance (Perry 2005). SPF45 has been also implicated in the neurodegenerative disease spinocerebellar ataxia type 1 (SCA1). Polyglutamine expansion of ataxin 1 ATXN1 is linked to the development of SCA1 (Banfi 1994); SPF45 directly interacts with ATXN1 through their C-termini (Lim 2008) and this interaction is necessary for SCA1 development.

SPF45 mechanism of action

Our understanding of how SPF45 is utilized by the cell for alternative splicing is limited. The first target of SPF45 to be studied is the sex lethal (sxl) mRNA in *Drosophila melanogaster* (Lallena 2002) in the lab of Juan Valcarcel. Sxl has a very peculiar property that sets it aside from most canonical intron sequences in the fact that intron 2 has two potential AG dinucleotide around the 3' splice site that are termed AG_p and AG_d for proximal and distal, respectively. SPF45 is thought to bind directly to the proximal AG dinucleotide, priming it as the 3' splice site to be utilized. Later studies from the lab of Juan Valcarcel into the binding partners of SPF45 in the alternative splicing of Fas, show that the C-terminus of SPF45 harbors a UHM domain that interacts with members of the

constitutive splicing machinery, U2AF65, SF1 and SF3b155. NMR experiments show SPF45 to interact with the aforementioned splicing factors; however, no measurable interaction is observed between SPF45 and the pre-mRNA. Whether SPF45 interacts with the nascent pre-mRNA or is mainly influenced by interactions with neighboring splicing factors remains an important area to unravel the true mechanism of action.

Rationale, hypothesis and specific aims

Identifying MAP kinase(s) targets in their natural tissue specific expression pattern is essential for determining the impact of MAP kinases on their function(s). With emerging studies on the effects of signaling and phosphorylation, the link between MAP kinases and splicing factors, has been at best, very limited. Previous work from our lab showed an increase in ERK2 activity in SKOV3 cells upon detachment and growth under anchorage-independent conditions. Using an engineered ERK2 enzyme to determine MAP kinase-specific substrates in SKOV3 cells, we identify SPF45 as a novel substrate under conditions of adherence and suspension. In this dissertation, I investigate SPF45 expression in ovarian cancer cells, identify the phosphorylation residues and characterize this phosphorylation both *in vitro* and in cells by ERK, JNK and p38 MAP kinases. I also examine the effect of the phosphorylation on SPF45 splicing activity, determine genetic changes in response to SPF45 overexpression as well as identify novel splicing targets and binding partners. Finally, I will address the role of SPF45 on drug resistance in cancer. The long term objective of this study is to understand the biological role of SPF45 in ovarian cancer and establish a strong link between the MAP kinase pathways, pre-mRNA alternative splicing events and the regulation of aberrant genetic changes that occur in cancer cells in the scope of designing efficient therapeutic interventions.

Hypothesis

MAP kinase phosphorylation of SPF45 is responsible for altering SPF45 interactions within the spliceosome and modulates SPF45-induced genetic changes and splicing activity.

Specific Aims

Aim1 Identify SPF45 as a novel MAP kinase substrate. Characterize the phosphorylation of SPF45 by ERK, JNK and p38 MAP kinases *in vitro* and in cells.

Aim2 Determine the effect of MAP kinase phosphorylation on SPF45 splicing activity. Identify novel genetic and splicing events controlled by SPF45 as well as SPF45 interaction with constitutive splicing factors. Assess the role of SPF45 on drug resistance in SKOV3 cells. Determine the impact of modulation of SPF45 functions via mutations of the MAP kinase-targeted residues.

Chapter 2 Identification of SPF45 as a novel MAP kinase substrate

Introduction

MAP kinases are a ubiquitous family of protein kinases with important regulatory functions throughout the cell: they are involved in a diverse array of processes including but not restricted to, gene expression, differentiation, proliferation, migration and survival (reviewed in Pratilas 2010). Several MAP kinase subfamilies have been identified in eukaryotic cells, some of which are common to all eukaryotes while others are organism specific. All subfamilies share a common connected topology of activation: simplistically, an extracellular stimulus on the cell membrane activates a membrane receptor which leads to a sequential activation of a three-tiered cascade starting with MAP3K, followed by MAP2K and then the penultimate MAPK. Due to the similarities between the subfamilies, substrate promiscuity is not uncommon; hence identification of MAP kinase substrates in the cellular contexts and compartments is crucial to understand their functions (Eblen 2003). Earlier work from our lab (Eblen 2003, Kumar 2004, Zheng 2010) led to the generation of an engineered ERK2/MAP kinase capable of efficiently identifying specific substrates of ERK2 under physiological and pathological cell conditions.

Interests in MAP kinase pathways stem from their intricate involvements in several pathological conditions, especially cancer (McCubrey 2006). In ovarian cancer, ERK/MAP kinase pathway is indeed an important player (Thant 2000, Wong 2001, Givant-Horwitz 2003 and Ventura 2010). Previous work from our lab identified a novel form of sustained ERK activation in several ovarian cancer cell lines upon cellular

detachment that we showed to be necessary for anchorage-independent growth (Al-Ayoubi 2008). Using the engineered ERK2 kinase mentioned above to generate SKOV3-ERK2Q103 stable ovarian cancer cells, we identified Splicing Factor 45 KD (SPF45) as a novel substrate of ERK2 in ovarian cancer cells under anchorage-dependendent as well as -independent conditions.

SPF45 is an mRNA alternative splicing factor initially identified by mass spec analysis to localize and engage with the spliceosome complex (Neubauer 1998). Concomitantly, studies of anti-neoplastic agents' resistance identified SPF45 as a gene overexpressed in EMT-6 mouse mammary cancer cells selected for cyclophosphamide resistance (Perry 2005). Overexpression of SPF45 in HeLa cervical cancer cells confers drug resistance against doxorubicin and vincristine (Sampath 2003), while overexpression in A2780 ovarian cancer cells confers a multidrug resistance phenotype against carboplatin, vinorelbine, etoposide, mitoxantrone, doxorubicin and vincristine (Perry 2005). In A2780-SPF45 cells, treatment with the selective estrogen receptors modulators tamoxifen and LY117018 partially reverses mitoxantrone resistance, while SPF45 knock-down sensitizes the cells to etoposide (Perry 2005). Structurally, SPF45 has a large N-terminus that is unstructured, followed by a 10-residues SF (SPF45-like) motif, a G-patch motif of approximately 40 amino acid residues involved in protein-protein and protein-nucleic acid interactions and finally a C-terminus containing a modified RNA recognition motif (RRM) important for its intrinsic splicing activity (Corsini 2007). Crystallographic and NMR studies of the Toxoplasma gondii 55KDa DNA repair protein, TgDRE, which shares high homology to SPF45, show the capability of the G-motif to bind

small RNA oligonucleotides (as predicted for SPF45 G-domain) and that the RRM domain adopts a classical βαββαβ topology necessary for hydrophobic protein-protein interactions. Subsequent experiments by Corsini et al. resolved SPF45 RRM domain to adopt the same configuration (Corsini 2007). The SF motif is also involved in protein oligomerization (Frenal 2006). Besides its function in splicing and drug resistance, SPF45 interacts with DNA repair proteins RAD201 (Chaouki 2006) and RAD51B (Horikoshi 2009) indicating its involvement in DNA repair pathways and specifically through SPF45 N-terminal lysines (Horikoshi 2009). Additionally, SPF45 is implicated in the development of spinocerebellar ataxia through a protein complex interacting with the polyglutamine expanded protein ATXN1 (Lim 2008).

There is evidence that phosphorylation of splicing factors is implicated in the splicing machinery, regulating assembly, splice site selection and localization (Stamm 2008). Identification of the serine-arginine rich proteins (SR) and the heterogeneous nuclear ribonucleic proteins (hnRNPs) as regulators of splicing had a major impact on recognizing that splicing factors undergo post-translational phosphorylation important for regulation of splicing. SR proteins were initially identified by their immunoreactivity to an antibody that detects a phosphorylated epitope (Roth 1991, 1992, Zahler 1992). Characterization of SR proteins and hnRNPs regulation led to the identification of SR protein kinases (SRPKs), cdc2-like kinases (CLK/Sty) and DNA topoisomerase I as the main kinases regulating splicing, with a very limited contribution of MAP kinases to pre-mRNA splicing/alternative splicing. To date, Sam68, (Src-associated in mitosis, 68Kd), remains the only pre-mRNA alternative splicing factor to be a direct substrate of

ERK/MAP kinase; phosphorylation of Sam68 by ERK/MAP kinase improves its binding to an exonic enhancer region leading to increase inclusion of CD44-v5 exon (Matter 2002). Of note, $MKK_{3/6}$/p38 MAP kinase is also involved in the regulation of splicing factors, through Mnk1 activation (Guil 2006), via an inhibitory phosphorylation of the splicing repressor hnRNP A1, causing its cytoplasmic retention (van der Houven van Oordt 2000).

In this chapter we show that SPF45 is a novel ERK2/MAP kinase substrate with overlapping substrate specificity by JNK and p38 MAP kinase; we identify the targeted residues for phosphorylation by MAP kinases and characterize SPF45 phosphorylation on these sites in response to oncogenic and extracellular cues.

Materials and methods

Cell lines and cell cultures

ES-2, CaOV3, and OV2008 cells were a gift from Runzhao Li. SKOV-3 and Cos-1 cells were obtained from American Type Culture Collection (ATCC Manassas, VA). OVCAR3 cells were a gift from Kristen Atkins, OVCAR5 and A2780 cells were a gift from Thomas Hamilton. IOSE cells were a gift from Nellie Auersperg and were grown in a 50:50 mixture of media 105 and 199. Cos-1 cells were grown in DMEM (Invitrogen Carlsbad, CA). SKOV-3, and OVCAR5 cells were grown in McCoy's 5A (Invitrogen Carlsbad, CA). ES2, A2780 OVCAR3 and OV2008 cells were grown in RPMI 1640 (Invitrogen Carlsbad, CA). All cell lines were grown in 10% fetal bovine serum (FBS) (ThermoScientific Rockford, IL).

Plasmids and mutagenesis

SPF45 cDNA was obtained from ATCC in the pOTB7 vector, digested with EcoR1 and Xho1 restriction enzymes (New England Bio Ipswitch, MA) and subcloned into the multi-cloning site of pCMV3-2C vector encoding an N-terminal triple Myc-tag epitope downstream of the constitutive CMV promoter. PCR-based site-directed mutagenesis of Thr 71 and/or Ser222 was carried out using the following primers:

Thr71→Ala71 (GACCGGCAAATTGTGGACGCTCCACCGCATGTAGCAG & CTGGCCGTTTAACACCTGCGAGGTGGCGTACATCGTC)
Ser222→Ala222 (GAACAAGACAGACCGAGAGCTCCAACCGGACCTAGC & CTTGTTCTGTCTGGCTCTCGAGGTTGGCCTGGATCG)
Thr71→Asp71 (GACCGGCAAATTGTGGACGATCCACCGCATGTAGCAGC & CTGGCCGTTTAACACCTGCTAGGTGGCGTACATCGTCG)
Ser222→Asp222 (GAACAAGACAGACCGAGAGATCCAACCGGACCTAGCAAC & CTTGTTCTGTCTGGCTCTCTAGGTTGGCCTGGATCG).

Wild type or alanine-mutants SPF45 were subcloned from this vector into pET15b encoding a 6-Histidine tag and transformed into BL21 E. coli to generate recombinant His-SPF45 proteins that were purified with nickel agarose (Roche Indianapolis, IN).

Generation of SKOV3-QT8 cells

Retroviral plasmids (pQCXIP) encoding empty vector/Flag-ERK2/Flag-ERK2-Q103G were transfected into phoenix A cells along with VSV-G and Gag/Pol for virus production and packaging. Cellular media containing the viruses were harvested at 36 and 60 hours post transfection, filtered with 45 μm syringe filters (Corning Lowell, MA) and mixed with polybrene (Sigma St Louis, MO) to a final 2μg/ml concentration before infecting the SKOV3 target cells. Cells were selected with 1.5 μg/ml puromycin and several clones were generated. Individual clones were harvested and grown in 24 well plates, then screened by western blot for Flag-ERK2/or Flag-ERK2-Q103G expression.

^{32}P-Labeling of cyclopentyl ADP (cpADP)

Nucleoside diphosphate kinase (NDPK, 200 units, Sigma St Louis, MO) and 800 μCi of [γ-^{32}P]ATP were added in a 100-ml reaction containing HBS (150 mm NaCl, 20 mmHEPES, pH 7.4, and 5 mm $MgCl_2$). The reaction was allowed to equilibrate at 30°C for 5 min.^{32}P-Labeled NDPK was purified from free [γ-^{32}P]ATP by two successive rounds of Microspin G50 columns (Amersham Biosciences, Piscataway, NJ). 1000 pmol of cpADP was added to the ^{32}P-labeled NDPK in HBS, and the reaction was carried out for 20 min at 30°C. After the reaction, contents were transferred to a Microcon-YM30 (Millipore, Bedford,

MA) and centrifuged at 13,000 rpm for 15 min. With this procedure we usually obtained ~ 200 µCi of purified [γ-^{32}P]cyclopentyl-ATP ([γ-^{32}P]cpATP).

Identification of SPF45 as a novel ERK2 substrate

Four 150-mm dishes of SKOV3-QT8 cells (4× 10^6 cells/dish) expressing Flag-ERK2-Q103G were used. Cells were lysed in M2 buffer (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM HEPES, pH 7.4) supplied with protease inhibitors mix (100 µM Na-*o*-vanadate, 50 mM NaF, 40 mM β-glycerophosphate, 5 mM Na-pyrophosphate, Aprotinin and 2 mM PMSF). Immunoprecipitation was performed using M2-Flag antibody (Sigma St Louis, MO). Immunoprecipitated ERK2-QG and associated proteins were eluted from the M2-agarose beads with FLAG peptide and separated on 8% SDS-PAGE. Labeled proteins were excised from the gel and microsequenced using a Finnigan LCQ ion trap mass spectrometer with a protana nanospray ion source (W. M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia).

Generation of antibodies – α-total SPF45, α-pThr71-SPF45 and α-pSer222-SPF45

Total SPF45 antibody and phospho-specific antibodies to pThr71-SPF45 and pSer222-SPF45 were generated in New Zealand White rabbits by Pacific Immunology, who assisted in the design and synthesis of immunogenic peptides, as well as generation and purification of the antibodies. Peptides used were: SPF45, PYEEDSRPRSQSSKAC; pThr71-SPF45, SDDRQIVD-T(PO_4)-PPHVAAGC; pSer222-SPF45, YEEQDRPR-S(PO_4)-PTGPSNSFC.

ERK binding assays

His-ERK2 or buffer was added to 500 µg of nuclear extract from HeLa cells and mixed overnight at 4°C. His-ERK2 was isolated with nickel agarose and washed three times with wash buffer (20 mM Hepes pH 7.4, 2 mM EDTA and 2 mM EGTA). The pull-downs were run on a gel and immunoblotted for SPF45 and ERK2.

Transfections

1.5 million Cos-1 cells were plated the night before transfection on 10 cm cell-culture dishes (Corning Lowell, MA) and transfected the following day with 6 µg total of DNA. Briefly, 6 µg total DNA and 24 µl of Lipofectamine 2000 were suspended in 1.5 ml of Opti-MEM (Invitrogen Carlsbad, CA) each for 5 minutes at RT, then mixed together for a total of 20 minutes at RT before drop wise addition onto cells. The media and transfection mixture were recollected 5~6 hours following transfection, washed once in 1X PBS and incubated in fresh media supplied with 10% FBS. The cells were collected 24 hours post-transfection.

In vitro kinase assay and phosphoamino acid analysis

0.5 µg of recombinant SPF45 was incubated with the kinases of interest (His-ERK2, GST-p38α and GST-JNK1 (Invitrogen Carlsbad, CA) at an equal activity of 500 nmol/min in 30 µl of kinase buffer (20 mM Hepes pH 7.4, 10 mM Mg acetate, 1 mM dithiothreitol (DTT)), 100µM ATP and 10µCi of γ ^{32}P-ATP (Perkin Elmer Waltham, MA) at 30°C for 10minutes. The reaction was quenched by addition of 2X Laemmli sample buffer and the phosphorylated protein (His-SPF45) was resolved by SDS-PAGE, transferred by western blot into PVDF (Biorad Hercules, CA) membranes and exposed on HyBlot CL films

(Denville Scientific South Plainfield, NJ) for autoradiography. Radioactive bands were excised, quantified by Cerenkov count and analyzed for phosphorylated residues by phosphoamino acid analysis (Boyle 1991). Briefly, radioactive bands were excised, hydrolyzed using 6N HCl (Sigma St Louis, MO) at 110^0C for 1h, washed and lyophilized in water than suspended in buffer 1.9 (2.5% v/v 88% Formic acid, 7.8% v/v Acetic acid, 89.7% v/v H_2O). 1000 cpm of the final suspension were mixed with standard threonine, serine and tyrosine amino acids for detection before spotting and 2-D electrophoresis on a TLC plate. The standard amino acids were stained with 0.2% ninhydrin in ethanol (Sigma St Louis, MO) and the plate was developed on Hyblot CL films a week after exposure.

Immunoprecipitation

Cells were lysed in M2 buffer (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM HEPES, pH 7.4) supplied with protease inhibitors mix (100 µM Na-*o*-vanadate, 50 mM NaF, 40 mM β-glycerophosphate, 5 mM Na-pyrophosphate, Aprotinin and 2 mM PMSF). 500 µg of lysate were incubated with 2 µl of Myc-antibody (Sigma St Louis, MO) conjugated to protein A agarose with constant mixing for 2 hours at 4^oC. The precipitates were then washed 3X in M2 buffer followed by suspension in 1X Laemmli Sample buffer or washed 2X in kinase buffer in preparation for *in vitro* kinase reaction.

In vitro kinase reaction of SPF45/alanine mutants

Empty vector/Myc-SPF45/alanine mutants were transfected into Cos-1 cells, Myc-immunoprecipitated 24 hours post-transfection then suspended in 30 µl of 1X phosphatase buffer (NEB buffer3: 50mM Tris-HCl, 100 mM NaCl, 10mM $MgCl_2$ and 1mM

DTT) with 2 µl of calf intestinal phosphatase CIP (New England Bio) at 37°C for 1 hour followed by 2 washes in kinase buffer prior to *in vitro* kinase phosphorylation as described above.

SPF45 dephosphorylation - Phosphatase Assay

His-SPF45 (0.5 µg) was recollected following the kinase assay (with ERK2) by Ni^{++}-agarose binding with rocking for 2 hours at 4°C. The SPF45-conjugated beads were washed twice in 1X phosphatase buffer, then re-suspended in 30 µl of 1X phosphatase buffer supplied with 2 µl of Calf Intestinal Phosphatase (New England BioLabs Ipswitch, MA) and incubated at 37°C for 1 hour. The reaction was quenched by addition of 10 µl of 4X Laemmli Sample buffer. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane and visualized using pThr71-SPF45, pSer222-SPF45, total SPF45 and ERK2 antibodies by western blotting.

Immunoblotting

Cells were lysed and sonicated in M2 buffer with 0.5% SDS and protease inhibitors mix. Lysates were normalized using BCA protein assay kit (Pierce Rockford, IL). Each sample (100 µg) was run on a 10% polyacrylamide gel (SDS-PAGE) under denaturing conditions and transferred to Protran nitrocellulose membrane (Whatman-GE Healthcare Piscataway, NJ). After blocking with 5% milk for 1 hour at room temperature, the membranes were incubated with relevant primary antibodies (total-SPF45, pThr71-SPF45, pSer222-SPF45, Myc, ERK, pERK, p38 MAP kinase, p-p38 MAP kinase, JNK, p-JNK and Actin) in 1X PBS with 5% BSA and 0.1% Tween-20, 0.1% sodium azide (Sigma St Louis, MO) at 4°C overnight. The blots were washed three times in 1X PBS + 0.1%

Tween-20 and incubated with the appropriate secondary HRP-conjugated antibody (Mouse, Rabbit (Biorad Hercules, CA)) in PBS-0.1%T with 1% milk for 1 hour at room temperature. The membranes were then washed 6 times with PBS-0.1%T for a total of 1 hour at room temperature. The membranes were incubated with femto-ECL (Pierce Rockford, IL) for 2 minutes at room temperature and the bands were exposed on Hyblot CL films (Denville Scientific South Plainfield, NJ).

Results

Identification of SPF45 as a novel substrate of ERK2

In previous works, we reported the generation of a mutant ERK2/MAP kinase (ERK2-Q103G) capable of identifying specific substrate of the enzyme under the desired cellular conditions (Eblen 2003), and show activation of the ERK2/MAP kinase pathway in several ovarian cancer cells under anchorage-independent conditions (Al-Ayoubi 2008). Using SKOV3-QT8, an ovarian cancer cell line stably expressing ERK2-Q103G (Fig 2.1 *A&B*), we screened for potential ERK2-associated substrates in ovarian cancer cells. SKOV3-QT8 cells were either grown adherent or trypsinized then put in suspension for 3 hrs. Subsequently, the cells were lysed and Flag-ERK2-Q103G immunoprecipitated under mild conditions to preserve protein-protein interactions prior to incubation for 3 min in kinase buffer containing [32P-γ]-N6-cpATP. The kinase reaction was run on a gel and we detected radiolabeled bands by autoradiography showing labeling of a 47 kDa band under both conditions (Fig 2.1 *C* left). A parallel immunoprecipitation and kinase reaction using more cell lysate from suspended cells and unlabeled cyclopentyl ATP displayed a silver-stainable band at the same molecular weight (Fig 2.1 *C* right). Mass spec analysis of the band identifies several proteins including SPF45, a pre-mRNA alternative splicing factor.

SPF45 is phosphorylated by ERK2, JNK and p38 MAP kinases on threonine and serine

Since MAP kinases are serine/threonine proline-directed kinases that phosphorylate the canonical T/SP or PXT/SP sequences, overlapping substrate specificity is not uncommon among the different subfamilies. In all that follows, SPF45 cDNA was

obtained and subcloned into pCMV3-2C (adds a Myc-tag) and pET15b (adds a His- tag) for mammalian and bacterial expression, respectively. Accordingly, we tested whether phosphorylation of SPF45 by the three MAPK subfamilies occurs, and subjected His-SPF45 to an *in vitro* kinase reaction with active recombinant ERK2, p38α and JNK1 in the presence of [^{32}P-γ]-ATP. Protein fractionation by SDS-PAGE showed a radiolabeled band around 50 KDa corresponding to phosphorylated His-SPF45 (Fig 2.1 *D&E*). The bands were excised and processed for phosphoaminoacid analysis (PAA) identifying phosphorylated threonine and serine residues on all three bands (Fig 2.1 *F*).

Generation of SPF45 antibodies: α-total-SPF45, α-pThr71-SPF45 and α-pSer222-SPF45

Among the 401 amino acid residues that make SPF45, only two residues conform to the MAPK substrates criterion. Namely, they are Thr71 and Ser222, which - by sequence analogy - appear to be highly conserved among mammalian species, suggesting a role for their phosphorylation in regulating the function of this protein (Fig 2.2 *A*). As there are no commercially available total and phospho-specific antibodies to SPF45, we generated polyclonal antibodies in New Zealand White rabbits. To test their ability to recognize total-SPF45 as well as pThr71-SPF45 and pSer222-SPF45 by western blot, we subjected SPF45 to three different treatments: no ERK2 phosphorylation, with ERK2 phosphorylation and ERK2 followed by phosphatase treatment (Fig *2.2 B*). Anti-total-SPF45 antibody was able to detect His-SPF45 both in the presence and absence of ERK2 kinase reaction, but not the empty SPF45 lane. On the other hand, anti-pThr71-SPF45 and anti-pSer222-SPF45 antibodies were weakly able to detect SPF45 before it was phosphorylated and their specificity was strongly enhanced following incubation

with active ERK2. Phosphatase treatment of the Ni^{++}-purified SPF45 after the kinase reaction showed a decrease in phospho-specific antibodies detection of SPF45 but not of the total-SPF45 antibody. Altogether, these data support our prediction that Thr71 and Ser222 are ERK2-targeted residues, at least *in vitro*. To validate the specificity of the phospho-specific antibodies, we generated single and double mutants of Thr71 and Ser222 to either alanine (Fig 2.2 *C*); single and double aspartate mutants were equally generated for subsequent experiments. As anti-total-SPF45 was mounted against an epitope that does not encompass the phosphorylation residues, it equally detected SPF45-mutants ($SPF45^{T71A}$, $SPF45^{S222A}$ and $SPF45^{AA}$) both before and after ERK2-induced phosphorylation. Anti-pThr71-SPF45 failed to detect His-$SPF45^{T71A}$ and His-$SPF45^{AA}$ but not His-$SPF45^{S222A}$, while anti-pSer222-SPF45 detects His-$SPF45^{T71A}$ but not His-$SPF45^{S222A}$ and His-$SPF45^{AA}$ following *in vitro* kinase reaction with ERK2, confirming their efficacy in specifically identifying SPF45 and its phosphorylated forms (Fig 2.2 *D*). It is noteworthy to mention that phosphorylation of SPF45 on Thr71 causes a mobility shift (Fig 2.2*D* S222A+ERK2); in a later experiment we show a similar mobility shift for $SPF45^{T71D}$. Finally, we examined and showed SPF45 phosphorylation on Thr71 and Ser222 in response to JNK and p38 MAP kinases (Fig 2.2 *E*). Unfortunately, the ability of these antibodies to immunoprecipitate SPF45 and immunofluorescence cytochemistry is not successful (data not shown). ELISA and immunohistochemistry (IHC) were not tested.

SPF45 and ERK2 are binding partners *in vitro* and in cells

SPF45 was initially identified as an ERK2-associated substrate in our search for potential ERK2 targets in SKOV-3 ovarian cancer cells. MAP kinases are known to bind their targets in their non-phosphorylated state prior to activation and detachment from the protein complex, potentially through interactions with the D-domain in the target protein (Cobb 1995). Although not tested, the amino acid sequence of SPF45 exhibits a putative D-domain (in green) ^{136}KRRKDRHEASGFARR^{151} and a potential overlap with a nuclear localization signal (NLS) which can possibly explain the nature of interactions between ERK2 and SPF45. To support the hypothesis of binding between ERK2 and SPF45, His-ERK2 was incubated overnight at 4^0C with nuclear extracts from HeLa cells, pulled-down the following day with Ni^{++}-conjugated agarose beads solubilized in 1X sample buffer and fractionated by SDS-PAGE. Immunoblotting with anti-total-SPF45 antibody shows SPF45 in the ERK2-pull-down, but not with Ni^{++} beads alone (Fig 2.3 *A*), and the amount of SPF45 recovered from the nuclear extract was dose-dependent to the amount of His-ERK2 in the pull-down assay (Fig 2.3 *B*), indicating a stoichiometric binding of ERK2 to SPF45. Efforts to show the reciprocal binding of recombinant His-ERK2 with recombinant SPF45 were not successful since both proteins are His-tagged and efforts to generate GST-SPF45 were futile possibly due to solubility issues (data not shown). Using SKOV3-pQCXIP and SKOV3-pQCSPF45, two cell lines stably expressing empty vector and Myc-SPF45 respectively generated in the next chapter, we showed that ERK2 co-immunoprecipitates with SPF45 but not the empty vector (Fig 2.3 *C*). Taken

together, we confirmed that ERK2 and SPF45 bind together both *in vitro* and in cells supporting a genuine interaction between ERK2 and its target, SPF45.

Phosphorylation of SPF45 in Cos-1 cells in the presence of constitutive activation of the ERK2/MAP kinase pathway

To characterize SPF45 phosphorylation in cells in response to continuous MEK1/ERK2 activation, we transfected Myc-SPF45/mutants (to alanine and aspartate) into Cos-1 cells along with constitutively-active MEK1-S222/2224D (CA-MEK1(DD)) and ERK2 (Fig 2.4 *A*). Twenty four hours hours post-transfection, the cells were lysed and Myc-SPF45/mutants were Myc-immunoprecipitated. Immunoblotting against anti-pThr71-SPF45, anti-pSer222-SPF45 and anti-Myc antibodies showed that SPF45 is phosphorylated on both Thr71 and Ser222 in agreement with the *in vitro* kinase assay. Mutations of Thr71 to either alanine or aspartate inhibited its phosphorylation but not Ser222. Similarly mutations of Ser222 to either alanine or aspartate prevented its phosphorylation but not Thr71. To note, we also observed a shift in SPF45 molecular weight with Thr71 phosphorylation that corresponds to the mobility shift displayed by $SPF45^{T71D}$ mutant. Although CA-MEK1(DD) causes a permanent ERK2 activation, no such mutation is observed to occur naturally in cells. Hyperactivation of the MAP kinase pathways is linked to several mechanisms, including but not limited to: overexpression of receptor tyrosine kinases (RTKs), pathophysiological processes such as inflammation and stress, and in cancer due to activating mutations in the upstream signaling cascade of ERK2 such as K-Ras, H-Ras and B-Raf (see the "Introduction" chapter for more details). We transfected Myc-SPF45 into Cos-1 cells along with K-Ras-G12V, B-Raf-V600E

or CA-MEK1(DD) with or without ERK2 (Fig 2.4 *B*). Co-transfection of each member of the pathway stimulated SPF45 phosphorylation on both Thr71 and Ser222 above basal levels which was further enhanced on Ser222 phosphorylation and greatly induced Thr71 phosphorylation by addition of exogenous ERK2. Using Cos-1 cells as a "neutral" milieu for studying SPF45 phosphorylation, we showed that hyperactivation of ERK2/MAP kinase pathway results in Thr71 and Ser222 phosphorylation indicating that SPF45 could be phosphorylated on these two residues in cancer cells that have elevated ERK2/MAP kinase expression and activation levels as demonstrated by the assay above. We further examined the validity of this hypothesis below in ovarian cancer cell lines.

Phosphorylation of endogenous SPF45 in ovarian cancer cells following extracellular stimulation of MAP kinases pathways

Phosphorylation of endogenous SPF45 has not been reported. We showed above that Thr71 and Ser222 are MAP kinase targets for SPF45 phosphorylation *in vitro* and by forced phosphorylation of exogenous SPF45 in Cos-1 cells. Previously published data (Sampath 2003) showed SPF45 to be overexpressed in several solid cancers including ovarian cancer. Since SPF45 is an ERK2/MAP kinase substrate in SKOV3 cells, we sought to validate Thr71 and Ser222 as genuine MAP kinase targets on endogenous SPF45 and to characterize the pattern of SPF45 phosphorylation in cells. Screening for SPF45 expression levels in several ovarian cancer cells and IOSE cells, a benign immortalized ovarian cell line, revealed the following: IOSE expressed little SPF45 and would accordingly serve as a negative control while the A2780 cancer cell line showed the highest levels of SPF45 expression followed by ES2, OV2008, OVCAR-3 and OVCAR-5

cells, in no specific order (Fig 2.5 *A*). We observed basal levels of Thr71 and Ser222 phosphorylation in all cell lines tested (Fig 2.5 *A*). Immunoblotting with anti-phospho MAP kinase antibodies revealed variable expression/activation of ERK, p38 and JNK kinases, with most exhibiting elevated ERK1/2 activation. Based on these results, we speculate that SPF45 maintains a basal phosphorylation level required for its ubiquitous functions depending on cellular conditions; additionally, an elevation in Thr71 and/or Ser222 phosphorylation levels in response to MAP kinase(s) activation can potentially reflect increased cellular requirement of SPF45 functions, which remains to be demonstrated.

We originally identified SPF45 as an ERK2-associated substrate from anoikis-resistant SKOV-3 cells grown in suspension. Therefore, it was natural to test the phosphorylation of endogenous SPF45 in SKOV-3 cells that are either grown adherent or detached (Fig 2.5 *B*). Endogenous SPF45 was phosphorylated detached but not adherent cells, suggesting that SPF45 phosphorylation, like ERK2 activation is anchorage-independent in ovarian cancer cells (Al-Ayoubi 2008).

In what follows, we describe several experiments performed to identify extracellular stimuli that induce SPF45 in ovarian cancer cells and to identify the MAP kinase pathway(s) responsible for SPF45 phosphorylation. For these experiments we used A2780 (Fig 2.5 *C*), ES2 (Fig 2.5 *D*) and SKOV3 (Fig 2.5 *E*) cells as they had three different levels of SPF45 expression: high, medium and modest, respectively. After serum starvation overnight the cells were treated with agents that stimulate the various MAP kinase pathways, including fetal bovine serum, phorbol myristate acid (PMA),

H_2O_2, anisomycin, EGF, HGF, sorbitol and ultraviolet (UV) light. In A2780 cells, PMA - an activator of ERK and p38 - stimulated high Thr71 phosphorylation and moderate Ser222 phosphorylation. Anisomycin - an activator of JNK - stimulated high Thr71 phosphorylation, with little to no increase in Ser222 phosphorylation. Similar results were observed with UV treatment, which activated both JNK and p38. H_2O_2, an acute stimulator of intracellular ROS and an activator of both p38 and ERK, induced phosphorylation of both sites equally. In ES2 cells, no change in Ser222 phosphorylation was observed with the different stimuli tested. Thr71 on the other hand exhibited elevated phosphorylation levels that were more pronounced an hour following UV, anisomycin, sorbitol, H_2O_2 and EGF stimulation. Finally, in SKOV3 cells, both Thr71 and Ser222 phosphorylations were observed; Thr71 phosphorylation was more pronounced under serum starvation, H_2O_2 and UV stimulation while Ser222 phosphorylation was highest following UV, EGF and HGF stimulation.

To determine which MAP kinase pathway(s) was responsible for endogenous SPF45 phosphorylation in cells, A2780 cells were serum starved, pretreated for 30 min with a specific MAP kinase pathway inhibitor and then stimulated as above in the presence of the inhibitor (Fig 2.5 *F&G*). The MAP kinase pathway inhibitors used were U0126 for the ERK pathway, SB203580 for the p38 pathway and SP600025 for the JNK pathway. U0126 inhibited SPF45 phosphorylation in response to PMA and H_2O_2; on the other hand, SP600025 inhibited SPF45 phosphorylation in response to UV light, while SB203580 inhibited Ser222 only in response to UV, suggesting that ERK, JNK and p38 MAP kinase are able to phosphorylate endogenous SPF45 in response to extracellular

signals in a stimulus-dependent manner. Although we were unable to find a stimulus that significantly induced SPF45 phosphorylation on both Thr71 and Ser222 in a p38-dependent manner, we have observed SPF45 phosphorylation on Thr71 and Ser222 when SPF45 was co-expressed in Cos-1 cells along with MKK3 and p38 (data not shown). Accordingly, SPF45 phosphorylation in a p38-dependent manner, if it were to happen, is more likely to be stimulus and cell type-dependent.

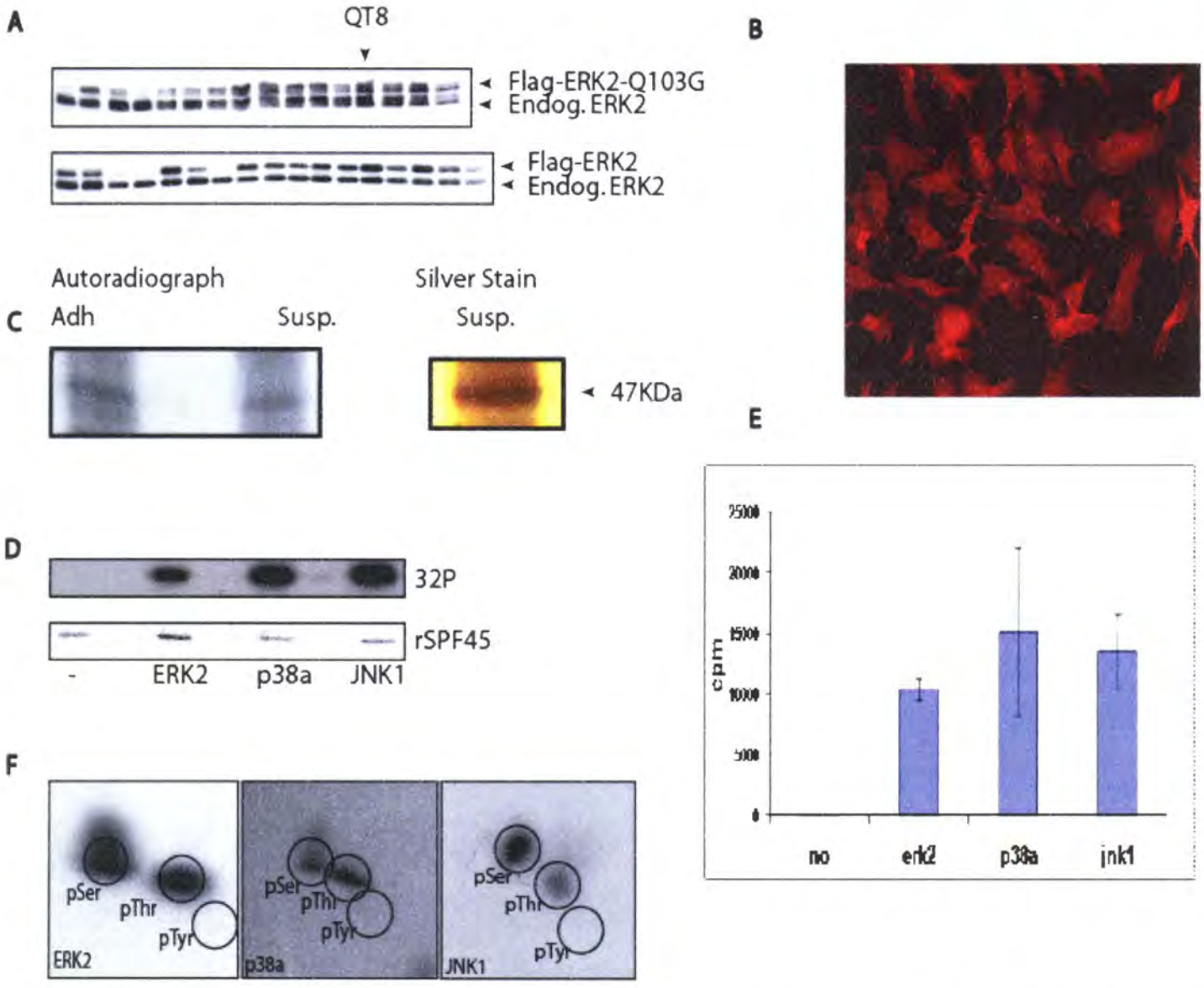

Fig 2.1 SPF45 is a novel MAP kinase substrate *A*. FLAG-ERK2-Q103G or FLAG-ERK2 is stably expressed in SKOV-3 cells via retroviral infection. Cell lysates are used to detect exogenous and endogenous ERK2 expression in several clones. *B*. Immunofluroescence using Flag antibody and clone QT8 demonstrates efficient ERK2-Q103G expression. *C*. FLAG-ERK-Q103G is immunoprecipitated from SKOV-3 clone QT8 either growing adherent (Adh) or anchorage-independent (Susp) for 3 hr. Co-immunoprecipitating substrates are labeled in an *in vitro* kinase reaction with [32P-γ]-cyclopentyl ATP (left). A silver-stained scaled-up non-radioactive kinase assay is performed in parallel (right) and SPF45 is identified by mass spectrometry from this protein band among several other targets. *D. In vitro* MAPK kinase reaction using active recombinant ERK2, JNK1 or p38α, and [32P-γ]-ATP and His-SPF45 as substrate. The reactions are run on a gel and exposed for autoradiography. *E.* Histogram of counts per minutes (cpm) obtained from the phosphorylated SPF45 bands. *F.* Phosphorylated His-SPF45 from the reactions in *D* are digested with HCl and processed for 2-dimensional phosphoamino acid analysis before exposure for autoradiography. *Experiments performed with Scott Eblen.*

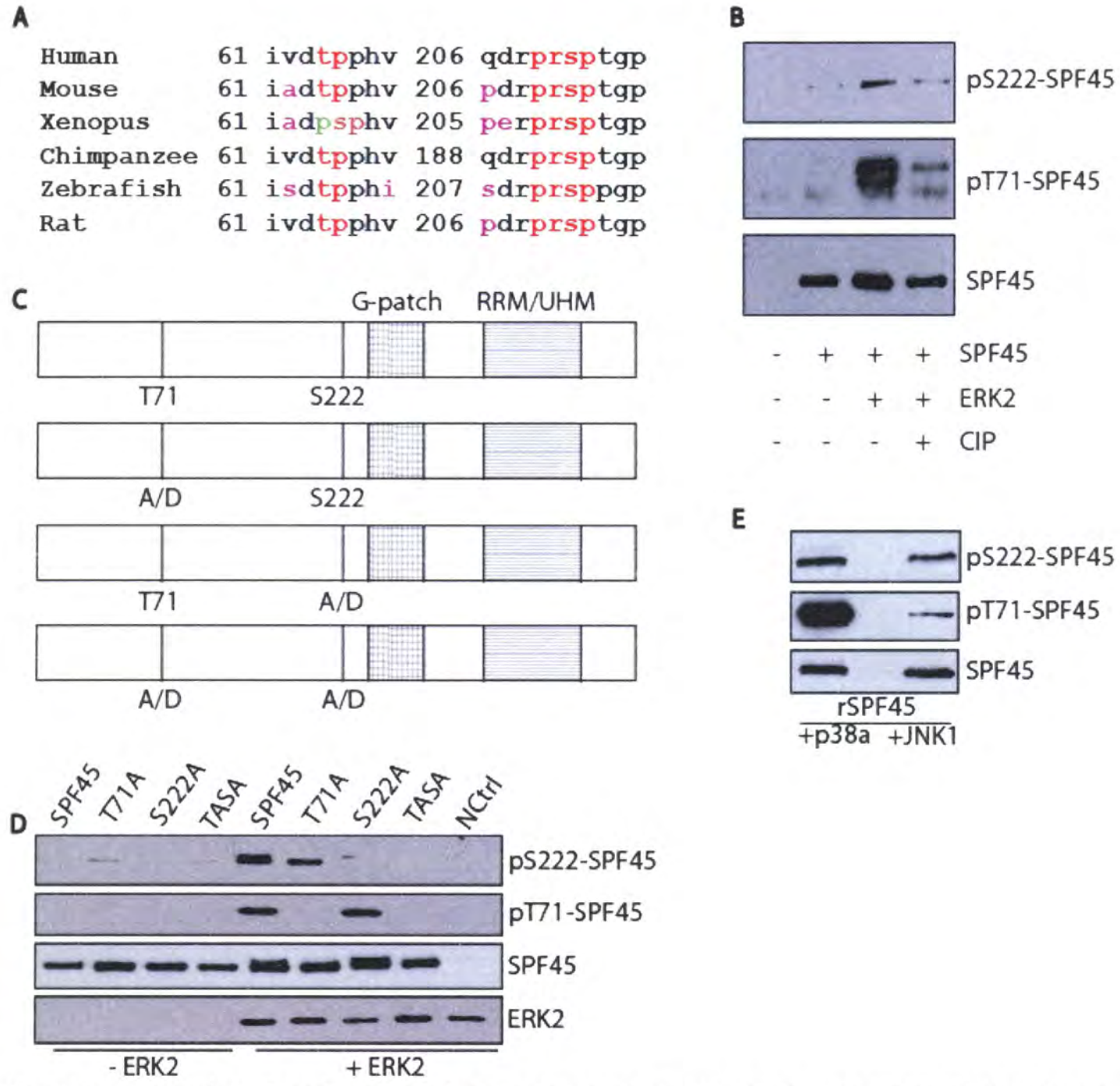

Fig 2.2 SPF45 is phosphorylated on Thr71 and Ser222 *A.* SPF45 has two potential MAP kinase phosphorylation sites well conserved among mammalian species. *B.* His-SPF45 is phosphorylated *in vitro* with ERK2 followed by CIP treatment. pSer222- & pThr71-specific antibodies show SPF45 phosphorylation that is diminished after phosphatase treatment (last lane to the right.) *C.* Cartoon depicting the different combinations of alanine and aspartate mutants of SPF45. *D.* Total-SPF45 antibody detects SPF45 *before* and *after* ERK2 phosphorylation. pThr71- and pSer222-specific antibodies detect SPF45 *after* ERK2 phosphorylation but not the corresponding alanine mutants. *E.* His-SPF45 is phosphorylated *in vitro* by p38 and JNK MAP kinases on both Thr71 and Ser222. *Experiments performed with Scott Eblen.*

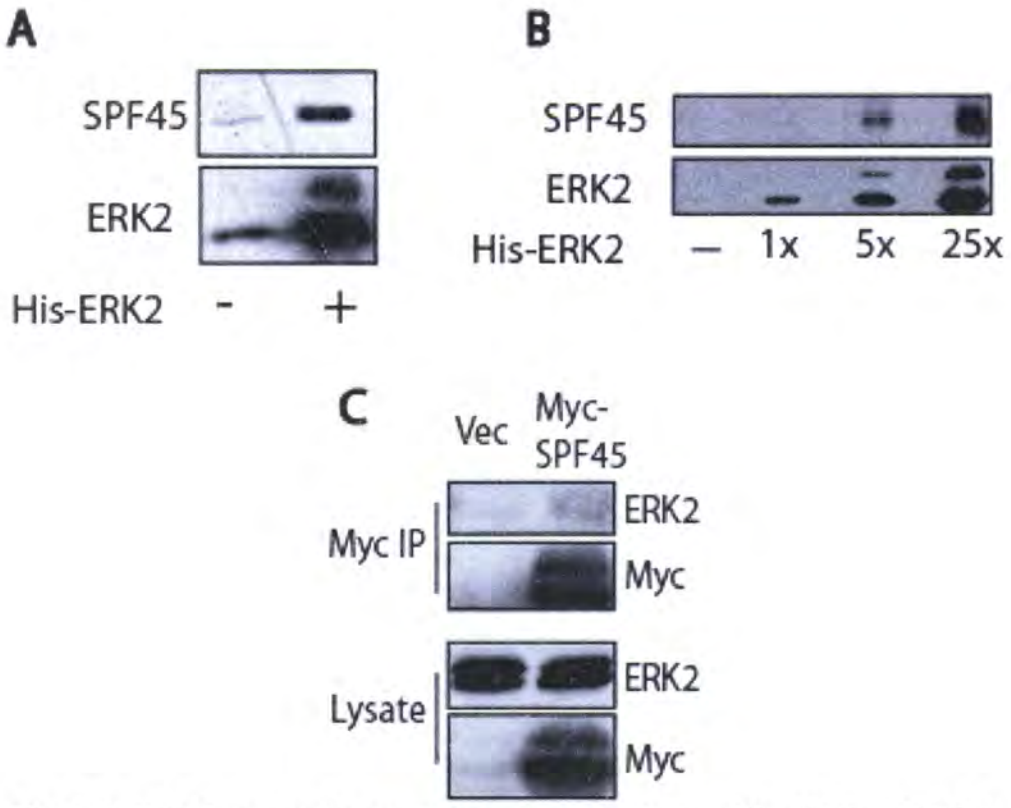

Fig 2.3 ERK2 associates with SPF45 *A.* Purified His-ERK2 is mixed overnight with HeLa cell nuclear extracts. His-ERK2 is pulled down with nickel agarose, washed, run on a gel and transferred to nitrocellulose. The membrane is immunoblotted with anti-SPF45 and anti-ERK antibodies. *B.* Increasing amounts of His-ERK2 are mixed overnight with HeLa cell nuclear extracts and processed as in *A*. *C.* ERK2 co-immunoprecipitates with Myc-SPF45 pulled down from SKOV3-pQCSPF45 with protein A agarose conjugated to Myc antibody following 2 hr incubation at 4°C. IP is processed as in *A*. *Experiments performed with Scott Eblen.*

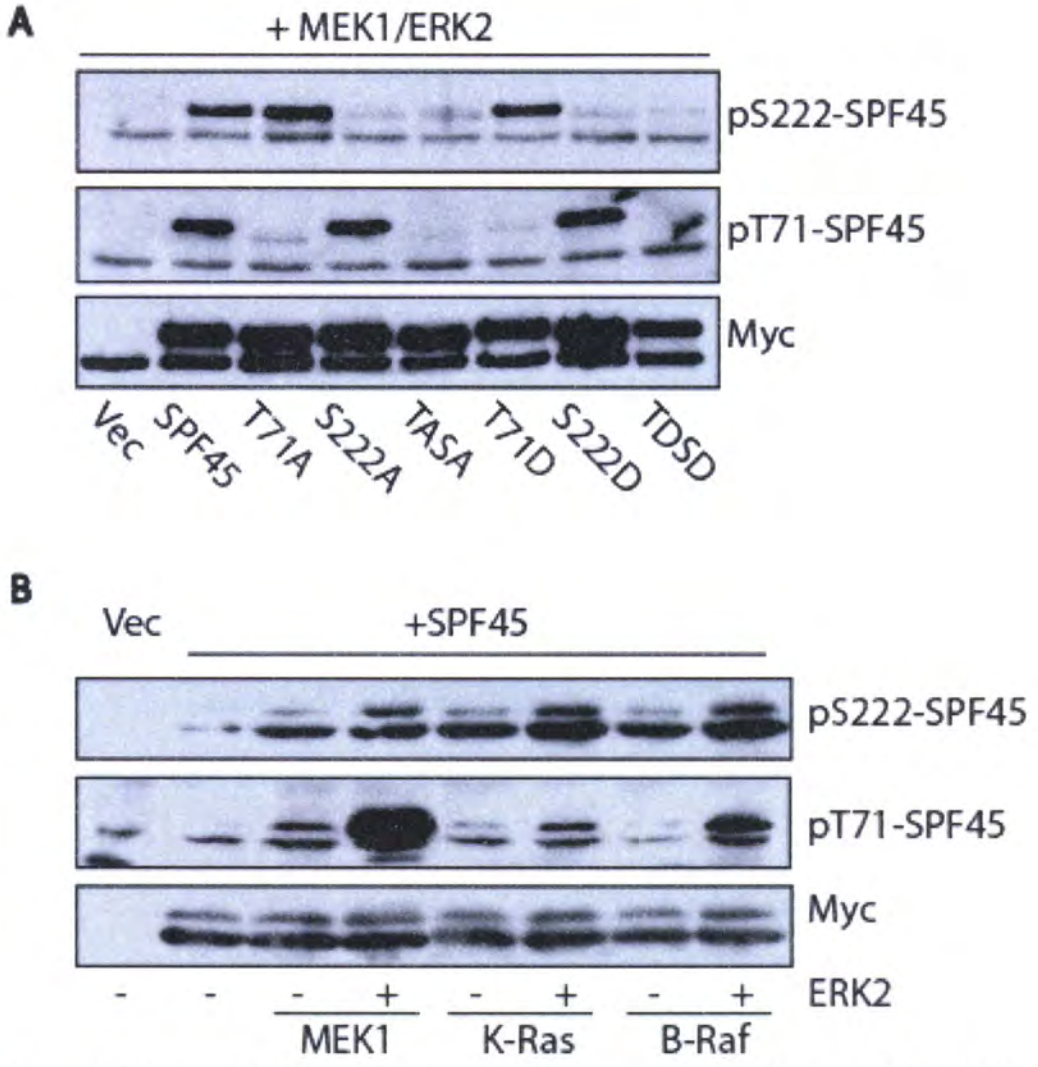

Fig 2.4 Phosphorylation of SPF45/mutants in Cos-1 cells by ERK2 MAP kinase *A.* Myc-SPF45/mutants are transfected into Cos-1 cells with ERK2 and CA-MEK1(DD). Anti-Myc immunoprecipitates are immunoblotted with anti-phospho-SPF45 and anti-Myc antibodies. *B.* Myc-SPF45 is transfected into Cos-1 cells with mutationally-activated MEK1, B-Raf or H-Ras -/+ ERK2. Anti-Myc immunoprecipitates are immunoblotted with anti-phospho-SPF45 and anti-Myc antibodies. *Experiments performed with Scott Eblen.*

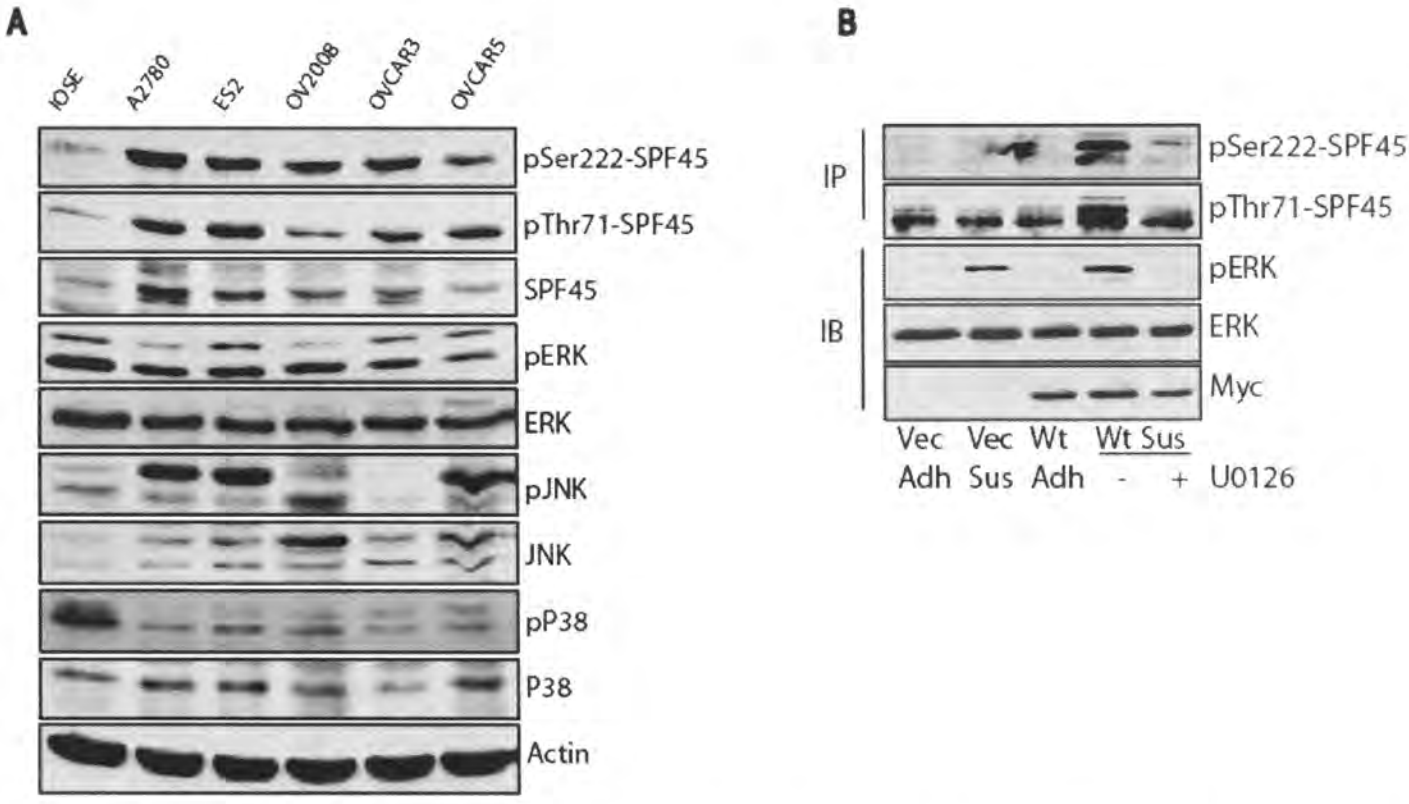

Fig 2.5 Endogenous SPF45 is primarily phosphorylated by ERK and JNK in cells *A.* Cell lysates are prepared from ovarian benign immortalized and cancer cell lines. The lysates are run on a gel and endogenous proteins immunoblotted as indicated in the methods. *B.* SKOV-3 cells are grown adherent or trypsinized and put in cell suspension for 24 hr. Cell lysates are run on a gel and immunoblotted for endogenous SPF45 with anti-SPF45 and anti-phospho-SPF45 antibodies, along with actin as a loading control. *C, D & E.* A2780 (*C*), SKOV3 (*D*) and ES2 (*E*) cells are serum-deprived overnight and then treated with serum, PMA, H_2O_2, EGF, HGF, sorbitol, anisomycin or UV light for 15 min or 1 hr. Cell lysates are prepared, run on a gel and endogenous proteins immunoblotted with the indicated antibodies. *F & G.* A2780 cells are put in serum free media (SF) overnight and then treated with either DMSO (DM), the MEK1 inhibitor U0126 (UO), the p38 inhibitor SB20358 or the JNK inhibitor SP600025 (SP) for 30 min prior to stimulation with PMA, H2O2 or UV for 15 min. The cells are lysed and equal amounts of protein run on a gel, transferred to nitrocellulose and immunoblotted with the indicated antibodies. *Experiments performed with Hui Zheng and Yuying Liu.*

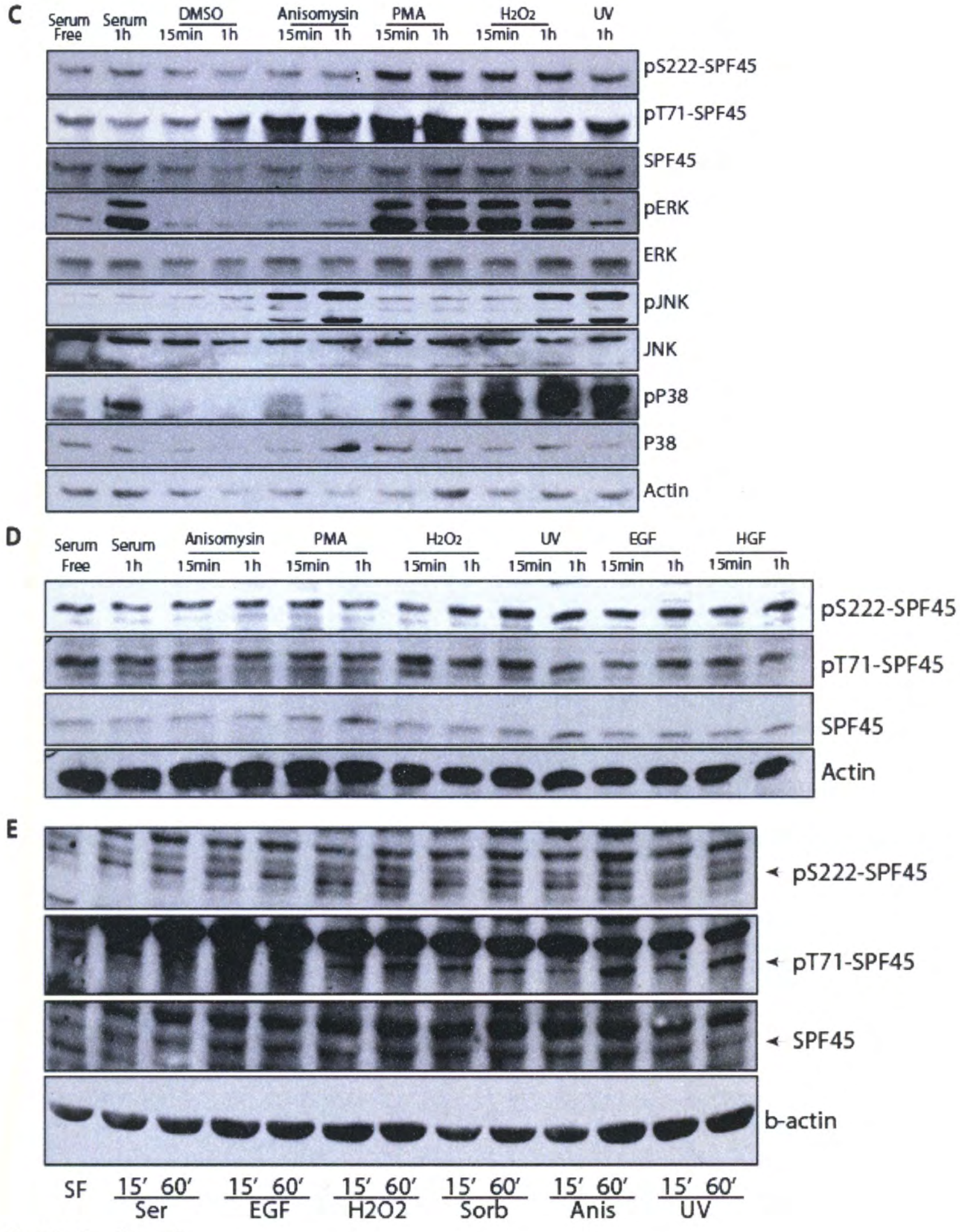

Fig 2.5 *Continued.*

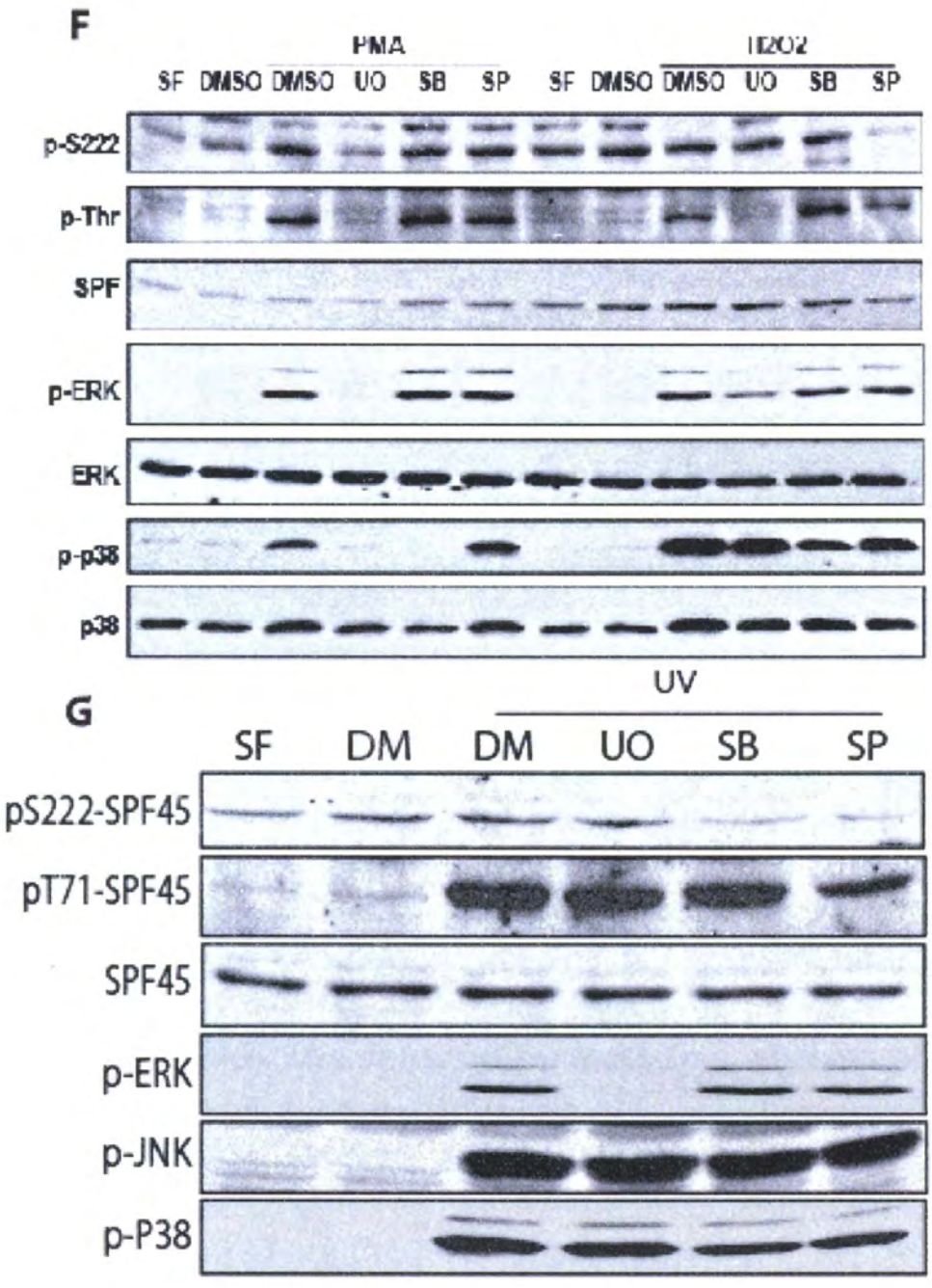

Fig 2.5 ***Continued.***

Discussion

Identification of MAP kinase substrates in their tissue-specific expressions as well as their spatio-temporal regulation is an important task in order to dissect the exact roles the different MAP kinases play in controlling cellular functions. Using the approach developed by Shokat (Shah 1997) and modified for ERK2 by our group (Eblen, 2003), we report the detection of SPF45, a pre-mRNA alternative splicing factor, as a novel substrate of ERK2 in SKOV3 cells, an ovarian cancer cell line that we previously showed to strongly activate ERK2 pathway under anchorage-independent conditions (Al-Ayoubi 2008). Radio-labeling of ERK2-associated substrates with a ^{32}P isotope under anchorage-dependent and –independent conditions revealed a phosphorylated band at 47Kda corresponding to SPF45, which was detected by mass spec analysis of a silver-stained equivalent band. We further showed, using phospho-amino acid and mutational analyses, that Thr71 and Ser222 are the MAP kinase-target residues. We also confirmed that SPF45 is similarly phosphorylated by JNK and p38 MAP kinases, in vitro, on Thr71 and Ser222. We generated polyclonal and phospho-specific antibodies against the total protein as well the phosphorylated epitopes, and used them to determine the phosphorylation of transfected SPF45 in Cos-1 cells in response to co-expressing activators of the ERK2/MAP kinase pathway which had the highest response upon co-expression of activated MEK1 and ERK2, simulating a continuous upstream signaling to ERK2. We also explored the phosphorylation of endogenous SPF45 in several ovarian cancer cell lines in response to stimulation with known activators of the different MAP kinase pathways. In A2780, ES2 and SKOV3 cells, SPF45 phosphorylation was mainly

induced following ERK (serum, PMA, EGF and HGF) and JNK (anisomycin and UV irradiation) activation but weakly in response to p38 (sorbitol and H_2O_2). While p38 MAP kinase strongly phosphorylated SPF45 in vitro, the response is, at best, modest in these cells in response to the stimuli tested. Additionally, using chemical inhibitors of the different kinase pathways supported our observation that ERK and JNK were more involved in SPF45 phosphorylation, at least in response to the stimuli we used, even stimulation by H_2O_2 – a strong p38 activator – was rather mediated by ERK. While it does not rule out the possibility that p38 MAPK has the potential of regulating SPF45 phosphorylation (such as Ser222 phosphorylation in response to UV light), it clearly indicated the specificity of the different kinases in controlling SPF45 functions. In fact, both Thr71 and Ser222 were phosphorylated upon ERK activation while JNK had a more pronounced effect on the phosphorylation of Thr71 compared to Ser222. Taken together, we predict that the phosphorylation pattern of SPF45 coupled with the spatio-temporal activation of the responsible kinase to dictate the function of the protein in a stimulus-dependent fashion.

SPF45 is a highly conserved protein among vertebrates, exceeding 80% homology. Interestingly, peptide sequences surrounding Thr71 and Ser222 are conserved as well, suggesting an old and preserved role of the phosphorylation in regulating the functions of SPF45. In Xenopus, the homolog of Thr71 is however replaced by a serine followed by a proline, which actually retains the ability to be phosphorylated by MAP kinases and therefore potentially preserves its function. As Thr71 lies in the N-terminal domain whose structure has not been resolved yet, it is hard

to speculate on the role that the phosphorylation may impart on the protein. Ser222 lies in the SPF45-like (SF) motif, in the vicinity of the G-patch domain. It is unclear what a phosphorylation in the vicinity of a G-patch motif or within the SF-domain will do to the protein's functions as there are no accounts linking these domains and phosphorylation. The SF motif is involved in protein oligomerization (although no oligomerization of SPF45 has been described yet) and the G-patch is linked to protein and ribonucleic acid interactions. Accordingly, we suspect that the phosphorylation on Ser222 to play an important role, possibly more so than Thr71, in modulating SPF45's interactions within the spliceosome, as well as any other protein complex it can be involved with. Previous studies linked SPF45 to DNA repair pathways, multidrug resistance, apoptosis regulation, spinocerebellar ataxia as well as β-thalassemia. As diverse as these functions may be, determining SPF45 targets and the effect that the phosphorylation plays is needed to better understand how it can be mediating some or all of these effects. Similar to the MAP kinase-targeted splicing factor Sam68, these functions can be mediated by the impact of more than one kinase, while the same phosphorylation may have different outcomes based on several factors - including but not limited to - the cellular distribution of SPF45 and its interaction with spliceosomal protein complexes. In fact, regulation of splicing factors by MAP kinase pathways is a rare event. Evidence of involvement of signaling pathways in regulating splicing factors are accruing; however, Sam68 remains the only splicing factor which is known to be under direct regulation of ERK/MAP kinase pathway to date (Matter 2002). Forced expression of Sam68 leads to an increase in inclusion of CD44 variable exon v5 in an ERK-dependent fashion. Fyn, a

src-tyrosine kinase, also phosphorylates Sam68, causing an increase in the pro-apoptotic Bcl-x(s) isoform (Paronetto 2007), but it does not have any effect on CD44 v5 inclusion, indicating the specificity of the different phosphorylations in modulating the splicing activity. HnRNP A1, a general repressor of splicing, is phosphorylated by Mnk1 in response to $MKK_{3/6}$/p38 activation following osmotic response, inducing its detainment in the cytosol lifting the ban on splicing (Guil 2006). Although not directly phosphorylated by p38, this is another example of the implication of MAP kinases in splicing, a function that is mainly performed by splicing factors specific kinases, such as SRPKs, CLKs/STY and Topo1. In a similar analogy, SPF45 phosphorylation in a MAP kinase-dependent manner is expected to be involved in the regulation of many downstream targets, the identification of which and determining the role of the splicing in modulating their expression patterns are crucial to understand the effect that SPF45 induces in the biology of the cells in general and cancer cells more specifically.

Incessant and aberrant proliferations as well as altered genomic profiles are among the hallmarks of cancer cells (Hanahan 2000). An interesting question that arises in this context is the meaning of increase in splicing factors levels and activities. For example, is SPF45 over-expression in ovarian cancer cells a cellular necessity imposed by the new status of the cells or is it a mere bystander that evolved in the middle of the genomic chaos of cancer? The fact that the levels of SPF45 are increased in many types of cancer compared to their normal counterparts (Sampath 2003), as well as our observation that - although not consistent in all tested cell lines -, there is an obvious increase in SPF45 expression in ovarian cancer cells compared to the benign

immortalized IOSE, suggests that SPF45 is a necessary component of what one might call "cancerome". Additionally, the different potential combinations of splicing factors which in part depend on the balance of their expression levels can possibly outweigh the number of transcription factors; as such, an increase in a specific splicing factor such as SPF45 must have its tremendous effects on the cellular biology. Hence, identifying the downstream targets of SPF45, as well as SPF45 interacting partners and their post-translational modifications, becomes necessary to determine its role and to develop successful chemotherapeutic strategies. In the next chapter, we discuss the effect of SPF45 phosphorylation on its splicing activity, identify genetic changes induced by SPF45 overexpression, novel SPF45-splicing targets and address their impact on ovarian cancer biology.

Chapter 3: Regulation of SPF45 splicing activity by MAP kinase phosphorylation. Role of Thr71 and Ser222 in modulating SPF45 functions.

Introduction

In analogy with electronic circuits, post-translational modifications serve as molecular switches that regulate proteins' functions, turning them "on/off" or even changing their function radically. In that sense, protein phosphorylation is regarded as one of the main regulatory molecular switches, and splicing factors are no exception. Phosphorylation of SR proteins and hnRNPs has been extensively studied and shown to regulate the formation of the mRNP-spliceosome complex -the major structure that determines the outcome of the splicing reaction. It also controls the spatial localization of the splicing factors in the nucleus and the cytosol (Stamm 2008). Phosphorylation of SR proteins is central for homophilic and heterophilic interactions; for example, phosphorylation of SR proteins is important in determining the outcome of alternative splicing. Tripathi et al. show that a long nuclear noncoding RNA, MALAT-1, regulates alternative splicing through modulating the phosphorylation of SR proteins, influencing their subnuclear localization (Tripathi 2010). Phosphorylation of SF2/ASF splicing factor has a wide array of effects on its interactions: no effect is seen on binding to $U2AF^{35}$, but it positively reinforces the interactions with U1-70K (Xiao 1997) while it negatively affects its interactions with TAP/NXF1, SRp40, hTra2 and itself (Xiao 1997, 1998). PI3K/Akt, CLKs and Topo I phosphorylation of SRp75, SRp55 and SF2/ASF enhance human tissue factor (HTF) mRNA splicing (Eisenreich 2009 a/b). Phosphorylation of SRp38 on the other hand, changes its function from a repressor to activator of splicing (Feng 2008).

Another class of alternative splicing factors, hnRNPs, commonly identified as repressors of splicing, can also be under the control of phosphorylation. SF1, a member of the hnRNP K family, is phosphorylated by PGK-I which inhibits its interactions with $U2AF^{65}$ preventing spliceosome assembly (Wang 1999). Casein kinase II-type activity is responsible for hnRNP C phosphorylation, affecting the RNA binding capacity (Fung 1997). Fas-activated Ser/Thr kinase (FAST) phosphorylates the hnRNP protein TIA-1, strengthening its interactions in the spliceosome and enhancing its pro-apoptotic effects (Tian 1995). Phosphorylation of hnRNP A1 by Mnk1 in a p38 MAP kinase-dependent manner in response to osmotic stress causes its localization to the cytoplasm (van der Houven van Oordt 2000), and induces senescence in normal human fibroblast cells (Shimada 2009). Changes in localization are also observed with SR protein phosphorylation, such as the changes in associations between SR1 and importin and transportin families (Kataoka 1999, Lai 2000, 2001).

The mechanism by which SPF45 concerts its effects is still under investigation. Although initially found to be recruited by the polypyrimidine-tract binding protein (PTB) to the cryptic 3' AG-dinucleotide of the *Drosophila* Sxl pre-mRNA (Lallena 2002), SPF45 exerts more complicated interactions in the splicing of Fas exon 6. Since neither the G-patch nor RRM domains are able to bind a RNA oligonucleotide, SPF45 interactions with the pre-mRNA are expected to occur through binding to other factors within the spliceosome in a UHM-ULM fashion (UHM: U2AF Homology Motif; ULM: UHM Ligand Motif) (Corsini 2007). UHM-ULM interactions are not uncommon among proteins within the spliceosome. Indeed, the SPF45 RRM domain coincidently matches a

UHM domain and interacts with ULMs of the constitutive splicing factors U2AF^{65}, SF1 and SF3b155: isolated SPF45 UHM domain co-immunoprecipitates with GST-tagged isolated ULM domains of the aforementioned factors (Corsini 2007). By the same authors, they show using ULM-selective SPF45 mutants that these interactions are necessary for SPF45 splicing activity in HeLa cells, while mutations that do not disrupt the interaction perform equally to the wild type in splicing. Interestingly, they suggest that phosphorylation events on SF1 and SF3b155 to be important in regulating a potential sequential mode of binding and interactions.

Our interest in SPF45 stems from its involvement in cancer, and ovarian cancer in particular, where it was shown to confer a multi-drug resistance phenotype in an ovarian cancer cell line (Perry 2005). Additionally, it is implicated in DNA repair (Chaouki 2006) and alteration of splicing (Lallena 2002, Corsini 2007), yet very little is known about the mechanism(s) of its actions. Although some splicing factors, such as NOVA, work in a tissue specific manner (Buckanovich 1993), the majority of these proteins have universal tissue-independent functions. The balance of the splicing factors expressed in cells dictates the general pattern of genetic and, consequently, proteomic events (Mayeda 1993). Accordingly, identifying the targets of SPF45 in ovarian cancer cells is a crucial step in unveiling its involvement in the pathobiology of the disease. In this chapter, we determine the effect of phosphorylation of SPF45 by ERK2 and p38MAP Kinase on Thr71 and Ser222 on its splicing function using a minigene reporter construct. We also generate stable SKOV3 cell lines expressing ectopic wild type SPF45 or mutants (SPF45^{AA} and SPF45^{DD}) and investigate their drug resistance profile. Using the stable

cells, we perform a gene and exon-array to identify novel downstream genetic and splicing targets of SPF45 that are dysregulated by SPF45 expression and assess the impact of the mutation on Thr71 and Ser222 on these targets. Molecular changes associated with these genetic changes are investigated as well. Finally, we use the stable cells to determine the role of the phosphorylation sites in SPF45 binding to constitutive splicing factors, in an effort to understand the mechanism of regulating the interactions which are believed to dictate SPF45 outcomes.

Materials and Methods

Plasmids and minigene system

Genomic DNA was isolated from IOSE cells by phenol/chloroform extraction followed by ethanol precipitation. Fas genomic DNA spanning exon 5 through 44 base pairs into exon 7 were amplified by PCR using the following primers: Fas5_primer AGATGTGAACATGGAATCATCAAGGA & Fas3_primer TTTCCTTTCTGTGCTTTCTGCATGTT. The amplicon was subcloned into Topo 2.1 vector (Invitrogen Carlsbad, CA) and the following primers: FasInRe5_primer GTAAGAATGAGGCAAATCTTTGTGA-3' and FasInRe3-_primer 5'-CCTTCTTATATTTCTCTTAGTGTGAAAGTA-3' were used to amplify the whole plasmid leaving out 1105 bp spanning nucleotides 53 through 1183 from the 5'-end of intron 6 and the resulting amplicon was incubated with T4 DNA ligase (New England BioLabs Ipswitch, MA) overnight at 16°C for blunt ligation. Fas DNA was cut using EcoRI (New England BioLabs Ipswitch, MA) and ligated into the multi-cloning sequence of pCDNA3 downstream of a CMV promoter to generate the Δ*fas* minigene.

Generation of SKOV3 stable cell lines

Retroviral plasmids (pQCXIP) encoding empty vector/SPF45/SPF45^{AA}/SPF45^{DD} were transfected into 293T cells along with pAMPHO, VSV-G and Gag/Pol for virus production and packaging. Cellular media containing the viruses were harvested at 36 and 60 hours post transfection, filtered with 45 μm syringe filters (Corning Lowell, MA) and mixed with polybrene (Sigma St Louis, MO) to a final 2μg/ml concentration before infecting the SKOV3 target cells. Cellular populations expressing the genes of interest were pooled

and selected with 1.5 µg/ml puromycin for 2 weeks. Following the 2 weeks period, puromycin is added at half strength as a maintenance dose.

SPF45 binding assays

SKOV3-Vec/-SPF45/-SPF45^{AA}/-SPF45^{DD} cells were grown to confluence in a 10 cm cell culture dish (Corning Lowell, MA) at 37°C, 5% CO2 and harvested with M2 lysis buffer (150 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 50 mM HEPES, pH 7.4) on ice. Homogenized lysates were incubated on ice with occasional vortexing followed by centrifugation for 15 min at 15000 rpm 4°C to separate the debris from the soluble phase. Standard BCA (Pierce Rockford, IL) assay was used to quantify protein concentrations. 1000 µg of protein lysates were incubated with protein agarose beads pre-conjugated to Myc antibody for 24 hours at 4°C. The beads were spun down quickly and washed three times with gentle vortexing three times using M2 buffer before adding 1X Laemmli sample buffer and boiling for 3 minutes. The proteins were separated on a 10% SDS-PAGE gel (Biorad Hercules, CA) and transferred onto nitrocellulose membrane.

Δfas alternative splicing assay

A- Transfections

2.5x10^5 Cos-1 cells were plated the night before transfection on 6-well plates (Corning Lowell, MA) and transfected the following day with 1.2 µg total of DNA. Briefly, 1.2 µg total DNA and 4.8 µl of Lipofectamine 2000 (Invitrogen Carlsbad, CA) were suspended in 0.25 ml of Optimem (Invitrogen, Carlsbad) each for 5 minutes at RT, then mixed together for a total of 20 minutes at RT before drop wise addition onto cells. The media

and transfection mixture were recollected 5~6 hours following transfection, washed once in PBS and incubated in fresh media supplied with 10% FBS. The cells were harvested in TRIzol (Invitrogen Carlsbad, CA) 24 hours post-transfection.

B- RNA isolation

RNA was isolated from cells by phase separation using TRIzol (Invitrogen Carlsbad, CA) followed by chloroform (Sigma St Louis, MO) extraction and isopropanol (Sigma, St Louis) precipitation. The RNA was washed twice in 75% ethanol and re-suspended in 25 µl of DEPC (Fisher Pittsburgh, PA)-treated water. Total RNA purity and concentration were assessed by UV-spectrophotometry.

C- RT-PCR and gel-electrophoresis

2.0 µg of total isolated RNA were reverse-transcribed in a 20.0 µl reaction using the High Capacity cDNA Reverse Transcription Kit (Applied Biosyst Carlsbad, MA) according to the manufacturer's protocol. Cycling parameters were the following: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min then hold at 4°C. Randomly-primed cDNA was used for subsequent amplification. 1.0 µl cDNA was mixed with 200nM of each of the forward and reverse primers and 23.5 µl PCR supermix (Invitrogen Carlsbad, CA). Cycling parameters were the following: 94°C for 5 min, followed by 22 cycles of 94°C, 58°C and 72°C for 30 sec each, followed by 10 min extension at 72°C then hold at 4°C. PCR products were separated and quantified on an ethidium bromide-stained 2% agarose gel.

Real time qRT-PCR

Total cellular RNA for use in quantitative RT-PCR assay was extracted from confluent SKOV3 cells stably expressing empty vector/SPF45/SPF45^{AA}/SPF45^{DD} using TRIzol (Invitrogen Carslbad, CA) lysis followed by chloroform/isopropanol extraction and precipitation and ethanol washes. RNA samples purity was assessed by an A_{260}/A_{280} ratio between 1.9 and 2.1 and total RNA was quantified by UV-spectrophotometry by absorbance at 260nm wavelength. Total RNA (2.0 µg) was transcribed to random-primed cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosyst Carlsbad, CA) according to manufacturer's protocol in a 20.0 µl reaction. The resulting cDNA was subjected to real-time PCR using RT^{2}SYBR Green dye (Qiagen Valencia, CA) and pre-validated primers for the following genes: erbB2, FN1, TGF-β and periostin (Qiagen Valencia, CA) while 18s rRNA (Qiagen Valencia, CA) was used as an internal control. 1.0 µl of the first-strand cDNA product was mixed with 12.5 µl of RT^{2}SYBR Green qPCR Master Mix (Qiagen Valencia, CA), 10.5 µl of H_2O and 1.0 µl of gene-specific 10uM PCR primer pair stock in a 25.0 µl reaction. Each sample was run in duplicate and a total of 3 independent repeats were performed. Cycling parameters were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C and 60°C for 15 and 60 seconds, respectively. A single band for each of the PCR products was observed on an ethidium bromide-stained 2% agarose gel electrophoresis assessing products integrity. Threshold cycle (C_T) values were determined for each gene and the corresponding 18srRNA. Fold change in gene expression was calculated by the $\Delta\Delta C_T$ method.

Cell proliferation rate

SKOV3 stable cell lines ($1x10^5$) were plated on 6 well plates on day 1 and counted at 24 hr intervals. Briefly, the cells were gently washed with PBS, trypsinized in 100 µl, and collected with 100 µl McCoy's supplemented with 50% FBS. Cells were homogenously suspended and manually counted using a hemocytometer.

Cellular adhesion assay

96-well tissue culture dishes were coated with fibronectin (20 µg/ml), laminin-1 (10 µg/ml), poly-L-Lysine (10 µg/ml) or nothing overnight at 4°C. Plates were washed twice with washing buffer (0.1% BSA in PBS) and blocked with blocking buffer (0.5% BSA in PBS) at 37°C for 60 min, followed by washing buffer and chilled on ice. Stable SKOV3 cells were suspended at $4x10^5$ cells/ml and 50 µl of the suspension were aliquoted into the wells. Plates were incubated at 37°C, 5% CO2 for 30 min, then placed on a plate shaker at 2000 rpm for 15 sec. Unattached cells were removed by aspiration and the wells were gently washed with washing buffer, stained with crystal violet (5 mg/l in 2% ethanol) for 10 min, washed gently three times and dried at room temperature. Crystal violet stain was solubilized in 2% SDS and plates were read at 550 nm.

MTS cytotoxicity/proliferation assay

SKOV3-pQCSPF45 or control were seeded on 96 well-plates ($3.0x10^3$ cells/100µl/well) and treated the following day with increasing concentrations of the following chemotherapeutic agents: cisplatin, doxorubicin, etoposide, mitoxantrone, paclitaxel and vincristine (Sigma, St Louis). 72 hours later, cell viability/proliferation was measured in a colorimetric assay using the tetrazolium salt 3-(4,5-dimethylthiazole-2-yl)-2,5-

diphenyltetrazolium bromide MTS kit (Promega Madison, WI) as described by manufacturer and analyzed on a bioard 96-well plate spectrophotometer. All assays were performed in triplicate with three independent experiments.

Immunoblotting

Cells were lysed and sonicated in M2 buffer with 0.5% SDS and protease inhibitors mix (100 μM Na-*o*-vanadate, 50 mM NaF, 40 mM β-glycerophosphate, 5 mM Na-pyrophosphate, Aprotinin and 2 mM PMSF). Lysates were normalized using BCA protein assay kit (Pierce Rockford, IL). Each sample (100 μg) was run on a 10% polyacrylamide gel (SDS-PAGE) under denaturing conditions and transferred to a Protran nitrocellulose membrane (Whatman-GE Healthcare, Piscataway, NJ). After blocking with 5% milk for 1 hour at room temperature, the membranes were incubated with relevant primary antibodies (Myc, erbB2, SF1, SF3b155 and Actin) in PBS with 5% BSA and 0.1% Tween-20, 0.1% sodium azide at 4°C overnight. The blots were washed three times in 1X PBS + 0.1% Tween-20 and incubated with the appropriate secondary HRP-conjugated antibody (Mouse, Rabbit (Biorad Hercules, CA)) in 1X PBS-0.1%T with 1% milk for 1 hour at room temperature. The membranes were then washed 6 times with PBS-0.1%T for a total of 1 hour at room temperature. The membranes were incubated with femto-ECL (Pierce Rockford, IL) for 2 minutes at room temperature and the bands were exposed on Hyblot CL films (Denville Scientific South Plainfield, NJ).

Immunofluorescence

$7.5x10^4$ SKOV-3 stable cells were plated on glass cover slips pre-coated with fibronectin (10ug/ml) and incubated at 37°C, 5% CO_2. 24 hours later, the cells were washed 1X with

PBS and fixed with 4% formaldehyde for 30 min at room temp. The cells were then permeabilized with ice cold 1% Triton-X on ice for 5 min followed by blocking with 5% BSA (Fisher Pittsburgh, PA) for 30 min at room temp. The glass cover slips were incubated with Myc-antibody (1:1000 dilution) for 1 hour at room temperature in the dark, followed by another hour of incubation with secondary anti-rabbit antibody conjugated to FITC. The cover slips were washed in 1X PBS then ddH_2O and mounted on microscope glass slides. Nuclei were stained with DAPI. Slides were visualized using epi-fluorescence on a Nikon microscope.

Gene- and exon-array analysis

Total RNA was amplified using the NuGEN™ Applause™ WT-Amp Plus ST Amplification System. First-strand synthesis of cDNA was performed using a unique first-strand DNA/RNA chimeric primer mix, resulting in cDNA/mRNA hybrid molecules. Following fragmentation of the mRNA component of the cDNA/mRNA molecules, second-strand synthesis was performed and double-stranded cDNA was formed with a unique DNA/RNA heteroduplex at one end. In the final amplification step, RNA within the heteroduplex was degraded using RNaseH, and replication of the resultant single-stranded cDNA was achieved through DNA/RNA chimeric primer binding and DNA polymerase enzymatic activity. A modification step targeting the amplified single-stranded cDNA resulted in the generation of sense-strand cDNA product compatible with the Affymetrix GeneChip® Exon arrays. The ST-cDNA was purified for accurate quantitation of the cDNA and to ensure optimal performance during the fragmentation and labeling process. The ST- cDNA was assessed using spectrophotometric methods in

combination with the Agilent Bioanalyzer. The appropriate amount of amplified ST-cDNA was fragmented and labeled using the Encore™ cDNA Biotin Module. The enzymatically and chemically fragmented product (50-100 nt) was labeled via the attachment of biotinylated nucleotides onto the 3'-end of the fragmented ST-cDNA. The resultant fragmented and labelled ST-cDNA was added to the hybridization cocktail in accordance with the NuGEN™ guidelines for hybridization onto Affymetrix GeneChip® arrays. Following the hybridization for 16-18 hours at 45°C in an Affymetrix GeneChip® Hybridization Oven 640, the array was washed and stained on the GeneChip® Fluidics Station 450 using the appropriate fluidics script, before being inserted into the Affymetrix autoloader carousel and scanned using the GeneChip® Scanner 3000.

Statistical Analysis

Statistical analysis was performed at $p < 0.05$ level of significance and determined using one-way ANOVA with Tukey's post-hoc test.

Results

SPF45 causes increase in Δfas splicing in a dose-dependent manner

Regulation of alternative splicing of Fas cassette exon 6 is influenced by the increase in SPF45 expression based on a screen of SPF45 splicing targets in HeLa cells (Corsini 2007). Although the splicing activity has been shown to be confined to its C-terminal RNA recognition motif (RRM) (Corsini 2007), it remains necessary to identify the factors that influence SPF45-alternative splicing function. The *in vivo* model of alternative splicing using a Fas minigene represents an ideal approach to study the effect of *trans* factors on regulation of splicing, as it preserves the cellular splicing machinery in its optimal milieu. Genomic *fas* DNA purified from IOSE cells was used to create a minigene labeled Δ*fas* (Fig 3.1 *A*) (Cascino 1995), spanning exon 5 through 44 base pairs of exon 7 and including introns 5 and 6 with a 1105 bp deletion in intron 6, previously shown not to hinder the ability of Fas to undergo alternative splicing (Cascino 1997). To determine the appropriate dose of SPF45 to be transfected into the cells along with Δ*fas*, increments of SPF45 DNA were transfected into Cos-1 cells with a fixed Δ*fas* amount (0.3 μg): 0.4, 0.8, 1.2 and 1.6 μg of SPF45 DNA were co-transfected with Δ*fas*; the total DNA mix was kept at a constant amount of 2.0 μg by adding the appropriate amount of empty pCMV3-2c vector (Fig 3.1 *B*). RT-PCR analysis of the resulting splicing reaction showed a dose-dependent increase in exon 6 exclusion, reaching around 50% between 0.4 and 0.8 μg of transfected SPF45. Accordingly, we considered 0.6 μg of SPF45 as the optimum dose in our splicing model to study the influence of phosphorylation of SPF45 on its alternative splicing function.

ERK phosphorylation of SPF45 reduces its alternative splicing activity towards Δfas

Post-translational modifications (PTMs) of proteins play an important role in determining their functional outcomes and phosphorylation is one of the most studied and investigated PTMs on proteins, as it mediates the effect of the majority of kinase signaling cascades. Phosphorylation of spliceosomal proteins and their associated proteins has been extensively studied and remains mostly carried out by spliceosomal protein-specific kinases such as SRPKs, CLKs and topoisomerase I. Besides Sam68, no other splicing factor protein has been identified as a direct target for phosphorylation by a MAP kinase. As we showed in the previous Aim, SPF45 is a target for phosphorylation by ERK2/MAP kinase as well as JNK and p38 MAP kinases. We also showed that co-expressing constitutively active MEK1, CA-MEK1-(DD) and ERK2 along with SPF45 caused phosphorylation of SPF45 on both Thr71 and Ser222 residues. To investigate the role of ERK2/MAP kinase phosphorylation of SPF45 on its splicing activity, we made use of the *in vivo* minigene system described above using the ratio of short/long Δ*fas* isoforms as a measure of splicing function. CA-MEK1-(DD) and ERK2 were transfected into Cos-1 cells along with SPF45 and Δ*fas*, and total RNA was isolated 24 hours post-transfection. We also used the MEK inhibitor U0126 to potentially reverse the role of phosphorylation. RT-PCR analysis of the spliced Δ*fas* showed an average of 40% decrease in the ratio of short/long Δ*fas* isoforms in the presence of constitutive ERK2/MAP kinase pathway activation as compared to vector control (Fig 3.1 *C&D*). This effect was partially reversed using U0126 showing only 17% decrease in splicing. Altogether, this experiment

demonstrated that a continuously maintained phosphorylation of SPF45 is responsible for decreasing its alternative splicing activity (Fig 3.1 *C&D*).

P38MAP Kinase phosphorylation of SPF45 strongly reduces its alternative splicing activity towards Δfas

In a similar attempt to determine the impact of p38MAP kinase phosphorylation on SPF45 splicing function, we transfected Cos-1 with MKK3(E) and p38α MAP kinase along with SPF45 and Δ*fas*, and total RNA was isolated 24 hours post-transfection for analysis. In this assay, we used the p38MAP kinase inhibitor SB203580 to determine the role of p38 inhibition on splicing. RT-PCR analysis of analysis of the spliced Δ*fas* showed a 65% decrease in the ratio of short/long Δ*fas* isoforms compared to SPF45 alone. Inhibition of p38 with SB203580 completely returned SPF45 splicing function to basal level (Fig 3.1 *E&F*). While p38MAP Kinase phosphorylation caused a more prominent effect on SPF45 function, the impact of this finding is still unclear. Experiments performed in our lab have determined stronger binding to and phosphorylation of SPF45 by p38 MAP kinase compared to ERK in co-immunoprecipitation and co-transfection assays to assess SPF45 phosphorylation (data not shown), which can potentially explain the results of the splicing assay.

While we showed in the previous chapter that JNK1 phosphorylates SPF45 *in vitro*, our efforts to replicate the splicing assay with activated MKK4 and JNK1 expression along with SPF45 and Δ*fas* were unsuccessful. JNK1 and MKK4 displayed poor expression in Cos-1 cells upon transfection and were not usable to study the effect of JNK phosphorylation of SPF45 on its splicing function (data not shown.) We believe

that MKK4/JNK1 transfection in Cos-1 cells induces an apoptotic effect. Further experiments are required to determine the role of JNK phosphorylation on SPF45 splicing functions.

Mutation of SPF45 phosphorylation enhances its alternative splicing activity and provides rescue from the decrease induced by MAP Kinase phosphorylation

As continuous phosphorylation of SPF45 caused a decrease in its alternative splicing activity, we speculated that mutation of both Thr71 and Ser222 into either alanine or aspartate would mimic a non-phosphorylated and a continuously phosphorylated state, respectively. Vector control, wild-type SPF45, $SPF45^{AA}$ and $SPF45^{DD}$ were transfected separately into Cos-1 cells along with Δ*fas*; the cells were harvested 24 hours post-transfection to assess the extent of alternative splicing (Fig 3.1 *G&H*). Compared to wild-type SPF45, $SPF45^{AA}$ showed a 16% increase in the ratio of short/long Δ*fas* isoforms ($p<0.05$) while $SPF45^{DD}$ showed a significant 37% increase ($p<0.01$) in its alternative splicing activity. Despite that $SPF45^{AA}$ has a relatively modest increase in splicing activity, it is consistent with our previous finding that continuous phosphorylation of SPF45 causes a decrease in alternative splicing; hence, inhibiting the phosphorylation by mutation to alanine shows the predicted positive effect on splicing. We expected $SPF45^{DD}$ - by virtue of the negative charges on the aspartate residues - to mimic a continuously phosphorylated state of the protein; surprisingly, it displayed a more robust increase in alternative splicing. Unlike the native SPF45 protein, which is subjected to dynamic and continuous upstream phosphorylation by MAP kinases and downstream de-phosphorylation by cellular phosphatases, $SPF45^{DD}$ is locked in a

"pseudo-phosphorylated" state. In fact, it has been proposed that a "balanced" reversible phosphorylation of splicing factors is crucial for their adequate function and is almost entirely disrupted under "hypo-" or "hyper-" phosphorylated states (Cao 1997, Xiao 1998, Dieker 2008, Chen 2009). Based on these experiments, $SPF45^{DD}$ failed to mimic the dynamic phosphorylation/dephosphorylation cycles required by SPF45 to carry its functions properly. Therefore, when we compared the splicing activity of wild-type SPF45 to that of $SPF45^{AA}$ in the presence of CA-MEK1(DD)/ERK2, $SPF45^{AA}$ (Fig 3.1 *I&J*), it showed a 32% increase in activity compared to wild-type SPF45, which is an apparent increase due to the attenuating effect of phosphorylation on the native SPF45 but not $SPF45^{AA}$.

SPF45 slows cellular proliferation in a phosphorylation-dependent manner

To further understand the role of SPF45 overexpression in ovarian cancer cells pathobiology, we generated stable populations of SKOV3 ovarian cancer cells expressing Myc-SPF45/mutants/empty vector. We used the SKOV3 cell lines for the following two reasons: i. SPF45 was identified in a screen of ERK2 associated substrates in SKOV3 cells and ii. SPF45 was expressed at lower endogenous levels in SKOV3 cells compared to the ovarian cancer cell lines examined. Pooled populations of SKOV3 cells expressing native SPF45 or mutants were generated by retroviral infection and puromycin selection to avoid clonal variation. Immunofluorescence microscopy of the different generated stable cell lines showed populations of cells with a quasi-equal expression levels (Fig 3.2 *A*). Western blot using Myc antibody showed close expression levels among the different cell lines (Fig 3.2 *B*). Comparing the levels of exogenous SPF45 to endogenous

levels with a western blot using total-SPF45 antibody revealed a 2:1 ratio of exogenous : endogenous SPF45 (Fig 3.2 *C*). Cell proliferation rates of the stable cells were determined by manual count at 24 hours interval, with an initial number of 10,000 cells plated per well per cell line. SKOV3-pQCXIP displayed an accelerated proliferation rate compared to SKOV3-pQCSPF45 which followed an indolent course. Interestingly, we observed a divergence in the behavior of SPF45 mutants on cellular phenotype in this assay. SKOV3-pQCSPF45^{AA} proliferation was similar to the control group while SKOV3-pQCSPF45^{DD} behaved like SKOV3-SPF45 (Fig 3.2 *D*). Although both SPF45^{AA} and SPF45^{DD} cause an increase in Δ*fas* splicing, the discordance in their effect on cellular proliferation suggests that SPF45^{DD} retains a selective phospho-mimetic phenotype depending on the choice of function involved. We show in a subsequent section of this chapter the molecular events that regulate SPF45-mediated cellular proliferation.

SPF45 overexpression in SKOV3 cells does not increase drug resistance

Since SPF45 overexpression in A2780 ovarian cancer cells and HeLa cervical cancer cells confers a multidrug resistance phenotype, we sought to determine the drug sensitivity/resistance of SPF45 in SKOV3 cells. SKOV3-pQCSPF45 vs SKOV3-pQCXIP against cisplatin, doxorubicin, etoposide, mitoxantrone, paclitaxel and vincristine, and then assess the role of SPF45 phosphorylation in drug resistance, if any. We examined the drug sensitivity/resistance in an MTS colorimetric assay using cellular proliferation in a 96 well plate exposed to stepwise 10-fold increasing doses of chemotherapeutic agents as a measure of survival (Fig 3.3). No difference between SPF45 overexpressing cells and control cells was observed. While these results were un-expected, it is

imperative to realize that developing resistance could be cell-type specific based on the genetic and proteomic makeup. Further experiments investigating the role of SPF45 drug resistance in different cell lines might be required to explain this discrepancy.

Gene- and exon-array analysis

To identify novel SPF45 targets that can explain the implications of this splicing factor in the pathobiology of ovarian cancer, we performed an exon- and gene-array to using the stable cell lines generated above. Changes in gene expressions and exon splicing were compared between SKOV3-pQCXIP and SKOV3-pQCSPF45 on an Affymetrix Human Exon 1.0 ST gene chip. Differential gene expression analysis revealed thousands of transcripts to be altered between SKOV3-pQCXIP and SKOV3-pQCSPF45; we divided the results into two groups based on the stringency (low and high) of the statistical analysis. We identified 524 transcripts in the low stringency group (136 upregulated, 388 downregulated SPF45 vs. vector) and 158 transcripts in the high stringency group (39 upregulated, 119 downregulated). We also divided changes in exon expression into low and high stringency groups based on statistical analysis with 396 potential alternative splicing events in the low and 138 in high stringency groups.

Based on intensity signals, fold changes and statistical analysis, we concentrated our efforts in this work on four genes for further validation: ErbB2; FN1; TGF-β; and POSTN. We also explored their role in SPF45-mediated cellular phenotypes and their implication in ovarian cancer. The results are presented in a subsequent section of this chapter. Below we provide a summary of the top genetic and exonic changes we

identified in our analysis and then discuss in further detail the roles of the 4 genes mentioned above.

Clustering of the gene transcripts in the microarray

The low stringency list of differentially-expressed genes provided a richer content in the amount of transcripts included; therefore we used it to determine the functional clustering of these genes into specific pathways. Functional analysis was performed by Almac Diagnostics against a GeneGo annotated Pathway using MetaCore software system from GeneGo, Inc. The most enriched pathways from the list of differentially expressed genes are ranked based on the enrichment p-value, which corresponds to the probability of observing the number of genes in the differentially-expressed genes annotated to a particular pathway by randomly picking genes from the whole array. Furthermore, we divided the list into 2 groups of up- and down-regulated genes in SKOV3-pQCSPF45 compared to SKOV3-pQCXIP.

Up-regulated gene pathways clusters

Gene pathways with upregulated differentially-expressed genes components were grouped and ranked based on the multi-testing corrected - using the false discovery method - enrichment p-value. Twenty two pathways were found to be significant at an adjusted $p<0.05$. Of the 22 pathways, 7 are involved in G-protein coupled receptor signaling while another 7 are involved in immune response. Interestingly, SPF45-overexpressing cells upregulated genes in the AP-1 transcription pathway genetic cluster. Since AP-1 transcription factor is involved in a diverse group of functions such as proliferation, differentiation, migration, wound healing, inflammation

and apoptosis, affecting its regulation could potentially present as a key modulator of several cellular functions. Cytoskeletal remodeling, neuronal migration and cell cycle pathways were also upregulated as well as genes of mucin production associated with cystic fibrosis. Results are summarized in table 3.1.

Down-regulated gene pathways clusters

Similarly, gene pathways with downregulated differentially-expressed gene components were grouped and ranked based on FDR-adjusted $p<0.05$ identifying 20 pathways. We determined 6 pathways to be involved in nucleoside metabolism, 3 cell cycle regulation pathways and 4 pathways of the cystic fibrosis. One apoptosis related pathway was also among the top 20 downregulated clusters pointing out to the potential role of SPF45 in decreased cell death. Interestingly, since CFTR-related genetic pathways appear to be dysregulated both up and down suggests a potentially important implication for SPF45 in regulating cystic fibrosis; however, investigating this disease is beyond the purpose of this work. Oxidative phosphorylation, regulation of translation initiation, alternative complement clusters and antigen presentation by MHC class I were also downregulated. Results are summarized in table 3.2.

Genetic pathways with altered splicing events

Affymetrix Human Exon 1.0 ST gene chip is designed to handle individual exons or parts of exons as independent objects, allowing the detection of alternatively spliced events (inclusion or skipping of exons), and overcoming the limitations of classical expression arrays which concentrate on transcription events at the 3' end of a gene. Exon 1.0 ST employs empirical annotations, curated mRNA sequences and *ab-initio*

prediction computations of 5.4 million probes annotating 1 million exon clusters for detection of alternative splicing events at the exon levels. Despite the advanced technology of the array, it is amenable for a high rate of false positives and the rationale is summarized in the following points: i. alternative splicing is not an all-or-none phenomenon; subtle changes carry the potential of significant biological events ii. alternative splicing is a very common phenomenon, predictably accounting for 90% of genetic events iii. the exon array does not detect specific transcripts, but rather assembles several transcripts (defined by Probe Set Regions-PSR) to associated loci raising the possibility of linking unrelated transcripts and iv. differential gene expression needs to be accounted for in determining differential alternative splicing events. Although the rate of false positive might be substantial, the exon array provides a starting point for the identification of true changes in splicing events; therefore, further validation by physical and bioinformatics analyses is required. In comparing SKOV3-pQCSPF45 to SKOV3-pQCXIP, gene pathways with changes in exon expressions were clustered and ranked based on adjusted $p<0.05$. We identified thirty seven (37) pathways and the top 25 pathways are provided in table 3.3. Of the 25 top pathways, 8 are involved in cell adhesion, 3 in each of cytoskeletal remodeling and immune response, one pathway is involved in regulation of CFTR activity and one in DNA damage nucleotide excision repair. These results predict an association between SPF45 overexpression and cellular adhesion (attachment, spreading and migration) as well as cellular morphology and/or size. The identification of a cluster involving CFTR regulation was not surprising since CFTR-implicated pathways were shown to be dysregulated up

or down in the gene-array. As for DNA damage repair, SPF45 is evolutionarily linked (DRT111: SPF45 homolog in *A. thaliana*) as well as experimentally to DNA repair enzymes such as RAD 201 (Chaouki 2006) and RAD 51B (Horikoshi 2010) of the RAD51 family; additionally, mutations in the C-terminus of SPF45 cause a decrease in DNA repair in drosophila (Chaouki 2006). Hence, it was no surprise to identify DNA repair pathways affected by SPF45 overexpression. Further investigation of the role of SPF45 in these pathways, as well as possible interactions, are necessary to determine the impact of SPF45 in DNA repair, apoptosis and possibly drug resistance.

Validation of target genes expression levels

Based on the results obtained from the gene microarray we performed, we focused on four different genes: ErbB2, FN1, TGF-β and POSTN. The choice of the genes was based on several factors: i. fold change in the array ii. intensity signal iii. statistical analysis and iv. relationship to cancer progression and migration. Using pre-validated gene-specific primers and RT^2SYBR Green chemistry, we determined below the fold changes of each of the aforementioned genes in wild-type SPF45-overexpressing cells compared to vector group. Also, we determined the fold changes in $SPF45^{AA}$ and $SPF45^{DD}$ SKOV-3 stable cells. Total RNA was isolated from each of these cells grown in 6-well plates to 90% confluence. Gene-changes validation was performed on three-independent passages for each of the cell lines.

ErbB2

ErbB2 levels were decreased by 69% in SKOV3-pQCSPF45 and by 80% in SKOV3-$pQCSPF45^{DD}$ compared to vector control, while it was decreased only by 36% in SKOV3-

pQCSPF45^{AA}. We further validated ErbB2 expression by western blot (Fig 3.4 *A&B*). While ErbB2 protein expression was slighlthy decreased in SKOV3-pQCSPF45AA cells, it was dramatically decreased in SKOV3-pQCSPF45 and SKOV3-pQCSPF45^{DD}. Accordingly, ErbB2 expression represents an example of a SPF45-mediated event that is responsive to the phosphorylation of Thr71 and Ser222. We further determined that ErbB2 expression in these cells is regulated by Elf3, an Ets-family transcription factor, but not Elf1 or PEA3. Decreased ErbB2 expression correlated with decreased Elf3 expression but not Elf1 and PEA3 which maintained a relatively constant expression among the different cell lines (Fig 3.4 *C*). Interestingly, the decrease in Elf3 expression was translated by similar effects on ErbB2 expression and cellular proliferation, which actually paralleled SPF45 expression and phosphorylation pattern. Further experiments will be required to identify the link between MAP kinase activation, SPF45 expression/phosphorylation, and Elf3.

Fibronectin FN1

Fibronectin levels were roughly 5 folds higher in SKOV3-pQCSPF45 compared to vector control. Unlike with ErbB2 expression, mutation of Thr71 and Ser222 had no effect on SPF45-induced increase in fibronectin levels, as evidenced by near-equal fold change in FN1 levels in both SKOV3-pQCSPF45^{AA} and SKOV3-pQCSPF45^{DD}. We further validated the qRT-PCR results with a northern blot and a western blot for protein levels (Fig 3.4 *D,E&F*). FN expression correlated with SPF45 gene expression and was not affected by the phosphorylation sites mutations, indicating a non-phosphorylation-dependent SPF45 function and adding to the versatility of this protein.

TGF-β

TGF-β levels showed a trend towards decrease in SKOV3-pQCSPF45 compared to vector control while they were significantly doubled in the SKOV3-pQCSPF45^{AA} and SKOV3-pQCSPF45^{DD} groups. No further investigation of TGF- β was pursued (Fig 3.4 *G*).

Periostin

Based on the microarray data, we expected periostin - a protein involved in bone and teeth formation, but also has been shown to be overexpressed in several cancers and to be linked to migratory effects of some cancer cells (Tischler 2010, Michaylira 2010) -, to have the highest fold increase in wild-type SPF45 group compared to vector control. Validation by real-time PCR failed to show such a difference (Fig 3.4 *H*); additionally, we could not obtain consistent fold change levels in the four groups tested (large standard deviation). This is very likely due to the very low abundance of the mRNA and the lowest threshold cycle of detection is the 28th cycle. Accordingly, slight changes in the mRNA levels would translate as high fold changes and this is where the microarray technology fails. Although periostin seemed a luring candidate because of its predicted fold increase and its function in cancer cells, this case provided a clear example regarding the limitations of the microarray results and the necessity for further validation.

<u>Validation of FN-EDA as a splicing target for SPF45</u>

Three regions in the fibronectin mRNA are subject to alternative splicing: extra domain 3 A (EDIIIA), extra domain 3 B (EDIIIB) and the variable region (V or IIICS) (Fig 3.5 *A*). EDA and EDB are cassette-exons while the variable region spans two exons over 120

nucleotides in length. The variable region can generate up to 5 different splice forms that are very close in length (0 to 120 nucleotides). The combination of inclusion/exclusion of these different regions potentially generates up to 20 different isoforms of fibronectin (Schwarzbauer 1983). We showed above, using real-time PCR and northern blot, that fibronectin levels are increased in SKOV3-pQCSPF45 (and the mutants cell lines) compared to SKOV3-pQCXIP. Based on the results of the exon array, we also expected fibronectin to undergo alternative splicing in SKOV3-pQCSPF45. We employed two approaches to determine changes in EDA and EDB fibronectin splicing patterns in SKOV3-pQCSPF45 (as well as SPF45^{AA} and SPF45^{DD} mutant cell lines) vs. SKOV3-pQCXIP: end-point PCR and real-time PCR.

End-point PCR fails to validate FN alternative splicing

SKOV3-pQCXIP, SKOV3-pQCSPF45, SKOV3-pQCSPF45^{AA} and SKOV3-pQCSPF45^{DD} cells were grown to 90% confluence in 6-well plates prior to harvest for RNA extraction and synthesis of cDNA. Since EDA and EDB are spliced in/out in a cassette-exon splicing pattern, we used primer sets outside of the corresponding exonic sequences to detect both the (+) and (-) isoforms of fibronectin. As total fibronectin levels are higher in the SPF45-overexpressing cells, we used the ratio of EDA (+)/EDA (-) isoforms as well as EDB (+)/EDB (-), as a measure of change in FN splicing. PCR was first performed with 28 cycles of amplification and the bands were visualized on a 2% agarose gels (Fig 3.5 *B bottom*). EDA (+) bands (upper band) were observed in WT-SPF45, SPF45^{AA} and SPF45^{DD} lanes but not the vector lane, while EDA (-) was present in all lanes; with respect to EDB-FN splicing, EDB (-) and (+) isoforms were detected in wild-type SPF45, SPF45^{AA} and

SPF45^{DD} lanes but not the vector lane. Decreasing the amplification cycle number to 22, neither EDA (-) nor (+) bands were detected in the vector lane, while both were present in the wild-type SPF45 lane (Fig 3.5 *B middle*). Raising the cycle number to 35, both EDA (-) and (+) isoforms were detected in the vector and the wild-type SPF45 lanes (Fig 3.5 *B top*). Since the end-point PCR is not quantitative, no decisions could be made concerning a change in splicing of either EDA or EDB regions and further experiments were needed.

Real-time PCR validates FN alternative splicing

To overcome the hurdles of end-point PCR technique in validating a change in splicing, we employed real-time PCR to determine the fold inclusion of EDA(+) and EDB(+) isoforms (Fig 3.5 *C*). We isolated total RNA from SKOV3-pQCXIP, SKOV3-pQCSPF45, SKOV3-pQCSPF45^{AA} and SKOV3-pQCSPF45^{DD} cells grown to 90% confluence in 6-well plates and reverse-transcribed to synthesize cDNA. Three separate passages were harvested and ran on a real-time PCR plate in duplicate. Using RT^{2}SYBR Green chemistry, total fibronectin primer set and exon-specific primers to EDA and EDB regions were used to determine the fold inclusion of EDA(+) and EDB(+) isoforms. We used 18sRNA as a housekeeping gene control. The fibronectin primers were designed to detect a region common to all FN isoforms while EDA and EDB primers were specific to FN isoforms that contain their corresponding regions. As the fold increase of EDA(+) mRNA in the wild-type SPF45 (~ 2.8 folds) group compared to vector exceeds the fold increase of total FN in wild-type SPF45 compared to vector (~ 2.1 fold), we concluded that there is an increase in EDA exon inclusion concomitant with an increase in SPF45 expression levels. Fold increase in EDA(+) isoform in SPF45^{AA} and SPF45^{DD} groups

compared to vector was similar to that of total fibronectin, indicating that intact Thr71 and Ser222 were necessary for EDA inclusion. Fold increase in EDB exon in wild-type SPF45, SPF45^{AA} and SPF45^{DD} cell lines compared to vector control were similar to those of total fibronectin in their corresponding groups. As a result, no change in EDB exon splicing levels could be deduced. Based on these data, we conclude that fibronectin EDA exon is subject to alternative splicing regulated by SPF45. Although we showed that mutation of the MAP kinase target sites reversed the increase in EDA splicing, further experiments are needed to support the role of phosphorylation on these sites in controlling EDA splicing. Currently, minigene splicing assays are the standard of choice to study the effect of *trans* factors and their modification on splicing; using minigene plasmids of fibronectin EDA or EDB along with SPF45 plasmid in Cos-1 cells will likely support our findings on the effect of SPF45 on fibronectin splicing as well as determine further impacts of SPF45 phosphorylation.

To determine a potential role for the increase in EDA(+) inclusion in FN1, we investigated the effect of SPF45 and the phosphorylation mutants on cellular adhesion. The stable cell lines were plated onto the following matrices for 30 min: tissue culture plastic, laminin, poly-L-Lysine and fibronectin; followed by cell fixation, staining with crytal violet and measurement at 550 nm. While all cells displayed an equal and non-specific attachment to plastic, laminin and poly-L-Lysine, there was an overall increase in attachment to fibronectin. Furthermore, SKOV3-pQCXIP had the highest attachment with SKOV3-pQCSPF45, -pQCSPF45^{AA} and -pQCSPF45^{DD} having 29.6%, 15.8% and 19.5% decrease compared to SKOV3-pQCXIP, respectively. Of note, the stable cell lines

adhesion to fibronectin parallels EDA(+) FN expression suggesting a possible implication for changes in FN isoform and cellular attachment to extracellular matrix.

Mutations on Thr71 and Ser222 does not decrease SPF45 binding to SF1 and SF3b155

In order to understand the role of the phosphorylation sites in the mechanism of alternative splicing, we investigated the role of the phosphorylation sites and their mutants on SPF45 binding to two proteins in the spliceosome, SF1 and SF3b155, which are known SPF45 binding partners (Corsini 2007). Immunoprecipitation of Myc-SPF45, Myc-SPF45^{AA} or Myc-SPF45^{DD} after transient transfection did not show a difference in binding to SF1 or SF3b155 (Fig 3.6). While we did not observe an effect of the phosphorylation mutants on interaction with these splicesomal proteins, further experiments with protein-RNA assays might be warranted to better address this question.

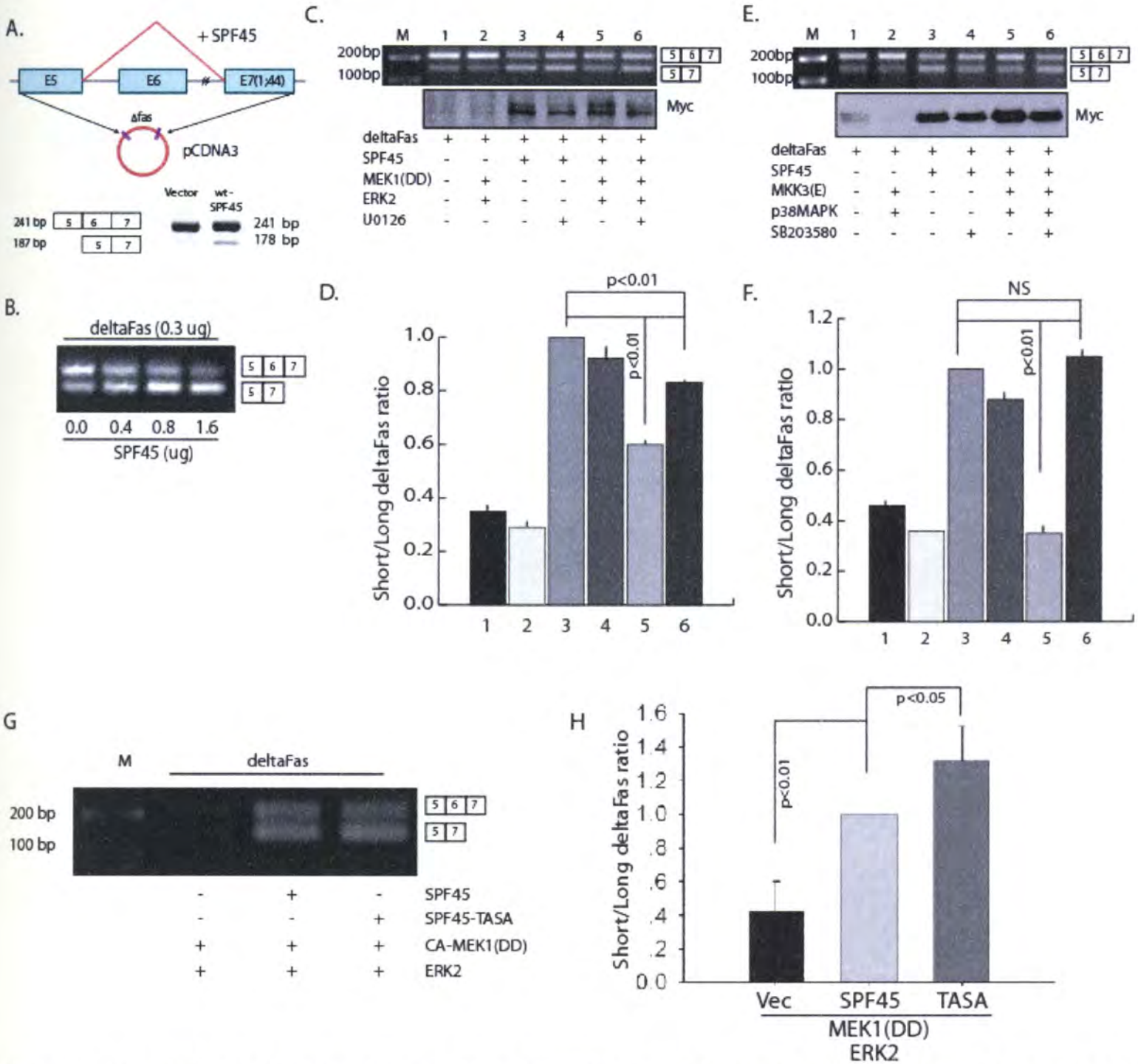

Fig 3.1 MAPK phosphorylation of SPF45 decreases its splicing function towards ΔFas *A.* Schematic of the ΔFas minigene system. *B.* 0, 0.4, 0.8 and 1.6 μg SPF45 were added to 0.3 μg of ΔFas and transfected to Cos-1 cells followed by Fas RT-PCR. *C&D.* ERK2 activation decreased SPF45 splicing activity and was partially reversed with MEK inhibtor U0126. A representative gel is shown above. Ratios were determined by densitometry; p values as indicated, n=3. *E&F.* p38MAP kinase activation strongly decreased SPF45 splicing function towards ΔFas and was completely reversed using p38 inhibitor SB203580. A representative gel is shown above. Ratios were determined by densitometry; p values as indicated, n=3. *G&H.* $SPF45^{AA}$ splicing function compared to SPF45 with active ERK expression ; p values as indicated on the histogram in H, (n=4).

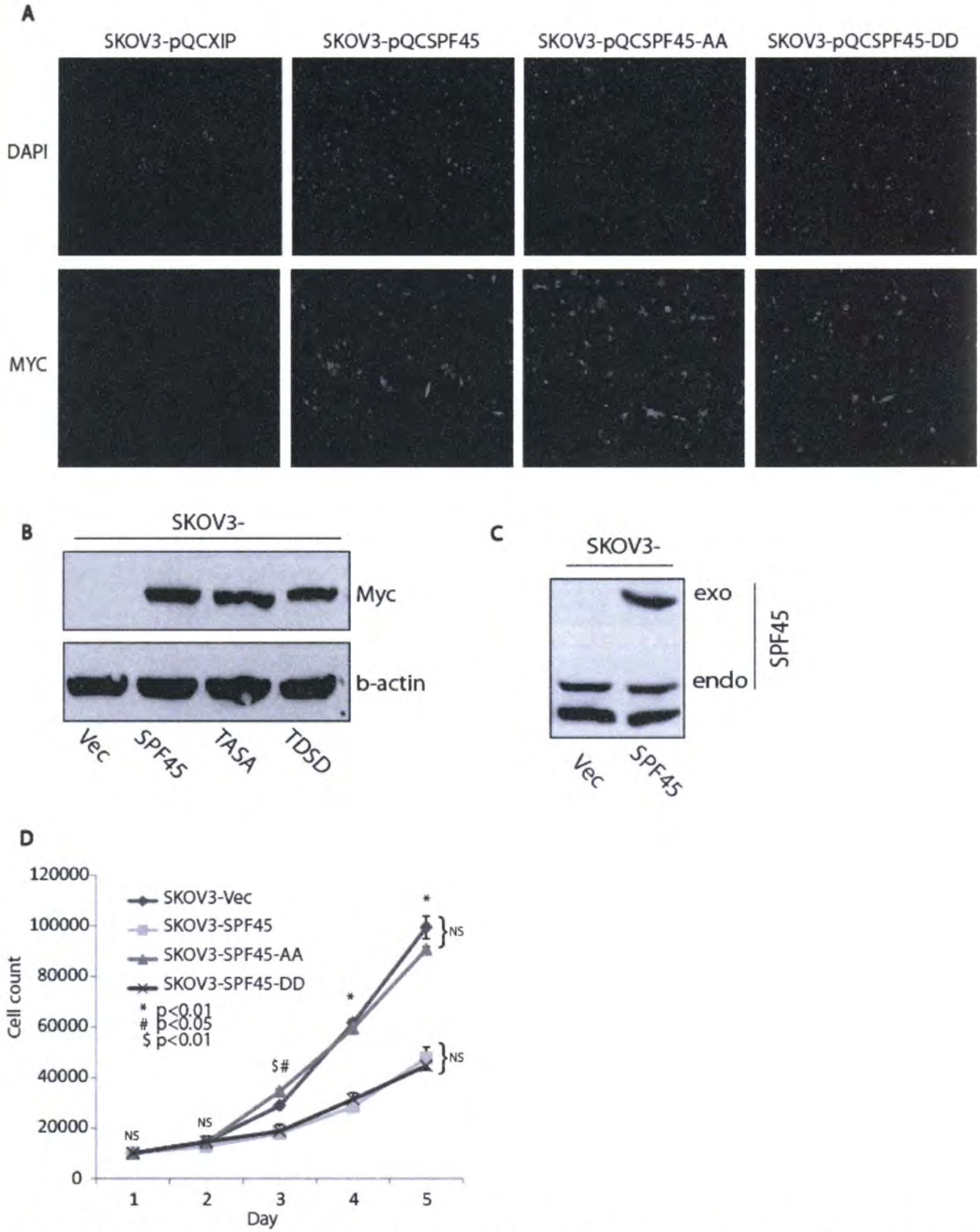

Fig 3.2 Generation of SKOV3 stable cells overexpressing SPF45 or mutants *A.* Immunofluorescent staining using myc-antibody of SKOV3-pQCXIP, -pQCSPF45, -pQCSPF45^{AA} and –pQCSPF45^{DD}. Nuclei were stained with DAPI. Stable cell lines were generated with retroviral infection and puromycin selection. *B.* Cell lysates of SKOV3 stable cells resolved by 10% SDS-PAGE and immunoblotted with anti-Myc antibody; β-actin was used as a loading control. *C.* Relative expression levels of exogenous to endogenous SPF45 in SKOV3 stable cells. *D.* Cell proliferation assay of the four stable cell lines was performed using cell counting at 24 hours interval; NS, non-significant (n=3, triplicates).

Gene pathway cluster
1. β-adrenergic receptor transactivation of EGFR
2. Angiotensin signaling via Pyk2
3. Cytoskeleton remodeling via Activin A
4. Immune response
5. Gastrin in cell growth and proliferation
6. EphB receptor in dentritic spine morphogenesis and synaptogenesis
7. Mucin expression in cystic fibrosis via IL-6 and IL-7
8. Immune response
9. Immune response
10. Olfactory transduction
11. VEGF family signaling
12. ESR1 regulation of G1/S transition
13. Regulation of GnRH
14. Gastrin in inflammation – immune response
15. Immune response
16. Ligand-dependent activation of ESR1/AP-1 pathway
17. Transcription role of AP-1 in regulation of cell metabolism
18. Histamine – immune response
19. α_2-adrenergic activation of ERK
20. Metabolic pathway
21. Mucin expression in cystic fibrosis via TLRs
22. Muscle contraction-regulation of eNOS activity in endothelial cells

Table 3.1 Up-regulated genetic pathways induced by SPF45 overexpression in SKOV3 cells

Gene pathway cluster
1. GTP-XTP metabolism
2. RXR transcription
3. CTP-UTP metabolism
4. Chromosome condensation in metaphase
5. ATP/ITP metabolism
6. Oxidative phosphorylation
7. Apoptosis and survival – Granzyme A
8. CFTR translational fidelity
9. APC
10. Spindle and chromosomes assembly
11. ATP metabolism
12. Normal CFTR trafficking
13. Δ508 CFTR trafficking
14. Amino acid metabolism
15. Alternative pathway
16. Regulation of translation/initiation
17. Ubiquitin metabolism
18. Antigen presentation by MHC I
19. Regulation of CFTR activity
20. G-protein signaling – RAC1 in cellular processes

Table 3.2 Down-regulated genetic pathways induced by SPF45 overexpression in SKOV3 cells

Gene pathways with altered splicing events
1. Alternative complement pathway
2. Role of tetraspanins in the integrin-mediated cell adhesion
3. Endothelial cell contact by non-junctional mechanisms
4. Nucleotide excision repair
5. Endothelial cell contacts by junctional mechanisms
6. Histamine H1 receptor signaling in the interruption of cell barrier integrity
7. Regulation of CFTR activity (normal and cystic fibrosis)
8. ECM remodeling
9. Role of PKA in cytoskeleton reorganization
10. Ligand-dependent transcription of retinoid-target genes
11. Calcium signaling
12. Lectin induced complement pathway
13. Integrin-mediated cell adhesion and migration
14. Classical complement pathway
15. Angiotensin signaling via STATs
16. Cadherin-mediated cell adhesion
17. Netrin-1 in regulation of axon guidance
18. Cytoskeletion remodeling
19. Dopamine D2 receptor activation of PDGFR in CNS
20. Vitamin K metabolism
21. EPO-induced MAPK pathway
22. PDGF activation of prostacyclin synthesis
23. Vitamin B6 metabolism
24. Spindle assembly and chromosome metabolism
25. Chemokines and adhesion

Table 3.3 Genetic pathways with identified changes in splicing events accompanying SPF45 overexpression in SKOV3 cells

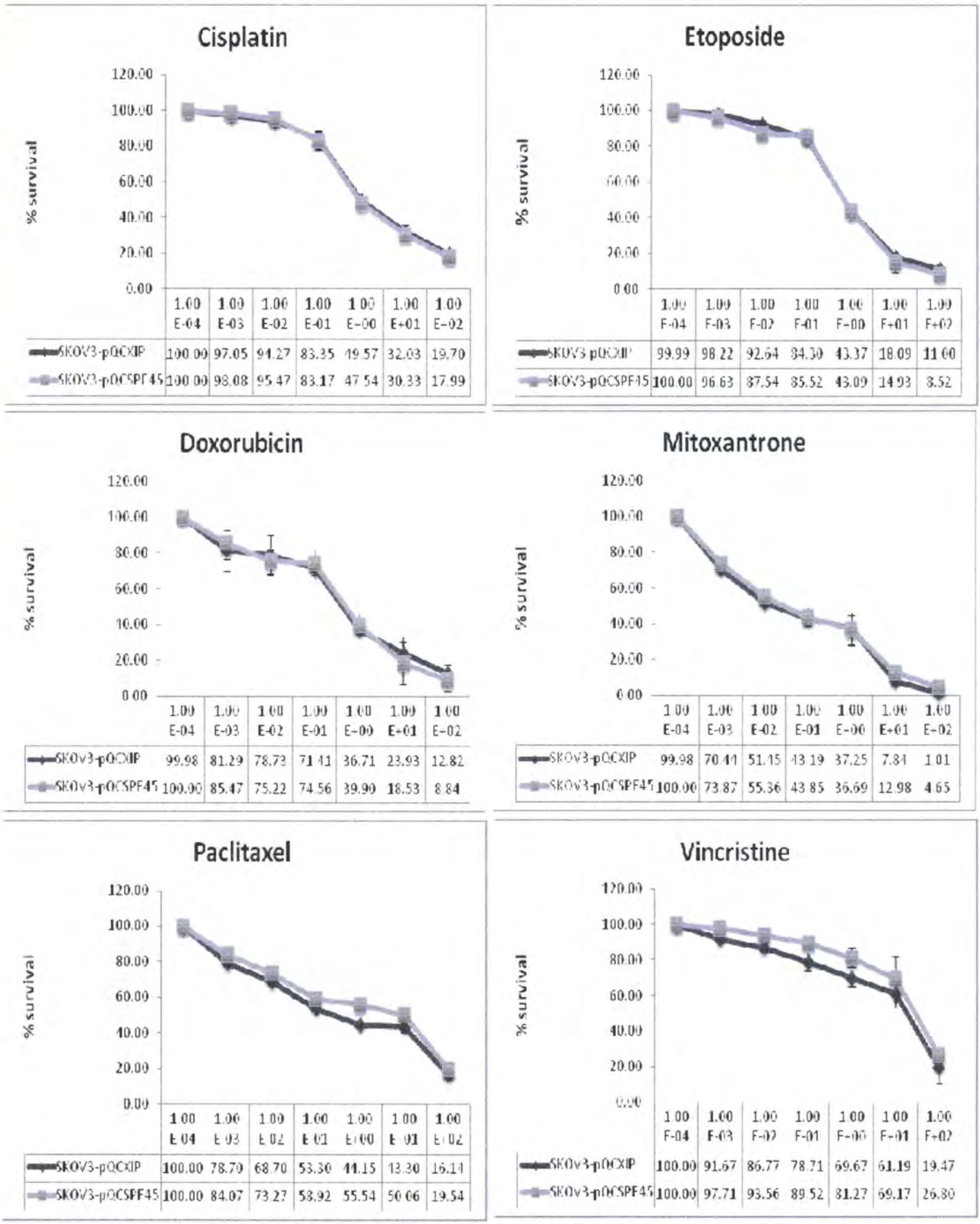

Fig 3.3 SPF45 does not confer drug resistance to SKOV3 cells MTS cytotoxicity assay in 96 wells plates of SKOV3-pQCXIP, -pQCSPF45 using cisplation, doxorubicin, etoposide, mitoxantrone, paclitaxel and vincristine. 3.0e+3 cells/well were plated onto separate wells and treated the following day with 10 fold increasing doses from 1.0e-4 μM to 1.0e+2 μM. 72 hrs post-treatment, MTS reagent was added and the cells were incubated for 2 hrs at 37°C and analyzed on a Biorad 96 well plate reader at 492 nm. (n=3, triplicates).

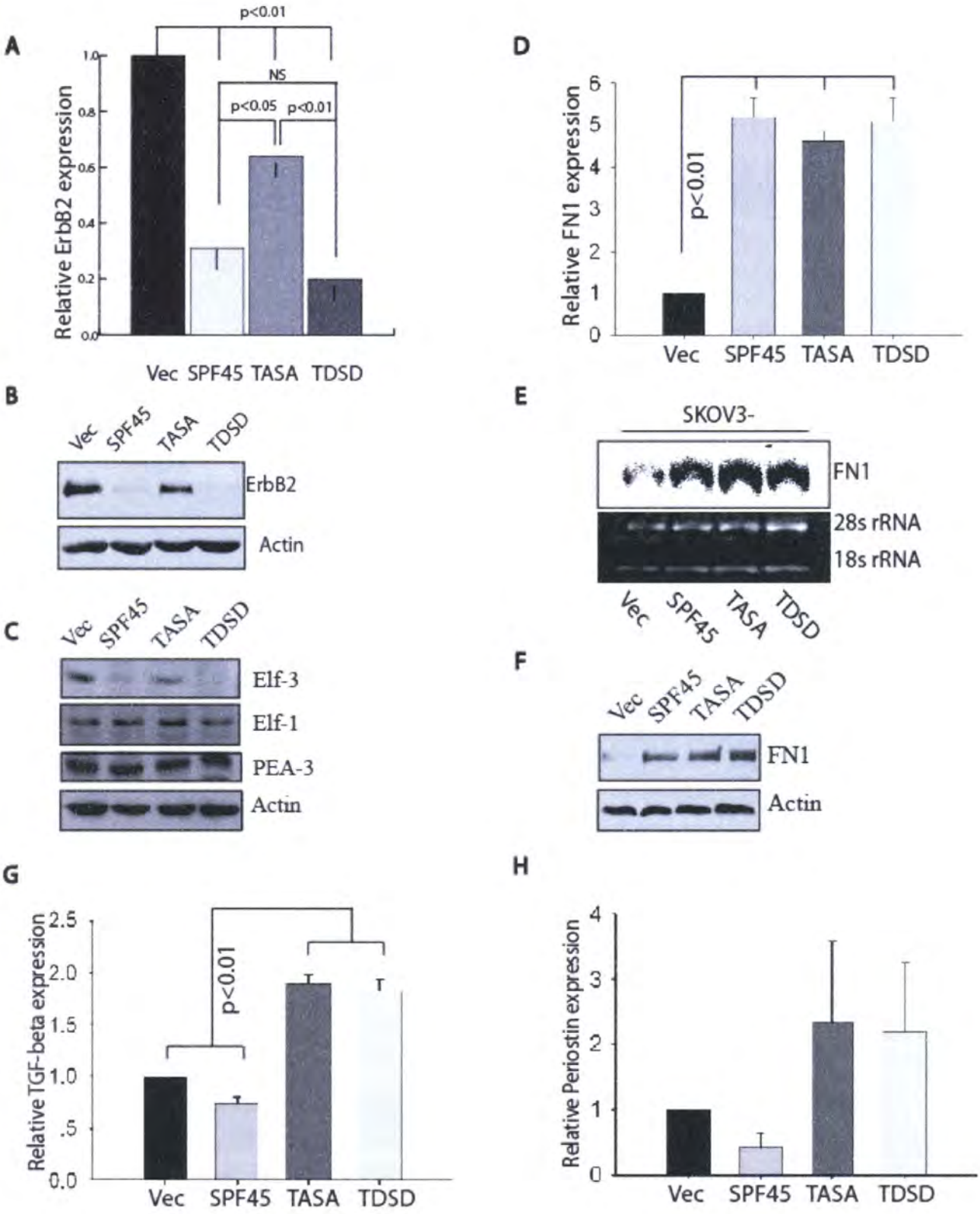

Fig 3.4 SPF45 induces genetic and transcriptional changes in SKOV3-SPF45 and mutants *A.* qRT-PCR of ErbB2 in in the four stable cell lines. ErbB2 level was set to 1 in SKOV3-pQCXIP and expressed relative to vector in the rest of the cell lines ($p<0.01$ n=3, duplicates). *B.* Western blot of erbB2 in cell lysates of the corresponding cell lines. *C.* Western blot of Elf1, Elf3 and PEA3 transcription factors in the corresponding cell lines (n=3). *D.* qRT-PCR of FN1 in the four stable cell lines. FN1 level was set to 1 in SKOV3-pQCXIP as in (A) ($p<0.01$ n=3, duplicates). *E.* Northern blot of FN1 using a ^{32}P-labeled single-stranded DNA probe produced by asymmetric PCR to FN exons 6&7. 28s and 18s rRNA were used as loading control. *F.* Western blot of FN1 in the corresponding cell lines using total cell lysates. *G&F.* qRT-PCR of TGF-β and Periostin in the corresponding cell lines treated as in (A) ($p<0.01$ n=3, duplicates).

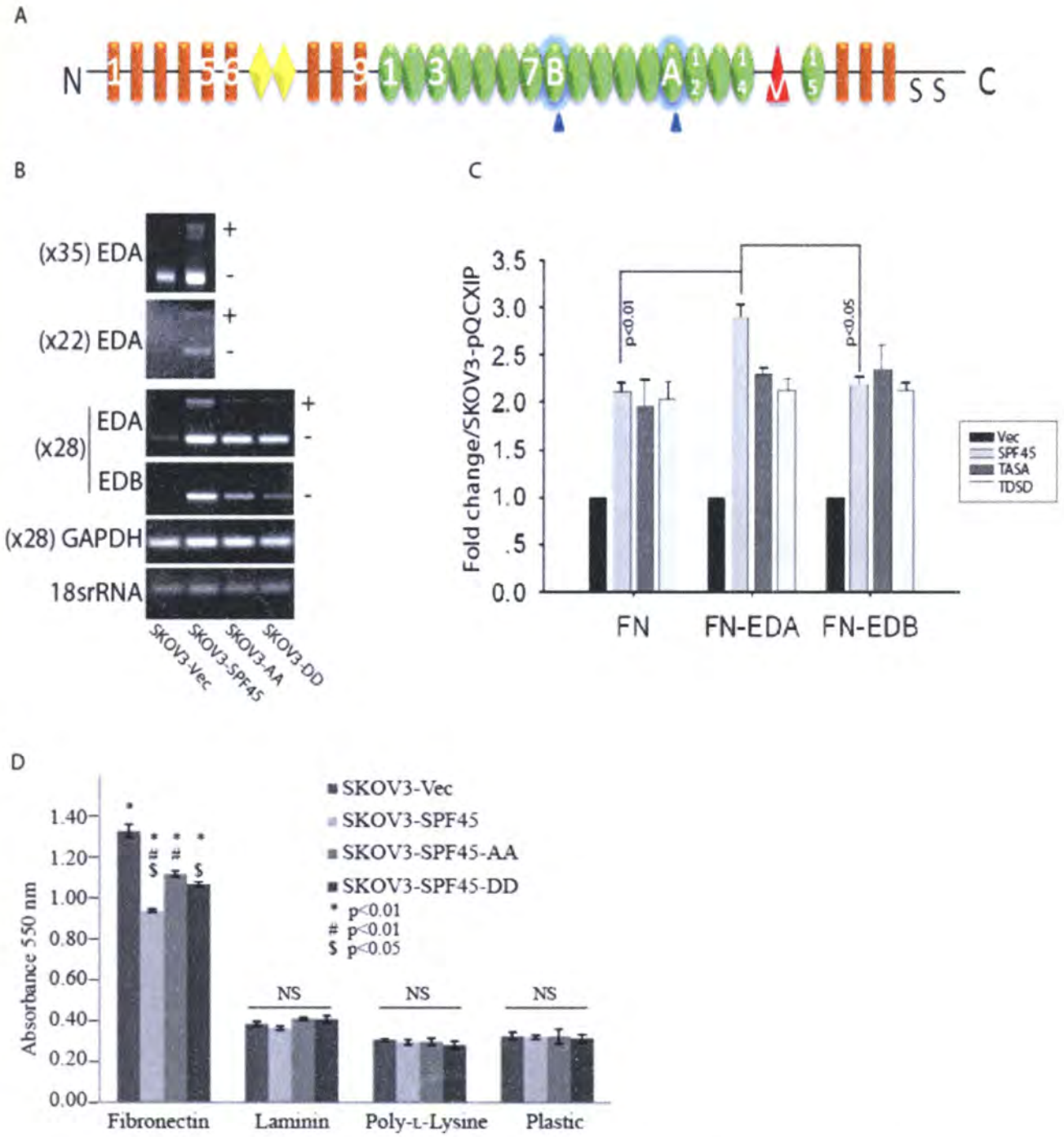

Fig 3.5 SPF45 regulates fibronectin splicing, increases EDA inclusion and decreases adhesion to fibronectin matrix *A*. Schematic diagram of fibronectin domains consisting of three different repeats. Regions B, A and V correspond to the alternatively spliced domains. *B.* 28 cycles RT-PCR (bottom) of FN-EDA and FN-EDB regions using RNA isolated from the corresponding cell lines. EDA RT-PCR is repeated at 22 cycles (middle) and 35 cycles (top). *C.* qRT-PCR of FN, FN-EDA and FN-EDB with exon-specific primers, using RNA isolated from the corresponding cell lines. FN-EDA is 30% increased in SKOV3-pQCSPF45 compared to FN ($p<0.01$) and EDB ($p<0.05$) (n=3, in duplicate). *D.* Cell adhesion assay. Stable cells were plated in 96-well plates onto the matrices as per histogram, fixed at 30 min after plating and absorbance measured at 550 nm; p values as per graph, NS non-significant (n=3, octuplets).

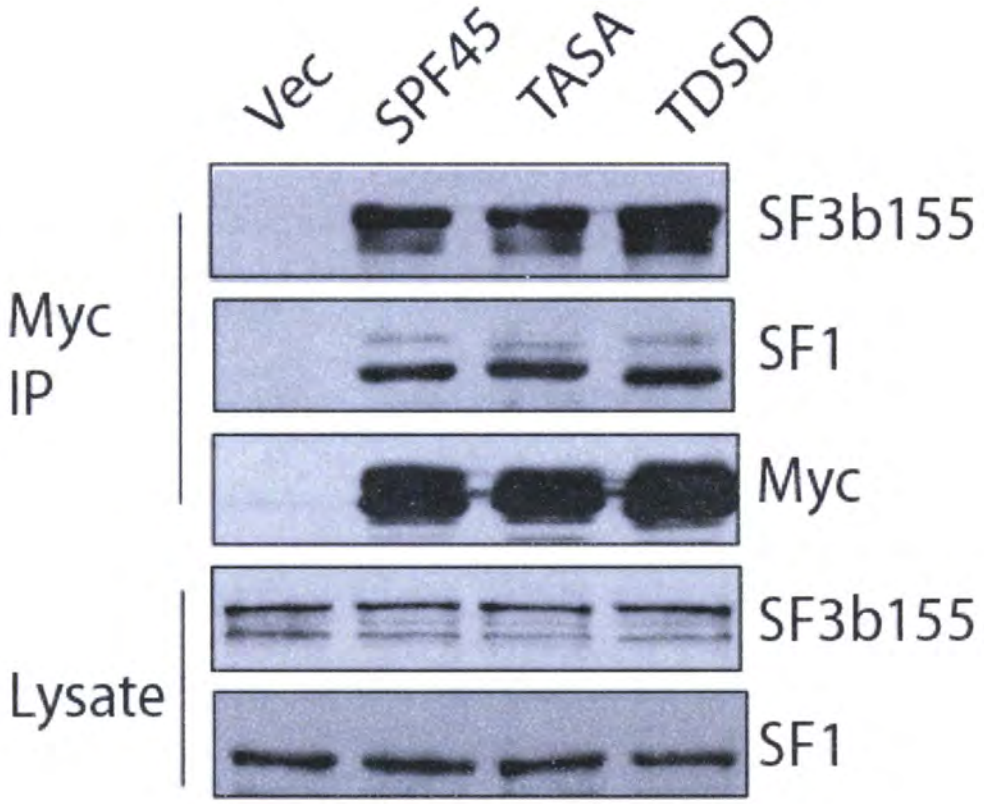

Fig 3.6 Mutations on Thr71 and Ser222 do not disrupt SPF45 binding to SF1 and SF3b155 SPF45 or mutants were myc-immunoprecipitated from the corresponding SKOV3 stable cells overnight at 4°C and resolved by SDS-PAGE. Immunoprecipitates were western blotted with SF1, SF3b155 and Myc antibodies.

Discussion

We showed in the previous aim that SPF45 is phosphorylated by MAP kinases on Thr71 and Ser222, *in vitro* and in cells. In this chapter, we determined the role of the phosphorylation on the alternative splicing activity of SPF45 *in vivo* using a minigene system of the death receptor Fas, transfected into Cos-1 cells. We first showed that SPF45 causes a dose-dependent increase in *fas* alternative splicing then used a dose that generates a 1:1 ratio of the long and short Fas mRNA isoforms in the ensuing experiments. Co-expressing SPF45 and activated ERK2/MAP kinase (using constitutionally-active MEK1(DD), we showed a decrease in Fas splicing compared to SPF45 alone, which was partially reversed when the cells were treated with MEK inhibitor U0126, reflecting a decrease in SPF45 alternative splicing activity towards Fas pre-mRNA, upon SPF45 phosphorylation. We also showed a similar and more pronounced effect of p38 MAP kinase phosphorylation of SPF45 on the alternative splicing acitivity. To support these findings, we impaired the ability of ERK2 to phosphorylate SPF45 using SPF45-alanine-mutant of Thr71 and Ser222 MAPK-phosphorylation targeted residues ($SPF45^{AA}$). We also generated a phospho-mimetic protein as well using SPF45-aspartate-mutant of the same residues ($SPF45^{DD}$). $SPF45^{AA}$ showed a modest increase in SPF45 alternative splicing activity compared to wild-type SPF45, while $SPF45^{DD}$ remarkably caused a strong increase in SPF45 alternative splicing activity towards Fas. Comparing wild-type SPF45 and $SPF45^{AA}$ in the presence of CA-MEK1(DD) and ERK2, demonstrated an increase of SPF45-alanine-mutant alternative splicing activity vs wild-type SPF45, and we attributed this observation to the

attenuating effect of the phosphorylation on wild-type SPF45 function, an effect that is not applicable to $SPF45^{AA}$. While $SPF45^{DD}$ was expected to behave similarly to $SPF45^{pT71pS222}$, it may not be necessarily true with respect to splicing. In fact, inducing phosphorylation of wild-type SPF45 by upstream signal activation is more likely to represent the true effect of phosphorylation on the protein's alternative splicing activity, as phosphorylated splicing factors are under a dynamic control of upstream phosphorylation and downstream de-phosphorylation (Cao 1997, Xiao 1998). A plausible explanation is that aspartate mutations of Thr71 and Ser222 influence SPF45 interaction(s) with other components of the spliceosome (eg SF1, SF3b155, U2AF), and the inability to get dephosphorylated causes the discrepancy observed between $SPF45^{DD}$ and MAPK-phosphorylated SPF45. Additionally, using $SPF45^{AA}$ to inhibit ERK2-mediated phosphorylation had the opposite effect of wild-type SPF45, reinforcing the role of Thr71 and Ser222 in regulating SPF45 function and supporting the observation of decreased Fas splicing upon SPF45 phosphorylation.

As we are interested in the biological role of SPF45 in ovarian cancer, our aim was and remains to identify novel targets of genetic and splicing modulation. Of the large list of potential substrates that were detected by gene and exon array, we focused on fibronectin and ErbB2 for the purposes of this dissertation. Fibronectin is involved in a wide array of cellular functions including adhesion, migration, growth and differentiation. In cancer, fibronectin is implicated in cellular mobility, metastasis and chemoresistance (Kaspar 2006). In development fibronectin occupies a central role, as FN-/- mice are embryonic lethal (George 1993, Watt 1994). Although complex in

function, this molecule's amino acid sequence is reduced to three types of repeating domains (also called FN repeats) totaling 29 to 31: 12 type I, 2 type II and 15-17 type III (Hynes 1990). Although sequence homology of each of the different repeats does not exceed 50%, structural homology is conserved. For instance, the different type III repeats are only 20-40% identical, but they all share similar β-sandwich structural topology (Main 1992, Leahy 1992). Two quasi-identical monomers of these different repeats are linked by disulfide bonds to create a more complex molecule. Fibronectin is encoded by a single gene but has varied expression and sequence patterns due to intense regulation by alternative splicing, which is tissue and species-dependent (Schwarzbauer 1983, Tamkun 1984, Gutman 1987). In humans, three fibronectin regions are known to undergo alternative splicing: EDA, EDB and the variable region V. EDA and EDB are included by the simplest form of splicing - ie exon-cassette inclusion. The variable region presents a more challenging splicing pattern, as it spans around 120 amino acids that are potentially spliced into 5 different sequences. Accordingly, four different forms of fibronectin could be formed by the inclusion/exclusion of EDA and EDB (EDA+/EDB+, EDA+/EDB-, EDA-/EDB+ and EDA-/EDB) and another five could be formed by the splicing arrangements of the variable region V leading to a total of twenty different potential isoforms (4x5 = 20). Both EDA and EDB are type III repeats known to influence their surrounding topology unveiling "cryptic" sites in the FN molecule (Muro 1999). The exact roles of these two domains remain elusive: they are both expressed in several solid cancers such as thyroid, lung, liver and breast (Muro 1999), although the impact of each domain alone on tumor progression remains unclear. EDB is associated

with angiogenesis (Khan 2005) and is expressed in the stroma and blood vessels of tumors. EDA is implicated in rheumatoid arthritis (Shiozawa 2001), tumor spreading (Manabe 1997) and invasions (Liu 1998) as well as proliferation of the blood vessel intima (Spargo 1994). While they differ in their spatiotemporal expressions in normal adult tissues, concomitant EDA and EDB expression is linked to fetal tissues and neoplastic masses, suggesting an active role of each of these two domains. FN-/- is embryologically lethal in mice (George 1993), but EDA-/- mice and EDB-/- mice are viable and fertile (Muro 2003, Fukuda 2002). In recent years, conditional knock-out mice of *both* EDA and EDB domains were generated and shown to be embryologically lethal (Astrof 2007), indicating the necessity of both domains in development. While fibronectin is known to be overexpressed in ovarian cancers and to be associated with a poorer prognosis (Ricciardelli 2006), nothing is known about EDA and EDB expression levels in ovarian cancer. In our experiments, we have observed an increase in fibronectin expression in SKOV3 cells overexpressing SPF45. Furthermore, we showed that expression of both the EDA and EDB domains was elevated in the presence of increased SPF45 expression independent of the mutations of Thr71 and Ser222. Also we showed that the inclusion of EDA domain in SKOV3-pQCSPF45 cells compared to SKOV3-pQCXIP is higher than that of total fibronectin in the same respective cell lines, suggesting that SPF45 enhances inclusion of EDA domain dependent on intact Thr71 and Ser222 residues. Additionally, we determined the adhesion profile of the different stable cells and showed that they adhere to plastic, laminin and poly-L-lysine non-specifically after a short 30 min time point. Adhesion to fibronectin was however

increased around 3 fold compared to the other surface coatings, with SKOV3-pQCSPF45 demonstrating the weakest attachment compared to SKOV3-pQCXIP, -pQCSPF45^{AA} and -pQCSPF45^{DD}, which can be explained by the increase in EDA-FN expression in SKOV3-pQCSPF45. The fact that SPF45 increases fibronectin expression and affects EDA splicing in SKOV3 cells raises several questions: does SPF45 increase fibronectin expression in a different cell line? What is the role of EDA and EDB inclusions in fibronectin in the context of ovarian cancer? Does it affect cellular growth, spreading, invasion and/or chemoresistance? While the answers to these different questions remain unanswered, it is appropriate to speculate that altered fibronectin levels in cells would be responsible of adhesive, proliferative and potentially invasive properties.

While FN expression levels were independent of SPF45 phosphorylation status, we demonstrated a decrease in ErbB2 in SPF45 expressing cells in a phosphorylation-dependent manner. A member of the EGFR family and lacking a physiological ligand, ErbB2 heterodimerizes with other EGFR members (Her1, Her3 and Her4) in order to activate its protein tyrosine kinase activity (Graus-Porta 1995, 1997; Karunagaran 1996) leading to activation of several transduction pathways including PI3K/Akt and MAPK (Campiglio 1999). ErbB2 is overexpressed in several solid cancers including colon (D'Emilia 1989), primary lung cancer (Shi 1992), breast cancer (Seshadri 1989) and ovarian cancer (Lichtenstein 1990). In ovarian cancer, ErbB2 has been found to be overexpressed up to 50% (Kury 1990). Unfortunately, this elevated expression of ErbB2 in advanced ovarian cancer is associated with a poorer prognosis for survival (Cirisano 1996). Several cellular and physiological disturbances are associated with ErbB2

overexpression. For instance, it modulates cellular adhesion through interaction with CD44s, thereby enhancing binding to hyaluronan (Bourguignon 1997). Not limited to the standard form of CD44, ErbB2 is recruited to another complex containing a variant CD44 (CD44-v3) and Vav2 in response to hyaluronan binding inducing Rac1 and Ras signaling in SKOV3-ipl ovarian cancer cells inducing cell migration and growth (Bourguignon 2001). In response to heregulin (an EGFR ligand), ErbB2 interacts with PAK1 and actin stimulating PAK1 activity in a PI3K/Akt manner, causing cytoskeletal reorganization and cellular migration of ErbB2-transfected MCF-7 breast cancer cells (Adam 1998). Heregulin and ErbB2 signaling induce an activating phosphorylation of c-Src in MCF-7 cells influencing their metastasis (Vadlamudi 2003). Also, overexpression of ErbB2 in another breast cancer cell line MCF-10A enhances cellular proliferation, migration and invasion (Seton-Rogers 2004). ErbB2 effects on cellular proliferation are not limited to ovarian and breast cancer cells, as it induces proliferation of head and neck squamous cell carcinoma in response to LPA (Gschwind 2002) and in prostate cancer cells in response to CXCL12/CXCR4 chemokine signaling (Chinni 2008). Beyond tumor growth and cellular migration, ErbB2 has been also been linked to decreased apoptotic response in MCF-7 cells by upregulating survivin and Bcl-2 through activation of ERK/MAPK and PI3K/Akt pathways (Siddiqa 2008). In clinical trials of platinum therapy of ErbB2 positive ovarian cancer cells, it has also been shown to decrease clinical outcome and enhance tumor resistance (Meden 1998, Verri 2005). Currently, several trials with ErbB2 monoclonal antibodies are being investigated for adjuvant therapy of advanced and chemotherapeutic resistant breast cancer (Romond 2005, Slamon 2006) and solid

tumors (Kaumaya 2009), as targeting ErbB2 harboring cells in athymic nude mice or in mouse model of tet-controlled ErbB2 cells leads to decreased tumor growth, enhanced apoptosis and tumor remission (Harwerth 1993, Schiffer 2003). Given the importance of ErbB2 in mitogenically modifying tumors, it was interesting to see it upregulated in response to SPF45 overexpression. Our results indicate that elevated SPF45 levels decreased ErbB2 expression in a phosophorylation-dependent manner: 69% decrease in SKOV3-pQCSPF45 and 80 % decrease in SKOV3-pQCSPF45^{DD}. As it caused an increase in cellular proliferation, ErbB2 expression levels parallel the proliferation rates of the corresponding stable SKOV3 cell lines, suggesting that SPF45 alters SKOV3 proliferation in an ErbB2-dependent manner. Furthermore, we showed that ErbB2 expression is regulated by SPF45 possibly in response to Elf3 but not Elf1 or PEA3 Ets transcription factors. Several questions arise to address the specific role of Erb2 in response to SPF45-mediated overexpression such as investigating the adhesive, proliferative and drug resistance phenotypes of these cells. While SKOV3 cells are ErbB2 positive irrespective of SPF45 overexpression, the role of added ErbB2 might be elusive. Interestingly, development of resistance to cisplatin is associated with decreased expression of ErbB2 (Langton-Webster 1994), which can partially explain the mechanism of SPF45-mediated drug resistance or alternatively, that the decrease in proliferation could result in the drug resistance. It will be necessary to determine ErbB2 changes with SPF45 overexpression in different cell lines to test this hypothesis. We are currently generating stable SPF45-expressing cells in HeLa (human cervical cancer cells) and MCF-7 (human breast cancer cells) both of which are ErbB2-negative, as well as several ovarian cancer

cell lines with both ErbB2-negative and –positive genotypes. Generation of these stable cells in tissues different than ovarian cancer cells will also help establish the role of SPF45 in cancer biology as well as address the role of ErbB2 in mediating SPF45 effects on cellular proliferation and drug resistance. If SPF45 does induce genetic changes in other cell lines similar to the ones observed in SKOV3 cells, it will reinforce our prediction of the "universal" role of SPF45; in other words, since splicing factors are not as ubiquitous as transcription factors, if it happens that they are overexpressed in cells they will probably induce similar changes. Biological systems tend to be more complex however, and the "universal" response might not be accurate. It will be interesting though to determine the percentage of agreement in genetic changes in these different systems.

Several clones of drug-resistant SKOV3 have been generated with introduction of increasing doses of chemotherapeutic agents such as cisplatin, taxol, doxorubicin and etoposide. The different mechanisms of resistance included decreased drug retention (Mistry 1992), increased BRCA1 and DNA damage repair (Husain 1998), P-glycoprotein expression (Bradley 1989, Yang 1995), Bcl-xL expression (Liu 1998), MRP-1 (Sharp 1998), decreased topoisomerase II activity (Kubota 1994), ErbB2 levels (Langton-Webster 1994) and more recently decreased RICTOR, RAPTOR and mTOR activities (Foster 2010). Unfortunately, we did not observe any of these changes with the gene array performed. While one or more of these genetic changes might have evaded detection, we also did not observe a change in cellular resistance. Generating different cancer cell lines,

ovarian and non-ovarian, overexpressing SPF45 will be necessary to better address the role of SPF45 overexpression on drug resistance.

Aside from the genetic changes induced by SPF45 and the effect of phosphorylation on its splicing activity, we sought to determine how the phosphorylation affects SPF45 splicing mechanism. For this end we focused on two splicing factors, SF1 and SF3b155, which are important for their role in constitutive splicing of pre-mRNA. SF1 consists of a single polypeptide of 75KDa and is necessary for the formation of the first ATP-dependent step in spliceosome assembly (Arning 1996). SF3b155 is a 155 KDa protein that exists in a complex structure of several Spliceosome-Associated Proteins (SAPs) and therefore is also known as SAP155 (the other proteins are known as SAP49, SAP130 and SAP145) (Das 1999). SF1 contains two [KH] RNA-binding domains and binds the adenine branch point; subsequent to ATP-hydrolysis, SF1 is replaced by SF3b155 (Thickman 2006). SF3b155 would then bind the pre-mRNA in the BPS recruiting U2sNRP forming the A complex (Gozani 1998). Recently, SF3b155 has been shown to regulate the selection of the 5' splice site of Bcl-x pre-mRNA inducing higher expression levels of Bcl-x(s) with its subsequent effect on potentiating the role of chemotherapeutic agents (Massiello 2006). Besides their role in directing the steps of splicing, little is known about SF1 and SF3b155. Manceau et al identified phosphorylations by KIS on a SPSP motif in SF1 and showed that phosphorylated SF1 has higher binding to U2AF65 (Manceau 2006). SF3b155 is known to be phosphorylated by NIPP-1 (nuclear inhibitor of protein phosphatase 1) which recruits PP1 to SF3b155 and subsequently inhibits splicing (Tanuma 2008), although the kinase(s) responsible

remain(s) elusive. In cancer, SF1 deficiency is associated with a decreased incidence of testicular germ cell tumors in 129-Ter and M19 mice (two strains with high incidence of spontaneous TGCT): while SF1-/- are embryologically lethal, SF1+/- mice show marked reduction, indicating a potential role of SF1 in cancer initiation (Zhu 2010). Interestingly, SF1 levels are inversely correlated with the tumorigenic potential of intestinal epithelial cells (Shitashige 2007). In our experiments, we investigated the binding of SF1 and SF3b155 to SPF45 in co-immunopreicipation experiments and the effect of mutating Thr71 and Ser222 on these interactions. While the SPF45 UHM domain interacts with ULMs in SF1 and SF3b155 (Corsini 2007), we provided the first proof of a total protein interaction between these proteins. However, we have not observed a change in binding with $SPF45^{AA}$ or $SPF45^{DD}$. While these findings show that SPF45 interaction with SF1 and SF3b155 is not dependent on intact phosphorylation sites, further experiments need to be performed to confirm it, such as isothermal calorimetric dissociation and NMR-based binding assays. The mechanism that explains how phosphorylation of SPF45 alters splicing remains unfortunately elusive. Whether it affects binding SF1 or SF3b155 or maybe RNA sequences remains to be investigated.

Chapter 4: Summary, limitations, contribution and future directions

Summary

The purpose of this dissertation is to examine the role of MAP kinase phosphorylation of SPF45 in modulating its function(s) in ovarian cancer cells. Our experimental approach is as follows: 1) to show that SPF45 is a substrate of MAP kinase in vitro and in vivo as well as identifying the targeted residues; 2) to determine the phosphorylation patterns of SPF45 in ovarian cancer cells in response to exogenous stimulation of MAP kinase pathways; 3) to determine the effect of the phosphorylation on regulating SPF45 alternative splicing activity towards ΔFas reporter system; 4) to identify genetic changes in SKOV3 ovarian cancer cells overexpressing SPF45 and the effect of phosphosite-mutants on these changes; 5) to identify novel pre-mRNA splicing targets of SPF45 and 6) to examine the role of SPF45 and the phospho-sites mutants in multi-drug resistance in ovarian cancer cells.

SPF45 is a novel substrate of ERK/MAP kinase

Previous work performed in our lab showed the generation of a mutant ERK2 kinase capable of identifying specific kinase targets under the desired cellular conditions (Eblen 2003). We have also published (Appendix I) a report showing increase in ERK2 phosphorylation in SKOV3 cells upon cellular detachment and anchorage-independent growth. Using these same conditions, we have identified SPF45 as a potential novel ERK2 substrate. To demonstrate SPF45 as a genuine MAP kinase substrate, we expressed and isolated SPF45 from Cos-1 cells as well as generated the recombinant protein and subjected both mammalian and bacterially expressed SPF45 to in vitro

kinase assays using not only ERK, but also JNK and p38 MAP kinase, as they can possibly share substrates. We showed SPF45 to be phosphorylated by the three MAP kinase subfamilies and phosphoamino acid analysis detected serine and threonine phosphorylations. As there are only two residues in SPF45 that satisfy the MAP kinase targets consensus sequence, namely Thr71 and Ser222, we generated alanine mutants of these two residues and repeated the kinase assays using active ERK2, which showed no phosphorylation on $SPF45^{AA}$, indicating that Thr71 and Ser222 are the unique MAP kinase target residues. Although not universally applicable, ERK/MAP kinase binds to its substrates prior to phosphorylation. We used three approaches to show that ERK2 and SPF45 exist in a binding complex. First, we identified SPF45 as an ERK2-binding protein in a co-immunoprecipitation of ERK2 from SKOV3 cells. Second, nuclear extracts from HeLa cells mixed with His-ERK2 showed SPF45 binding to ERK in a dose-dependent manner. Finally, using SKOV3 cells stably expressing Myc-SPF45, we showed ERK2 to co-immunoprecipitate with Myc-SPF45, but not empty-vector control. Altogether, we are confident that SPF45 is a genuine ERK2/MAP kinase substrate and we aimed to determine its phosphorylation pattern when exogenously expressed in cells, but more importantly the phosphorylation profile of endogenous SPF45 in ovarian cancer cells.

SPF45 is preferentially phosphorylated in ovarian cancer cells by ERK/MAP kinase and JNK in response to exogenous stimulation

Identification of the phosphorylation sites *in vitro* provided an important clue to generate total SPF45 antibody as well as anti-pThr71-SPF45 and anti-pSer222-SPF45,

since until the time of writing this dissertation these reagents remain commercially unavailable. These antibodies were crucial for the subsequent experiments as they reduced the demand for large amounts of radioactivity to study SPF45 phosphorylation in response to MAP kinase stimulation, as well as reduced the labor required to reach the same conclusions (such as using phosphoamino acid analysis and tryptic peptide mapping). In determining SPF45 phosphorylation in cells, we expressed Myc-SPF45 in Cos-1 cells along with activated upstream activators of ERK, namely KRas G12V, BRaf V600E or CA-MEK1(DD) in the presence or absence of exogenous ERK2. Our data showed phosphorylation of Thr71 and Ser222 under all conditions, however, co-expression of ERK2 increased their phosphorylation, especially Thr71. These results suggested that SPF45 is phosphorylated in cells similarly to *in vitro*, and we confirmed that SPF45 is phosphorylated on both Thr71 and Ser222 when CA-MEK1(DD) and ERK2 are co-expressed in Cos-1 cells, while the phosphorylation of these two residues is lost when they are replaced by either alanine or aspartate. It is interesting to note that aspartate mutants displayed an upward mobility shift; phosphorylated wild-type SPF45 equally displayed an upward shift. Prior to examining SPF45 phosphorylation in ovarian cancer cells, we screened SPF45 expression levels in several ovarian cancer cells and a benign transformed epithelial ovarian cancer cell line, to determine approrpriate cell lines for studying SPF45 phosphorylation in response to exogenous stimuli. Compared to malignant cell lines, IOSE, a benign transformed epithelial ovarian cell line, showed a low level of SPF45 expression. Of the different ovarian cancer cell line tested, A2780 had

the highest level; SPF45 expression in ES2 was moderate and SKOV3 had the lowest expression. All three cell lines were used to determine endogenous SPF45 phosphorylation patterns in response to stimulation of the MAP kinase pathways using known activators. Under the conditions of stimuli tested, SPF45 was phosphorylated primarily in response to ERK and JNK and weakly to p38 MAP kinase. While it does not rule out the possible involvement of p38 in regulating SPF45 phosphorylation, these results suggest that ERK and JNK to be more likely involved in SPF45 regulation. The fact that SPF45 is phosphorylated by ERK and JNK suggests that SPF45 phosphorylation occurs in response to both mitogenic and stress stimuli, which translates as a potential involvement of SPF45 in a wide array of functions. More importantly, it would be interesting to determine the interplay between different signaling cascades in regulating SPF45 activity. Additionally, it should not come as a surprise that SPF45 may be phosphorylated by other kinases; SPF45 amino acid sequence predicts several phosphorylation sites including, but not limited to, Akt and PKA kinases.

MAP kinase phosphorylation of SPF45 decreases its alternative splicing activity towards ΔFas

To examine the implication of SPF45 phosphorylation by ERK/MAP kinase on its alternative splicing activity, we utilized a ΔFas minigene reporter system. We optimized the transfection conditions to obtain, in the presence of SPF45, equal splicing of ΔFas into short and long isoforms; a 1:1 ratio of short:long isoform is used as the basal level of splicing. We showed that co-transfection of CA-MEK1(DD) and ERK2 with SPF45

causes 40% reduction in the ratio of the short/long ΔFas isoforms compared to SPF45 expression alone, and we partially reversed this decrease by using the MEK inhibitor U0126. Equally, we showed a similar effect by phosphorylating SPF45 using MKK3(E)/p38MAP kinase, while our efforts were unsuccessful in determining the impact of JNK overexpression on SPF45 alternative splicing function. To further support that inhibition of phosphorylation would produce the opposite effect, i.e. an increase in short/long ΔFas isoforms ratio, we compared the splicing activity patterns of wild type SPF45, $SPF45^{AA}$ and $SPF45^{DD}$. While $SPF45^{AA}$ induced a modest increase in short/long ΔFas ratio around 16% on average, $SPF45^{DD}$ unexpectedly displayed the strongest increase, an average of 37%. Nevertheless, the splicing activities of wild-type SPF45 and $SPF45^{AA}$ in the presence of CA-MEK1(DD) and ERK2 showed an average of 32% increase in $SPF45^{AA}$ activity. $SPF45^{DD}$ was generated in the scope of mimicking a continuously phosphorylated form of SPF45; however, our experimental data in splicing assays did not support it and $SPF45^{DD}$ failed to act as a phospho-mimetic of SPF45 toward fas splicing. A possible hypothesis that could explain this result is the dynamic nature of splicing factors regulation. Since alternating cycles of phosphorylation and dephosphorylation are thought to direct the function of splicing factors in the regulation of their activities (Prasad 1999, Shin 2004), SPF45 mutants cannot participate in these "regulatory" cycles; wild-type SPF45 on the other hand, is freely available for a continuous upstream phosphorylation by CA-MEK1(DD)/ERK2 or MKK3(E)/p38MAPK and downstream dephosphorylation by cellular phosphatases (yet to be determined),

hence subjected to "phospho-regulation". A potential alternative explanation could lie in the effect of the phosphorylation and/or the mutations in controlling SPF45 subcellular localization either within the nucleus or through altered nuclear-cytoplasmic shuttling. Unfortunately, subcellular localization experiments were not successful in our hands, and they remain potential unanswered questions to be investigated.

SPF45 induces genetic changes in SKOV3 ovarian cancer cells in both phosphorylation-dependent and –independent manners

As there are few known targets for alternative splicing by SPF45 and no reports describing the genetic changes imposed by its overexpression, we addressed these questions by performing an exon-array and a gene-array on SKOV3 cells stably overexpressing exogenous wild-type SPF45; SKOV3-pQCXIP (empty vector control cell line) was used as a control. We identified 158 potentially altered genes levels (high stringency criteria) and 139 potential novel splicing targets of SPF45. However, array data require physical confirmation and we focused on four different genes selected for their expression level changes in the array and their known involvement in cancer biology; they are: ErbB2, fibronectin, transforming growth factor-β and periostin. We also investigated their expression levels in SKOV3-SPF45^{AA} and SKOV3-SPF45^{DD} to assess the effect of Thr71 and Ser222 residues/mutations on SPF45 functions. ErbB2 was roughly 70% decreased in SKOV3-SPF45 compared to vector, while in SKOV3-SPF54^{AA} it was 36% decreased and SKOV3-SPF45^{DD} 80% decreased. ErbB2 mRNA levels were equally translated on the protein level in their corresponding cell lines. Fibronectin

expression was roughly 5 folds increased in wild-type SPF45, SPF45^{AA} and SPF45^{DD} compared to vector control. Real-time RT-PCR data were supported by northern blot of fibronectin and the mRNA levels were equally translated on a protein level (data not shown.) It is interesting to note that Thr71 and Ser222 mutations do not affect fibronectin levels compared to wild-type SPF45. Taken together, the role of mutations of Thr71 and Ser222 on ErbB2 and FN expression suggests the existence of phospho-SPF45-dependent and –independent effects. Two more genes were tested for their relative abundance in the generated stable cells: TGF-β and perisotin. No statistical difference was observed in TGF-β between SKOV3-pQCXIP and SKOV3-pQCSPF45 while both SKOV3-pQCSPF45^{AA} and SKOV3-pQCSPF45^{DD} showed 2 fold increase. Finally, periostin, which - based on the array results - was expected to show the strongest fold increase with SPF45 overexpression, failed to have any statistical difference among the four groups tested. Simply, the levels of periostin were so low, that any change in its concentration from one group to the other would show an "apparent" difference in the array analysis that may not necessarily materializes by physical approaches. In fact, the example of periostin represents one of the crucial reasons to address the results of gene-arrays cautiously and validate these observations with further experiments.

<u>EDA-Fibronectin but not EDB-Fibronectin is a novel splicing target for SPF45</u>

In addition to the genetic changes in fibronectin expression, exon-array analysis predicted several regions within FN to undergo alternative splicing in response to SPF45 overexpression. A caveat of the exon-array we used is its inability to point out to the

specific exons undergoing alternative splicing. Using literature search to check for reported alternative splicing regions of fibronectin, we identified three FN domains, extra domain III A (EDA), extra domain III B (EDB) and a variable (V) region that have been reported to undergo alternative splicing under different disease conditions and in a tissue specific manner. We directed our attention to EDA and EDB and designed primers to check for inclusion/exclusion of these regions in SKOV3-pQCXIP, -pQCSPF45, -pQCSPF45^{AA} and –pQCSPF45^{DD} using semi-quantitative reverse-transcriptase PCR. This method failed to show differences in inclusion/exclusion of these regions as RT-PCR lacks the ability to calculate with precision the ratios of the different bands when it is difficult to determine the reactions outcomes in a linear phase. To overcome this problem, we designed exon-specific primers to EDA and EDB and studied their relative abundance in the four different groups using real-time quantitative RT-PCR. Similar to our previous results, fibronectin was equally increased in SPF45 overexpressing cells (wild type and mutants) and the difference between the three groups was non-significant. Also, EDB-FN was equally elevated in the same groups compared to vector control, and showed a similar fold increase to total fibronectin. However, EDA inclusion was 37% increased in wild-type SPF45 compared to SPF45^{AA} and SPF45^{DD} as well as total fibronectin in wild-type SPF45 stable cells. These data showed that SPF45 is implicated in fibronectin alternative splicing, that mutations of the phosphorylation sites abrogate its effect on EDA inclusion, and that exon-array data, coupled with biophysical techniques, bioinformatics and literature search can be used as a tool to identify the

regulation of alternative splicing of specific genes. Although real-time RT-PCR is not the standard technique to address alternative splicing, it proved to be a useful approach to answer this question for the speed of the experiment as well as its high sensitivity and ability to render a quantifiable measurement. Generation of fibronectin-EDA minigene and/or a fibronectin-EDA pre-mRNA oligomer could be used to address the alternative splicing of this region in response to SPF45 overexpression *in vivo* and *in vitro*, respectively. Although these experiments are the gold standard techniques in studying alternative splicing, they both lack the specificity of studying the splicing in a biological tissue, as we did in the SKOV3 ovarian cancer cells.

Mutation of Thr71 and Ser222 does not affect SPF45 binding to constitutive splicing factors

SPF45-mediated alternative splicing has been explained, *in vitro,* through hydrophobic interactions with constitutive splicing factors in the initial steps of the spliceosome assembly, namely SF1 and SF3b155, via the RNA recognition motif in SPF45 C-terminus domain. To assess possible interactions of SPF45 with these proteins *in vivo*, we performed a co-immunoprecipitation of Myc-tagged SPF45 or mutants using the stable cell lines generated. Indeed, we show that both SF1 and SF3b155 co-immunoprecipitate with wild-type Myc-SPF45, demonstrating in cells a physical interaction between SPF45 and constitutive factors of the splicing machinery. While we expected that $SPF45^{AA}$ and/or $SPF45^{DD}$ to have different binding to SF1 and SF3b155, no difference was observed. Assays that address this process in a dynamic manner, looking

at spliceosome complex in its development and assembly might provide a better clue whether the phosphorylation of Thr71 and/or Ser222 is involved in these interactions.

SPF45 overexpression in SKOV3 ovarian cancer cells does not alter their cytotoxic response to chemotherapy

Although forced SPF45 expression in ovarian and cervical cancer cells is linked to drug resistance, we did not observe such a phenotype in SKOV3 cells. While it is unlikely that a single factor, such as the overexpression of a single protein, is responsible for the multidrug resistance phenotype, SPF45 is a splicing factor that has the potential to affect a large number of downstream targets, many of which could lead in a combined effort to the drug resistance phenotype. The only pathway that we identified in the genetic array to be related to drug resistance in SKOV3 cells involves ErbB2. In fact, ErbB2 levels were shown to decrease in a cisplatin resistant SKOV3 clone (Langston-Webster 1994). The decrease in ErbB2 level in response to SPF45 overexpression, might therefore account for the elevated drug resistance profile of SKOV3 cells. While Perry et. al (Perry 2005) showed that SPF45 induces a drug resistance phenotype in A2780 and HeLa cells, these studies were performed using few highly-resistant clones selected among multiple clones generated, not all of which display resistance; the stable cells we generated represent a heterogeneous population of cells pooled to overexpress SPF45 and hence might not display the similar outcome of individually selected colonies. Investigating the drug resistance phenotype of SPF45 in multiple cancer cell lines, ovarian and non-ovarian, is therefore necessary to address this question. Furthermore,

it would provide a platform to determine the common genetic and splicing events regulated by SPF45 both in a tissue-specific and –nonspecific manner.

Limitations of the study

This study is based in a large extent on the assumption that MAP Kinases phosphorylate SPF45 solely on Thr71 and Ser222. While it is typical to identify *a priori* phosphorylation sites using *in vitro* analysis of kinase-subjected proteins, *in vivo* determination of phosphorylation remains the gold standard for adequate functional analysis and for unraveling the impact the phosphorylation imparts on the substrate. Further experiments using in cells radioactive phosphate labeling of SPF45 for phospho-peptide mapping or alternatively mass spec analysis of SPF45 may possibly reveal phosphorylation sites not previously suspected or identified. In fact, mass spec analysis offers a powerful tool to determine multiple simultaneous post-translational modifications on SPF45 and has a strong potential for a wider insight onto phosphorylated SPF45 regulation. With this technique, the role of other kinases can be further investigated and SPF45 response to kinase activation under different extracellular cues, be assessed. A great advantage of mass spec analysis is its ability to analyze a large number of treatment in a fast and accurate way, generating a large amount of information in a timely and effective manner.

From another perspective, while we determined the phosphorylation of SPF45 in several ovarian cancer cells stimulated with known ERK, JNK and p38 MAPK activators, SPF45 phosphorylation under physiological and pathophysiological conditions was not

addressed. Given that the scope from this set of experiments was to show SPF45's phosphorylation *in vivo*, the question was answered appropriately. However, the study fails to investigate the functional significance of SPF45 phosphorylation under these conditions. It equally fails to address SPF45 phosphorylation under treatment with chemotherapeutic agents. Although drug resistance experiments are non-contributory in this work, investigating SPF45 response to anti-neoplastics adds additional information regarding SPF45 phosphorylation status in response to cellular treatment with chemotherapeutic agents. Unfortunately, the experiments performed in this thesis are limited to evaluating Thr71 and Ser222 solely and do not anticipate phosphorylation of SPF45 on different residues in response to activation of MAP kinase enzymes. Coupling these experiments with mass spec analysis will likely avoid the problem of unspecific bands due to the reactivity of the phospho-specific antibodies and will provide a wider view on the different possible post-translational modifications that occur on SPF45 in response to the different treatments employed.

The data we presented in this thesis showed a role for SPF45 phosphorylation by MAPKs in altering the splicing pattern of Fas. While we proposed in a previous section an explanation for the failure of $SPF45^{DD}$ to demonstrate a similar response to phosphorylated SPF45, we cannot exclude the possibility that increased MAPK activity in the cell can lead to increased Fas alternative splicing in a non-SPF45 mediated mechanism. Under this scenario, SPF45 overexpression facilitates Fas splicing in response to a yet-to-be-determined MAPK-dependent mechanism. Additionally, failure

of SPF45^{DD} mutant to behave similarly to phosphorylated SPF45 raises the suspicion that SPF45^{DD} might have a different impact on the cells than SPF45^{pT71pS222}. Also, since SPF45^{AA} and SPF45^{DD} display similar response in terms of the genetic changes on FN and TGF-β, which supports the importance of Thr71 and Ser222 residues in regulating the functions of SPF45, it may potentially indicate that SPF45^{DD} is not a true phospho-mimetic analog of SPF45^{pT71pS222} or that it selectively functions as a phosphomimetic. If this were to be true, caution is required when using SPF45^{DD} as a mimetic for SPF45^{pT71pS222}.

In this work, we showed that phosphorylation of SPF45 impacts its alternative splicing activity towards Fas. Due to the paucity of known pre-mRNA sequences influenced by SPF45 activity, the study is limited to fas gene spanning exons 5 through 7. While *in vitro* alternative splicing has fallen in favor compared to *in vivo* techniques using minigene systems and PCR approach, partly due to the convenience and simplicity of the minigene approach, and partly because splicing is studied in its quasi-native environment, using *in vitro* techniques offers control over the contents of splicing complex used, and can potentially substantiate the results of the minigene approach. Additionally, it can provide further information about the nucleic-proteomic interactions between SPF45 and the splicing complex, as well as SPF45 interactions with other splicing factors within the spliceosome. Given the large number of potential splicing events subjected to SPF45 regulation, as determined by the exon array analysis, generating minigenes of the predicted sequences will be necessary to further

understand the relationship between phosphorylation of SPF45 and alternative splicing. Alternatively, using the qRT-PCR approach that we use in this thesis to show the alternative splicing of the EDA region of FN, can be applied on a larger scale with specifically designed primers to determine the splicing changes of suspected alternative splicing events. Unfortunately, the array results do not point directly to the alternatively spliced sequences, and further bioinformatics analysis coupled with literature search will be necessary to determine these splicing events.

We have discussed previously the importance of the gene- and exon-array in determining novel genetic and splicing events regulated by SPF45; we equally discussed the limitation of this technique in terms of high rate for false positive results as well as the need for support using biochemical approaches. While we focused our efforts on four distinct genes from the gene array, the list of targets to be investigated is quite large and requires substantial dedication to sift through the data and validate the outcomes. Hence, bioinformatics tools will prove to be essential in speeding up the process. Functional map analysis is an example of linking different genes in a logical and biochemically acceptable pattern and has the potential to identify strong candidates for major molecular changes in the cells. DNA repair, regulation of apoptosis and cellular adhesion and migration are interesting areas for investigation, specifically in the context of cancer biology.

One of the major drawbacks of this study is its inability to reproduce the drug resistance phenotype imparted by SPF45 overexpression in ovarian cancer cells, as

published by Perry et al. (Perry 2005) and hence whether phosphorylation of SPF45 by MAP kinases has any effect on drug resistance. Several caveats can be accounted for to explain this failure. Our study is based on two publications by the same group, where the authors claim a link between SPF45 overexpression and multidrug resistance in A2780 and HeLa cells. Accordingly, the data on which the second aim of our hypothesis is based are weak, given the paucity of supporting evidence. While the rewards of this project can be remarkable, the lack of cumulative supporting data places this study in a high-risk category for failure upon addressing the possible link between phosphorylation of overexpressed SPF45 and drug resistance. Although we expect SPF45 to induce the drug resistance phenotype observed by Perry et al. (Perry 2005) in A2780 cells, it is possible to conclude that the choice of a different cell line, such as SKOV3 cells in this study, might not exhibit a similar behavior. Unfortunately, multiple efforts to generate A2780 cells that stably express SPF45 or its mutants were unsuccessful to test this hypothesis. Additionally, a major difference exists between our approach and that of the other group. Perry and his colleagues proposed a relationship between SPF45 and drug resistance based on few A2780 and HeLa clones - selected from a larger number of SPF45 expressing clones – because of their enhanced drug response profile, while we based our approach on generating pooled populations of cells stably expressing SPF45. As such, it may not come as a surprise the failure of our approach. If that turns out to be the true explanation for this disparity, it would likely be safe to assume that SPF45 does not have a role in drug resistance. However, until stable populations of cells in A2780

and HeLa overexpressing SPF45 are generated, this question remains unanswered. Using a lentiviral system for expression of SPF45 and its phosphorylation-sites mutants and/or an inducible expression system in the aforementioned cells may overcome the difficulties of infection using a retroviral plasmid and re-evaluation of drug resistance. If SPF45 is genuinely responsible for such a phenotype, investigating the mechanism(s) will be crucial. While glutathionylation, overexpression of MRP, PgP and MDR 1 are possible mechanisms for drug resistance, the data by Perry et al (Perry 2005) refute their involvement in SPF45 mechanism of resistance. Enhanced DNA repair and altered expression of apoptotic genes are common pathways to impart drug resistance to cisplatin, doxorubicin, etoposide and vincristine. If efforts to express SPF45 in different cell lines were to be successful and SPF45 does induce a drug resistance phenotype, investigating genetic changes of DNA repair pathways and altered apoptotic genes, presents a plausible starting point to understand its mechanism. Using the results of the gene array in SKOV3 and possibly repeating the gene array analysis among different cell lines may identify common pathways altered in response to SPF45. An interesting aspect of these experiments is the richness of information that can be construed regarding SPF45 roles and functions, regardless of its impact on drug resistance. As mentioned earlier, DNA repair pathways, altered apoptotic genes, cellular adhesion, spreading and migration are important venues in cancer biology and drug discovery worth to be investigated in their own regards.

Contribution and future directions

The importance of this study was identifying a novel mechanism of regulation of splicing through the MAP kinase signaling cascade. As mentioned earlier, there are few accounts of MAP kinase involvement in splicing regulation linking splicing mechanisms to extracellular cues. This fact can have dramatic results in the context of cancer.

We have determined SPF45 splicing activity towards Δ*fas* upon phosphorylation by ERK2 and p38 MAP kinase. It would be interesting to identify SPF45's splicing activity upon phosphorylation by JNK. Similar experiments with activated JNK will unveil the role of JNK on SPF45. Our findings that genetic changes and splicing events are affected by mutations of the MAP kinase target sites suggest a potential role of the different MAP kinase families in controlling SPF45 functions. In fact, such a response is very plausible especially that MAP kinases are activated in specific response patterns depending on the external and internal cellular conditions. Obviously, we are still at the beginnings of identifying the significance of this regulation, especially that SPF45 is overexpressed in, besides ovarian cancer, a broad range of solid tumors including breast, bladder, colon, lung and pancreatic cancer all of which display elevated expression and/or activation of one or more of the different MAP kinase subfamilies as well as several other signaling cascades secondary to oncogenic activation, environmental conditions of cancerous behavior or even chemotherapeutic agents. We show a change in the SKOV3-pQCSPF45 attachment to fibronectin coated wells compared to SKOV3-pQCXIP. Cellular adhesion is a complex process governed by multiple molecules and interactions between the extracellular matrix and intracellular/transmembrane proteins. Additionally, cellular

adhesion is of prime importance for cancer cells: migration, invasion and cellular proliferation are all affected by changes in cellular adhesive properties. Would the observed decrease in SKOV3-pQCSPF45 attachment within the first hour of seeding have any significance for cancer progression such as increased migration or invasion, remains to be determined. Alternatively, the observed increase in TGFβ together with fibronectin expression may enhance cellular compactness, increasing multicellular spheroid formation and invasive phenotypes (Sodek 2009). One approach to identify possible changes in adhesion is to perform a real-time gene array of adhesion pathways. In fact, we have recently obtained a pre-validated 96 well array (Qiagen) capable of detecting changes at the mRNA level of more than 80 different genes involved in cellular attachment to extracellular matrix; any changes identified will have a great impact in determining the role of SPF45 on cellular adhesion. Although not a comprehensive approach, it can provide an important lead into the molecules involved in this phenotype. Besides, attachment (a measure of bound cells) and spreading (a measure of the flattening of adherent cells) assays could be performed to better characterize this phenotype as well as determine the biochemical and structural regulation of the points of attachments also known as focal adhesions.

In understanding the functions and roles of SPF45, an important question always arises: are SPF45 effects universal? Would it confer similar genetic changes and phenotypes in different cell lines? It is actually quite difficult to answer this question. The fact that splicing factors are not as diverse as transcription factors suggest that they

tend to rely on the ratios of the different combinations of splicing factors as well as the strength of interactions with the *cis* sequences in the introns/exons of the pre-mRNA (Mayeda 1993, Faustino 2003, Shin 2004). Accordingly, it is almost impossible to predict the phenotypic outcome because of the heterogeneity of the different cell lines. For example, SPF45 confers drug resistance in HeLa and A2780 cells (Sampath 2003, Perry 2005); however we do not observe a similar phenotype in SKOV3 cells. If we simplistically assume that SPF45 induces similar genetic changes in SKOV3 cells that it induces in HeLa and A2780, then there must be other factors in these cells but not SKOV3 that causes the drug resistance. To answer this question, it would be imperative to investigate SPF45 effects when overexpressed in several cell lines. In fact, we are currently generating stable cell lines in several ovarian cancer cell lines as well as HeLa and MCF-7, a breast cancer cell line, of SPF45 and phospho-sites mutants to test this hypothesis. The importance of our approach is that we generate pooled population of heterogeneous cells that overexpress SPF45 to circumvent the problem of clonal variation. As such, if we fail to identify drug resistance changes in these cells, it will increase our confidence to assume that SPF45-mediated drug resistance is more likely an isolated event of individually selected clones rather than a true SPF45 effect. If on the other hand, drug resistance is enhanced in more than one of these cells, identifying the common genetic/proteomic changes will probably determine the mechanism of this resistance. Additionally, the cell lines generated in HeLa and MCF-7 will help answer several other questions; for instance, it will be interesting to compare the common

genetic changes induced by SPF45 overexpression. It will serve as a direct answer to the role of splicing factors as universal regulators of genetic outcomes. The higher the number of common changes observed, the stronger is the support for this hypothesis. Additionally, common genetic changes determined by gene and exon arrays in different cell lines are likely to be true events due to their overlap as well as decreased bias; it would generate higher likelihood of true genetic and splicing changes induced by SPF45. Testing the adhesive, migratory and invasive properties of the different stable cell lines would also assess the role of SPF45 in cancer.

As the role of SPF45 in cancer is still to be determined and studied, it would be necessary to generate knock-out mice of SPF45 to evaluate the role that it plays in cancer: initiation, progression, maintenance or suppression. Knockout mice of proteins of interest are an invaluable tool in singling out the properties of a protein. It would determine the role of SPF45 in development as well as the different phenotypic changes that could be imparted by lack of its expression. If the deletion turns to be embryonic lethal, conditional knockouts can be generated to study SPF45 functions in a tissue-specific pattern.

We have already mentioned that SPF45 is expressed in several solid tumors, which display varying levels of oncogenic signals and activate a multitude of signaling cascades. The amino acid sequence of SPF45 shows potential phosphorylation sites of several other kinases, many of which, such as PKC and Akt are of prime importance in cancer signaling. Determining the potential regulation of SPF45 phosphorylation by

these different cascades, as well as putative cross-talks with MAP kinase signaling will be exciting as well as important to better understand the link between signaling cascades and splicing machinery in regulating cancer cells and in generating efficient therapeutic interventions.

In conclusion, this dissertation investigated and confirmed the involvement of MAP kinase signaling in the functions of the splicing factor SPF45, regulating its splicing activity and potentially modulating its downstream genetic and splicing targets. SPF45 is but one player among a vast group of cellular factors that widely contribute to the aberrant nature of cancer cells. As our understanding of the molecular mechanisms that govern SPF45 interactions, as well as upstream events that modify its activities, we will get one step closer into developing sound strategies and efficient therapeutic interventions to overcome drug resistance or even cure cancer.

Appendix

ERK ACTIVATION AND NUCLEAR SIGNALING INDUCED BY THE LOSS OF CELL/MATRIX ADHESION STIMULATES ANCHORAGE-INDEPENDENT GROWTH OF OVARIAN CANCER CELLS

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ABSTRACT

Ovarian cancer metastasis involves the sloughing of epithelial cells from the ovary into the peritoneal cavity, where the cells can survive and proliferate in peritoneal ascites under anchorage-independent conditions. For normal epithelial cells and fibroblasts, cell adhesion to the extracellular matrix is required to prevent apoptosis and for proper activation and nuclear signaling of the ERK MAP kinase. The mechanisms of ERK regulation by adhesion have been determined by our lab and others. In this report, we elucidate a novel means of ERK regulation by cellular adhesion in ovarian cancer cells. We demonstrate that ERK and its activator MEK are robustly stimulated after cell detachment from a substratum in several ovarian cancer cell lines, but not a benign ovarian cell line, independent of serum and FAK or PAK activity. MEK and ERK activation was sustained for 48 hr after detachment, while activation by serum or growth factors in adherent cells was transient. Re-attachment of suspended ovarian cells to fibronectin restored basal levels of MEK and ERK activation. ERK activation in suspended cells was dynamically controlled through an autocrine stimulatory pathway and prevalent phosphatase activity. Suspended cells demonstrated higher levels of ERK nuclear signaling to Elk1 compared to adherent cells. Inhibition of ERK activation with the MEK inhibitor U0126 had minor effects on adherent cell growth, but greatly decreased soft agar colony growth. These data demonstrate a unique regulation of ERK by cellular adhesion and suggest a mechanism by which ERK may regulate anchorage independent growth of metastatic ovarian cancer cells.

Extracellular Regulated Kinases (ERKs) are members of the MAP Kinase family of protein kinases that regulate many aspects of cell biology including, proliferation, differentiation, migration, survival, and gene expression [Pearson et al., 2001]. Inactive ERKs reside in the cytoplasm, but following activation translocate to various cellular locations including the nucleus [Chen et al., 1993; Lenormand et al., 1993] where they phosphorylate a variety of substrates to affect gene transcription. ERK activation and nuclear translocation is required for cell proliferation [Brunet et al., 1999] and cell adhesion has been reported to be a requirement for ERK nuclear tranlocation and activation of the Elk1 transcription factor [Aplin et al., 2002]. Cell adhesion is also required for ERK activation by mitogens, as cells in suspension fail to activate ERK in response to growth factors [Lin et al., 1997; Renshaw et al., 1997]. Overexpression of an activated mutant of Focal Adhesion Kinase (FAK) [Renshaw et al., 1999] or p21 Activated Kinase (PAK) [Howe and Juliano, 2000] can stimulate anchorage independent activation of ERK, demonstrating that these proteins act as adhesion-dependent sensors for ERK activation. FAK is activated in adherent cells through autophosphorylation of Tyr397, which forms a binding site for Src [Calalb et al., 1996; Lipfert et al., 1992]. Activation of Raf, MEK and ERK and functional coupling between Raf and MEK and MEK and ERK upon cell attachment to fibronectin is dependent upon activation of PAK [Eblen et al., 2002; Edin and Juliano, 2005; Slack-Davis et al., 2003] through a FAK and Src dependent pathway [Slack-Davis et al., 2003]. This mechanism occurs in part through PAK

phosphorylation of MEK1 on S298, which is required for MEK1 activation and stimulates MEK1/ERK complex formation in newly-adherent cells [Eblen et al., 2002; Slack-Davis et al., 2003].

Ovarian cancer is the fifth leading cause of cancer deaths in women and the deadliest form of gynecological cancer. In humans most ovarian cancers originate from the ovarian surface epithelium (OSE), which is a poorly differentiated mesothelium with a tenuous attachment to the underlying basement membrane [Dietl and Marzusch, 1993]. Dissemination of ovarian cancer cells to the peritoneal cavity involves the loss of attachment of the OSE from the underlying basement membrane or their extravasation from inclusion cysts that form within the ovary [Auersperg et al., 1998]. Ovarian cancer patients often develop peritoneal ascites fluid containing metastatic cells growing individually or as multicellular spheroids, in addition to factors that promote tumor cell growth [Mills et al., 1990; Westermann et al., 1997; Westermann et al., 1998]. Ascitic cells survive and proliferate under anchorage-independent conditions until they attach to the mesothelium of the peritoneal cavity via fibronectin and hyaluronan produced by peritoneal mesothelial cells [Burleson et al., 2004; Casey et al., 2001; Lessan et al., 1999].

Mouse models have identified *ras* as a causative agent in the development of ovarian cancer [Liu et al., 2004; Orsulic et al., 2002]. Indeed, in humans two-thirds of low-grade ovarian tumors have mutations in the *k-ras* or *b-raf* oncogenes , while 16% of

high grade tumors carry *k-ras* mutations [Ho et al., 2004; Singer et al., 2003a; Singer et al., 2003b]. However, activation ERK is observed in 80% of low grade tumors, 40% of high grade ovarian tumors [Hsu et al., 2004], and 91% of pleural and peritoneal effusions from patients with metastatic disease [Davidson et al., 2003], suggesting that other factors also contribute to ERK activation in metastatic ovarian cancer. Downregulation of MAP Kinase Phosphatase-1, a negative regulator of ERK, has been observed in ovarian tumor samples [Denkert et al., 2002]. Overexpression of growth factor receptors that stimulate ERK activation, such as ErbB-2 and c-Met, is observed in ovarian tumors [Berchuck et al., 1990; Di Renzo et al., 1994]. In addition, ascites fluid is a complex milieu rich in mitogens such as lysophosphatidic acid (LPA) and Platelet Derived Growth Factor (PDGF) [Matei et al., 2006], among others, but the cellular origins of most of these factors are unknown.

We examined the regulation of ERK activation by loss of cellular adhesion, an early step in cancer metastasis, in several ovarian cancer cell lines. We demonstrate a strong, sustained activation of MEK and ERK in response to loss of cell/matrix adhesion. Re-attachment of cells restored basal levels of active ERK. Conditioned media from suspended cells could stimulate ERK activation in serum-starved adherent cells, suggesting an autocrine mechanism for ERK activation in suspended cells. ERK signaling to the nucleus was enhanced in suspended cells and inhibition of ERK signaling had profound effects on soft agar growth. These studies demonstrate a novel means of ERK regulation by cellular adhesion and suggest that upregulation of ERK activation through

an autocrine mechanism promotes the ability of ovarian cancer cells to grow anchorage-independent.

MATERIALS AND METHODS

Cells and Reagents. SKOV-3 cells were purchased from American Type Culture Collection (Manassas, VA) and grown in McCoy's 5A media supplemented with 10 % fetal calf serum. IOSE cells were a generously provided by Dr. Nellie Auersperg. CAOV3, ES2, TOV21G, and OV2008 cells were a kind gift from Dr. Runzhao Li. OVCAR3 cells were a kind gift from Dr. Kristen Atkins. ERK2 antibody was kindly provided by Dr. Michael J. Weber. Phospho-ERK antibody was purchased from Sigma (St Louis, MO). FAK and phospho-Y397 FAK antibodies were purchased from Cell Signaling (Danvers, MA). Phospho-MEK, MEK, phospho-AKT, AKT, tubulin and MKP-1 antibodies were purchased from Invitrogen (Carlsbad, CA).

Suspension assay. Six-well dishes coated with 1% agarose were prepared by melting 4% agarose into phosphate buffered saline (PBS). This solution was then mixed with 3 parts serum-free McCoy's 5A media and used to coat 6 well dishes. Adherent cells growing in media containing 10 % fetal calf serum were washed once with PBS and detached with trypsin-EDTA. The trypsin was inhibited with 1 mg/ml soybean trypsin inhibitor (Sigma) in PBS. The cells were collected, counted, pelleted by centrifugation, and resuspended in serum-free media at a concentration of 1 $X10^6$ cells / ml for all experiments. Two milliliters of cells were added to each well. The cells were then incubated for the indicated time at 37 ^{o}C and 5 % CO2 before harvest by gentle pipetting into 15 ml

conical tubes containing 10 ml of cold PBS on ice. Centrifuged cells were snap frozen and stored at -70 °C until lysis. For replating experiments, fibronectin was coated onto dishes overnight at 4 °C at a concentration of 10 □g/ml. Replated cells were put onto these dishes in the same serum-free media that they were suspended in and incubated at 37 °C and 5 % CO_2 until harvest. Citric saline experiments were performed in a similar manner, except that cells were washed, incubated with citric saline for 15 min and collected by gentle pipetting. The cells were centrifuged, washed in PBS, and resuspended in serum free media.

Western blotting. Cells were lysed in M2 lysis buffer [Eblen et al., 2001] and protein concentrations determined using the BCA kit (Pierce, Rockford, IL). Typically, 20 □g of protein was run on an SDS PAGE gel and transferred to nitrocellulose. The membranes were blocked in 5 % milk and incubated with antibodies from 1 hr to overnight. Secondary antibodies conjugated to horseradish peroxidase were followed by enhanced chemiluminescence (Pierce) to visualize proteins.

MTT assay. SKOV-3 or IOSE cells were plated in triplicate into 12 well dishes at a concentration of 1 X 10^3 cells / well. The following day (day 0) the cells were treated in duplicate with either 10 □M UO126 or DMSO vehicle control. On each of the next 4 days, triplicate wells of cells were washed and incubated in MTT reagent for 2 hrs before

harvest in DMSO. MTT activity was measured by reading the absorbance at 570 nm and reference wavelength of 630 nm. These experiments were performed 3 times.

Luciferase assays. SKOV-3 cells were transfected using LipofectAMINE (Invitrogen) with 1 μg of 5X Gal4 Luciferase, 50 ng of Gal4-Elk1 [Roberson et al., 1995], 100 ng of TK Renilla luciferase, and either 100 ng of empty vector or 100 ng of mutationally-activated MEK1 (MEK1 S218/222D) per 6 cm dish. After a 5 hr transfection in serum-free McCoy's 5A medium, the medium was removed, the cells were washed once with PBS and allowed to recover overnight in 10 % serum containing media. The day after transfection the cells were washed twice in PBS and either incubated in serum-free media or trypsinized and suspended in serum-free media, as described above. After 24 hr, both adherent and suspended cells were harvested, lysed and luciferase activity (Renilla and firefly) measured. The ratio of firefly:Renilla luciferase was determined for each sample. Activity in the adherent cells in the absence of activated MEK1 was set to 1 for each experiment. The results are a combination of 5 separate experiments all performed in triplicate. Statistical analysis was performed using the Student's *t*-test and significance determined as $p < 0.05$.

Soft agar assay. SKOV-3 cells were mixed at a density of 5 X 10^3 cells / ml in 0.4 % bacto agar, 10 % serum, and either 10 μM U0126 or DMSO control. One milliliter of this mixture was overlayed onto 35 mm dishes containing a 0.6 % agar plug. Assays were

performed in triplicate. The cells were incubated at 37 °C and 5 % CO_2 for 5 weeks. U0126 or DMSO was administered to the cells every 3 days by dissolving 1 μl DMSO or 1 μl of a 10 mM stock solution of U0126 into 100 μl of serum-free media and overlaying it onto the 1 ml of cells in agar, allowing for drug absorption at a final concentration of 10 μM. After 5 weeks, four random fields of cells from each plate were photographed under a 2.5X objective. Distance on the plate was determined using a known standard. The images were analyzed using Image J software (website: http://rsb.info.nih.gov/ij/) in order to quantitate colony number and individual colony size. Colonies above 400 μm^2 in 4 fields from each triplicate dish were counted. Results from each triplicate were averaged and the standard deviation determined. A Student's *t*-test was performed between DMSO control and U0126 treated dishes and significance determined as $p < 0.05$. The experiment was performed a total of 3 times with similar results. An identical experiment performed with IOSE cells did not yield any soft agar colony growth.

RESULTS

Loss of Cell Anchorage Activates ERK in Ovarian Cancer Cells

Cellular adhesion is required for efficient activation of ERK by mitogens in most epithelial cells and fibroblasts [Lin et al., 1997; Meredith et al., 1996; Renshaw et al., 1997]. To determine if serum could stimulate ERK activation in suspended ovarian cancer cells, adherent SKOV-3 cells were detached with trypsin followed by treatment with soybean trypsin inhibitor. The cells were then put in suspension culture for 3 hr in the presence or absence of serum. Cells under these suspended conditions remain rounded, do not attach to the agarose, and are easily collected by gentle pipetting (data not shown). Adherent SKOV-3 cells in serum had modest ERK activation, whereas cells suspended for 3 hr in serum had robust activation of ERK and MEK (Fig. 1A). Surprisingly, suspended cells incubated in serum-free media had a similar level of MEK and ERK activation as those suspended in serum. Release of cells by both typsin and citric saline and trypsin resulted in a loss of FAK phosphorylation, but an increase in ERK activation (Fig. 1B). In addition, we performed a short time course to look at ERK activation between 0-3 hr after trypsinization or citric sailine treatment. Activation of ERK did not occur until 1-2 hr after cells were in suspension culture under either condition (Figs. 1C and 1D).

To determine if activation of ERK also occurred in benign ovarian epithelial cells in suspension culture, we compared the level of ERK activation in detached SKOV-3 cells to IOSE cells, a benign cell line derived by SV40 large T antigen immortalization of normal ovarian surface epithelium [Auersperg et al., 1994]. After 3 hrs in suspension in serum free media, the basal level of ERK activation in adherent IOSE cells was lost, while ERK activation was strongly induced in SKOV-3 cells (Fig. 1E). To determine if activation of ERK upon detachment occurred in other ovarian cancer cell lines, we screened five other ovarian cancer cell lines for ERK activation in serum free media in response to cellular detachment. We observed an induction of ERK1 and ERK2 activation at 1 and 3 hr after detachment in four of the six cell lines tested (Figure 1F). ERK activation upon detachment was strongest in SKOV-3 cells, which have wild-type *ras* and *b-raf* genes [Yang et al., 2003] , but was also observed in OVCAR3, TOV21G, and CAOV3 cells. Like IOSE cells, ERK activation in adherent OV2008 cells was lost upon cell detachment, as has been reported in many benign and cancerous cell lines [Eblen et al., 2002; Fukazawa et al., 2002; Honma et al., 2006; Howe et al., 2002; Howe and Juliano, 2000; Kraus et al., 2002; Lin et al., 1997; Renshaw et al., 1997; Slack-Davis et al., 2003; Zhang et al., 2006] while ERK activation in adherent ES2 cells remained unchanged after cell detachment. Activation of ERK in suspension was also observed in cells incubated for 3 hr in the presence of 1 % bovine serum albumin, 4 mM EGTA, or a combination of the two, suggesting that ERK stimulation was not due to cell / cell contact or changes in osmolarity (Figure 1G).

Prolonged ERK Activation is FAK and PAK Independent

Growth factor stimulation of ERK in adherent cells is transient, often returning to basal levels within hours of stimulation. SKOV-3 cells are resistant to anoikis and can survive prolonged times in suspension culture [Frankel et al., 2001]. To determine the duration of ERK activation in suspended SKOV-3 cells, we performed a longer time course to determine the activation of ERK and its activator MEK in cells kept in suspension for a 2-day period. Activation of both MEK and ERK in suspended cells was sustained for the entire 48 hr time course (Figure 2A). Microscopic observation (data not shown) as well as immunoblotting for the phosphorylated, active form of focal adhesion kinase (FAK) demonstrated that the cells were not attached to the agar (Figure 2A), as Y397 phosphorylation of FAK only occurs in adherent cells [Calalb et al., 1996]. We have previously reported that MEK1 and ERK activation by cellular adhesion to the extracellular matrix requires phosphorylation of MEK1 on Ser 298 by PAK [Eblen et al., 2002; Slack-Davis et al., 2003], is negatively regulated by ERK feedback phosphorylation of MEK1 on T292 [Eblen et al., 2004] and that both were cell adhesion-dependent [Eblen et al., 2004; Slack-Davis et al., 2003]. To investigate whether activation of MEK1 and ERK in suspended ovarian cancer cells was controlled by enhanced phosphorylation of MEK1 S298 in the absence of T292 phosphorylation, we immunoblotted for both of these phosphorylation sites on endogenous MEK1 using phospho-specific antibodies. The results demonstrate that the prolonged activation of MEK and ERK in suspended SKOV-3 cells was independent of PAK signaling, due to the absence of Ser 298 phosphorylation

in suspended cells. Interestingly, Thr 292 phosphorylation of MEK1 had become anchorage independent due to the enhanced activation of ERK in suspended cells. These data demonstrate that MEK and ERK activation in suspended ovarian cells is independent of signaling from FAK and PAK and occurs even in the presence of a negative feedback loop. We next compared the duration of MEK and ERK activation in suspended cells in the presence or absence of serum. MEK and ERK activation were equally robust in suspended cells under both conditions at 3, 24, or 48 hr (Figure 2B). Phosphorylation of AKT, which was present in adherent cells, was not significantly enhanced upon cell suspension, while FAK activation was greatly reduced.

Acute Mitogen Treatment of Adherent Cells Stimulates Transient ERK Activation

Mitogen stimulation of cells typically results in robust ERK activation within minutes, returning to basal levels after several hours. To determine if the prolonged activation of ERK in suspension under serum and serum free conditions also occurred in adherent cells stimulated with mitogens, we first determined the ability of serum, epidermal growth factor (EGF), and hepatocyte growth factor (HGF) to stimulate ERK in adherent SKOV-3 cells. SKOV-3 cells overexpress both ErbB-2 [Wiechen et al., 1999] and c-Met [Corps et al., 1997], the HGF receptor, and both EGF and HGF have been shown to be important in ovarian cancer biology [Berchuck et al., 1990; Huntsman et al., 1999; Meden et al., 1998]. Cells were serum-starved overnight and then stimulated with increasing amounts of each mitogen for 10 min. (Figure 3A). Serum was a poor

stimulator of ERK activity at this time point, while EGF induced robust ERK activation at all concentrations. HGF was a moderate activator of ERK at the highest concentration tested. To determine the duration of ERK activation by each mitogen, a time course was performed after stimulation with 1 ng/ml EGF, 10 ng/ml HGF (Figure 3B) or 10% serum (Figure 3C). ERK activation with each mitogen was transient, returning to basal levels within several hours. These data demonstrate a fundamental difference in the duration of ERK activation stimulated by growth factor addition in adherent cells versus activation due to loss of cell attachment.

Cell / Matrix Re-attachment Restores Basal MEK and ERK Activation

To directly test the effects of extracellular matrix attachment on ERK activation, SKOV-3 cells were detached and put into suspension for 3 hr. The cells were then either left in suspension for additional time or allowed to re-attach to fibronectin-coated dishes (Fig. 4A). Fibronectin is one of the main extracellular matrix proteins that is produced by mesothelial cells of the peritoneal cavity and is important for attachment and migration of ovarian cancer cells to the mesothelium [Casey et al., 2001; Lessan et al., 1999]. Replating suspended SKOV-3 cells onto fibronectin resulted in a restoration of basal MEK and ERK activity within 2 hr, while both proteins remained active in parallel cultures of cells that were kept in suspension. Phosphorylation of MEK1 on Ser298 and FAK on Tyr397 were both restored upon cell adhesion. To compare the response of benign versus malignant ovarian cancer cells in this assay, IOSE and SKOV-3 cultures

were detached and suspended in parallel. The cells were then either kept in suspension or replated onto fibronectin-coated dishes, as in Figure 4A. MEK and ERK activation was enhanced in detached SKOV-3 cells (Fig. 4B), as above. Upon reattachment of the cells to fibronectin coated dishes, basal levels of MEK and ERK activation were restored within hours. Conversely, the level of activation of ERK and MEK in adherent IOSE cells was lost in detached cells, but restored upon adhesion of the cells to fibronectin. Phosphorylation of FAK and S298 of MEK1 were adhesion dependent in both cell lines. Protein levels of MKP-1, a nuclear MAP Kinase phosphatase, are down-regulated in ovarian cancer [Denkert et al., 2002], were much lower in SKOV-3 cells compared to IOSE cells, but did not significantly vary with the attachment state of the cells. Collectively, these data directly demonstrate that cellular adhesion to extracellular matrix and/or cell spreading acts to down-regulate ERK in malignant, but not benign, ovarian cells.

Dynamic Regulation of ERK Activation by an Autocrine Mechanism

We reasoned that if the activation of MAP Kinase in suspended ovarian cancer cells is due to low expression/ activity of ERK-specific phosphatases, such as MKP-1, suspended cells should have low MEK activity and there should be a slow turnover of ERK phosphorylation. Therefore, inhibition of upstream signaling with the MEK inhibitor U0126 should only decrease MAP Kinase phosphorylation after a prolonged treatment. We put SKOV-3 cells in suspension for 3 hr and treated them with U0126 for the last 2

min, 5 min, 10 min, 15 min, 30 min or the entire 3 hr before harvest (Fig. 5A). Interestingly, inhibition of MEK activity with U0126 for as little as 2 minutes before harvest caused the complete dephosphorylation of active ERK within the cell. Similar results were seen with 2 minutes of U0126 addition over a 48 hr time course in suspension (Fig. 5B). Therefore, even in the presence of reduced MKP-1 expression, the enhanced activation of ERK in suspended cells is not due to loss of phosphatase activity towards ERK, but is instead a very dynamic process involving activation by MEK followed by a rapid inactivation of ERK by phosphatases.

Since activation and inactivation of ERK in suspended cells was dynamic, we sought to determine if this activation was due to an autocrine stimulatory pathway. We collected conditioned media from serum starved cells suspended for either 3 hr or 24 hr (Fig. 6A). Activation of MAP Kinase in SKOV-3 cells was evident at both 3 and 24 hr in suspension, with greater activation at 24 hr. Either fresh serum-free media or the conditioned media from the suspended cells was then added to adherent, serum-starved SKOV-3 cells. Addition of conditioned media from suspended cells, but not fresh serum-free media, to adherent SKOV-3 cells resulted in a rapid activation of MEK and ERK in the adherent cells. A strong activation, equal to the level in suspended cells, was observed at 5 min after addition. Activation was reduced at 15 and 60 min after addition but was much higher than basal levels. Performing a longer time course of ERK activation using conditioned media from 24 hr suspended cells demonstrated that basal levels of ERK activation were restored between 3 and 6 hr after addition (Fig. 6B). These

data demonstrate that suspended cells produce a soluble factor that acts in an autocrine fashion to stimulate intracellular signaling to MEK and ERK.

Enhanced ERK Nuclear Signaling in Suspended Cells

Upon activation of adherent cells with a mitogen, ERK translocates to the nucleus [Chen et al., 1993; Lenormand et al., 1993], which is required for cellular proliferation [Brunet et al., 1999]. It has been reported that ERK cannot signal to its nuclear substrate Elk1 in the absence of cellular adhesion [Aplin et al., 2001]. We used an Elk1-responsive luciferase reporter system [Roberson et al., 1995] to test for the ability of ERKs to signal to the nucleus in suspended SKOV-3 cells. Cells were transfected with plasmids encoding a GAL4-Elk1 fusion protein, 5X GAL4 luciferase [Roberson et al., 1995], and TK Renilla luciferase. The following day the cells were either serum starved or put in suspension in serum free media. Adherent and suspended cells were harvested 24 hr later and luciferase activity determined (Figure 7). The TK Renilla reporter demonstrated an overall reduction in transcription in suspended cells (data not shown). Normalizing to Renilla luciferase, ERK-responsive firefly luciferase activity was 2.5 fold higher in suspended SKOV-3 cells compared to adherent cells (Fig. 7). Co-transfection of a mutationally activated MEK1 enhanced ERK nuclear signaling in both adherent and suspended cells, demonstrating that enhanced ERK activation in adherent cell lines could stimulate luciferase production and that the difference in endogenous signaling was due to the enhanced ERK activation in suspended cells. These results demonstrate

that cellular detachment in SKOV-3 cells enhances MAP Kinase signaling to the nucleus and regulation of gene transcription.

Enhanced Role of ERK in Suspended Cells

ERK activation has a known role in cell proliferation. To determine the effect of ERK activation on anchorage dependent versus anchorage independent cell growth, we first performed cell proliferation assays on adherent SKOV-3 and IOSE cells. Cells were plated and treated the following day with either 10 ?M U0126 or DMSO control. Cells were treated with MTT reagent 2 hr before harvest over the next 4 days (Fig. 8A). After 4 days of treatment, cell proliferation in U0126 treated cells was decreased 22 % in IOSE cells and only 12% in SKOV-3 cells, both of which, while small differences, were statistically significant. These results suggest that SKOV-3 cells have a reduced requirement for ERK activity in regards to adherent proliferation compared to IOSE cells. Since we have observed an enhanced activation of ERK in detached SKOV-3 cells, we hypothesized that ERK inhibition may have a greater effect on anchorage independent growth. Therefore, we determined the ability of IOSE and SKOV-3 cells to grow in soft agar in the presence of DMSO or the MEK inhibitor U0126. Cells were treated with DMSO or U0126 at the time of plating and every 3 days afterwards. IOSE cells demonstrated no growth in soft agar (data not shown), as expected. Conversely, SKOV-3 cells produced colonies in soft agar, as reported [Pegues et al., 1999; Popadiuk et al., 2006]. We observed an overall reduction in colony number and size in the presence of

U0126 (Figs. 8B and 8C). U0126 treatment decreased colony growth by 69% compared to DMSO treated cells, demonstrating that inhibition of ERK activation has profound affects on anchorage independent growth of ovarian cancer cells.

DISCUSSION

We present the novel finding of strong and sustained activation of MEK and ERK in ovarian cancer cell lines in response to loss of cell adhesion. To our knowledge, this is the first report of loss of cell adhesion stimulating an induction of ERK activity. ERK activation in suspension was dynamically regulated by autocrine activation and robust phosphatase activity. Re-attachment of suspended cells restored basal ERK activity. ERK signaling to the Elk1 transcription factor was enhanced in suspended cells and MEK activity was required for soft agar growth. These results demonstrate a unique mode of ERK regulation by cell attachment and demonstrate a novel mechanism of ERK activation that occurs specifically during loss of cell adhesion, which has implications for anchorage independent growth of ovarian cancer cells.

In benign epithelial cells and fibroblasts, ERK activation by growth factors requires cell adhesion to the extracellular matrix [Lin et al., 1997; Renshaw et al., 1997] Loss of cell attachment inactivates the ERK pathway due to inhibition of PAK by PKA [Howe and Juliano, 2000] and uncoupling of MEK and ERK [Eblen et al., 2002]. ERK inactivation in suspended cells has also been noted in several cancer cell lines, including breast cancer [Fukazawa et al., 2002], small cell lung cancer [Kraus et al., 2002], and in highly metastatic melanoma [Zhang et al., 2006] and hepatocellular carcinoma [Honma et al., 2006] cell lines. This suggests that loss of ERK activation in suspension does not just occur in benign cells, but also in cancer cells as well.

ERK activation in newly-adhering cells requires activation of the Rac / PAK pathway, which acts to enhance activation of the ERK pathway through PAK phosphorylation of Raf on Ser338, leading to Raf activation [Edin and Juliano, 2005]. We have shown that PAK phosphorylation of MEK1 on Ser 298 is required for MEK1 activation upon cell adhesion [Slack-Davis et al., 2003] and functional coupling between MEK1 and ERK, which are dissociated in suspended cells [Eblen et al., 2002]. Expression of mutationally-activated FAK [Renshaw et al., 1999] or PAK [Howe and Juliano, 2000] in suspended cells has been shown to result in ERK activation. However, we demonstrate that MEK and ERK activation in suspended ovarian cancer cells does not occur through a mechanism involving aberrant PAK activity leading to anchorage-independent phosphorylation of MEK1 on Ser298. These results suggest that signaling to MEK and ERK in suspended SKOV-3 cells is through a mechanism that does not require pathways that crosstalk for ERK activation in adherent cells. Indeed, inactivation of FAK was observed in suspended SKOV-3 cells and we have previously shown that PAK activation in newly-adherent cells requires FAK activity [Slack-Davis et al., 2003]. Interestingly, we observe that feedback phosphorylation of MEK1 on Ser292 [Mansour et al., 1994], which is normally adhesion dependent and acts as a negative regulatory mechanism for MEK1 activation during cell adhesion [Eblen et al., 2004], is not diminished in suspended SKOV-3 cells due to sustained activation of ERK. We currently show that it does not play an inhibitory feedback role in the context of loss of cell adhesion.

ERK activation in response to EGF stimulation in adherent SKOV-3 cells was robust and returned to basal levels within hours, as has been reported extensively in the literature for other cell types. However, ERK activation in newly-detached cells in the absence of any external mitogen stimulation occurred with slow kinetics accompanied by sustained activity. One possibility for this slow but sustained activation of ERK is the loss of ERK-directed phosphatase activity, through a mechanism in which basal activation of MEK slowly phosphorylates ERK and there is insufficient phosphatase activity to restore basal levels of ERK activity. Indeed, reduced expression of MKP-1 occurs in ovarian tumor specimens compared to normal ovarian tissue [Denkert et al., 2002] and we observed lower levels of MKP-1 in SKOV-3 cells compared to benign IOSE cells. Steinmetz [Steinmetz et al., 2004] demonstrated that forced overexpression of MKP1 in malignant granulosa cells, which normally express low levels of MKP1 and have elevated levels of active ERK, lowers basal levels of ERK phosphorylation to that seen in benign ovarian cells. They concluded that low levels of MKP-1 result in enhanced ERK activity in these cells, which was supported by undetectable levels of active MEK [Steinmetz et al., 2004]. However, we observed normal kinetics of growth factor stimulation of ERK in adherent SKOV-3 cells by EGF, HGF, and serum and no difference in MKP-1 protein levels in suspended versus adherent SKOV-3 cells. Moreover, we demonstrate a robust and sustained activation of MEK in suspended SKOV-3 cells and demonstrate that blocking upstream signaling from MEK to ERK by the addition of

U0126 for as little as 2 minutes resulted in a complete dephosphorylation of ERK. Thus, while MKP-1 levels are lower in SKOV-3 cells compared to benign IOSE cells, there is sufficient phosphatase activity present from either MKP1 or other ERK phosphatases [Camps et al., 2000] to stimulate rapid turnover of ERK phosphorylation. Our results demonstrate that ERK activation in detached SKOV-3 cells is dynamically regulated by a skewed balance between robust upstream activation by MEK through an autocrine mechanism, which is favored over and counteracted by prevalent phosphatase activity. This balance is shifted towards MEK and ERK inactivation in adherent cells to allow for inactivation of MEK and ERK after cell stimulation. The signaling pathways that restore this balance upon cell adhesion will be an interesting area to investigate, as they regulate production of the autocrine factor or the way that the cell responds to the factor.

Aplin et al. [Aplin et al., 2001] demonstrated a requirement for cellular adhesion in order for ERK to signal to the nucleus in NIH3T3 cells, correlating with an inhibition of ERK activity in suspended cells. While co-transfection of a mutationally-activated form of Raf or MEK could stimulate ERK activity in suspended NIH3T3 cells, signaling to Elk1 in the nucleus required cell attachment [Aplin et al., 2001]. We demonstrate that the enhanced activation of MEK and ERK in suspended SKOV-3 cells allows for enhanced nuclear signaling to transcription factors compared to adherent cells. Overall transcription was greatly reduced in suspended cells, but ERK signaling to Elk1 was 2.5

fold higher than in adherent cells. When endogenous ERK was artificially activated by co-transfection of a constitutively activated MEK, ERK nuclear signaling was not statistically significant between adherent and suspended cells. This suggests that some ovarian cancer cells have developed mechanisms to support nuclear translocation of activated ERK under conditions of anchorage independence. The enhanced gene transcription may then contribute to anchorage independent growth. The mechanism of ERK nuclear translocation under anchorage independent conditions has not been elucidated, but may provide novel targets for therapeutic intervention.

The effects of ERK signaling in suspended cells may not only be transcriptional, but non-genomic as well. In MCF7 cells, arrest of cells at G1/S results in transient activation of ERK in suspended cells, preventing apoptosis through suppression of Bim-EL, which is pro-apoptotic [Collins et al., 2005]. ERK phosphorylation of Bim-EL stimulates its degradation, promoting cell survival [Ley et al., 2004; Luciano et al., 2003; Marani et al., 2004]. However, the mechanism for anchorage-independent activation of ERK in ovarian cancer cells is apparently not due to G1 growth arrest, as MEK was inactive in the suspended MCF7 cells and activation of ERK was thought to occur through loss of phosphatase activity [Collins et al., 2005].

We have shown that conditioned media from serum-starved suspended cells was able to stimulate ERK activation in serum-starved adherent cells, demonstrating the

presence of an autocrine pathway activated in detached cells. At present, the identity of the autocrine signal is unknown. Several mitogens, such as lysophosphatidic acid (LPA), platelet derived growth factor (PDGF) [Matei et al., 2006] and others have been identified in ovarian ascites fluid. LPA was shown to be secreted by peritoneal mesothelial cells, stimulating the migration of ovarian cancer cells [Ren et al., 2006]. It is doubtful that LPA is responsible for the autocrine signal, as LPA is the main mitogen in serum and serum was a weak stimulator of ERK activation in adherent SKOV-3 cells; however, we have not ruled out this possibility, as high levels of LPA may be produced from suspended cells. Signaling through the EGF receptor by either direct or indirect means could also be a possibility, as SKOV-3 cells overexpress EGF receptor family members and EGF stimulates robust ERK activation in these cells. EGFR and ErbB-2 are both overexpressed in a significant percentage of ovarian tumors and correlate with poor prognosis [Berchuck et al., 1990]. The identification of the autocrine signal is currently under investigation in our laboratory, as it could serve as a marker for metastatic ovarian cancer. In addition, we are currently discerning the upstream intracellular signaling proteins that are utilized by the autocrine signal to enhance ERK activation, as these may give insight into signaling pathways induced by anchorage independent conditions of ovarian cancer cell growth in pleural and peritoneal ascites.

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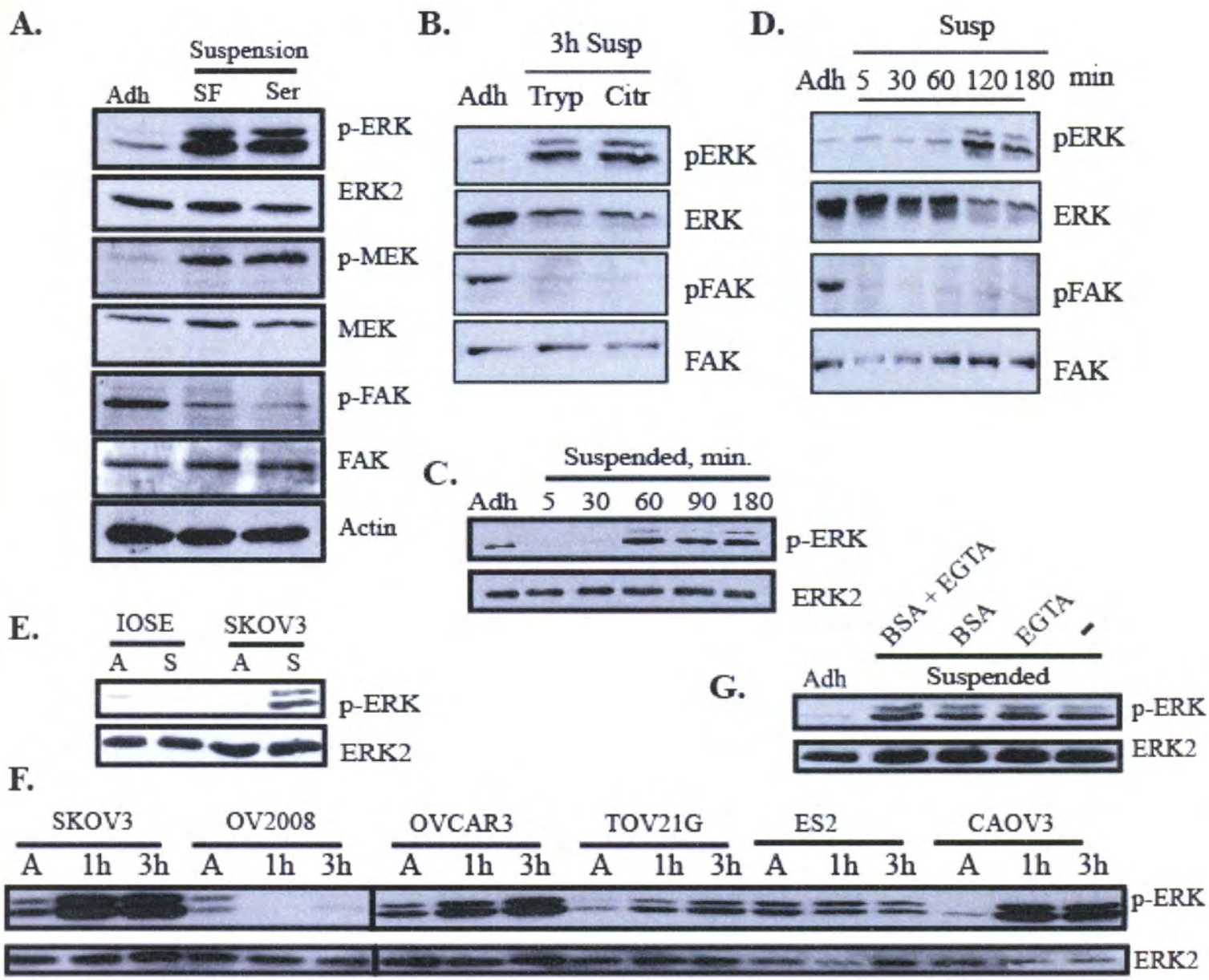
Figure 1


A.
Suspension
Adh
SF
Ser
p-ERK
ERK2
p-MEK
MEK
p-FAK
FAK
Actin
B.
3h Susp
Adh
Tryp
Citr
pERK
ERK
pFAK
FAK
D.
Susp
Adh 5 30 60 120 180 min
pERK
ERK
pFAK
FAK
C.
Suspended, min.
Adh 5 30 60 90 180
p-ERK
ERK2
E.
IOSE
SKOV3
A S A S
p-ERK
ERK2
G.
BSA + EGTA
BSA
EGTA
-
Adh
Suspended
p-ERK
ERK2
F.
SKOV3
OV2008
OVCAR3
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ES2
CAOV3
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p-ERK
ERK2

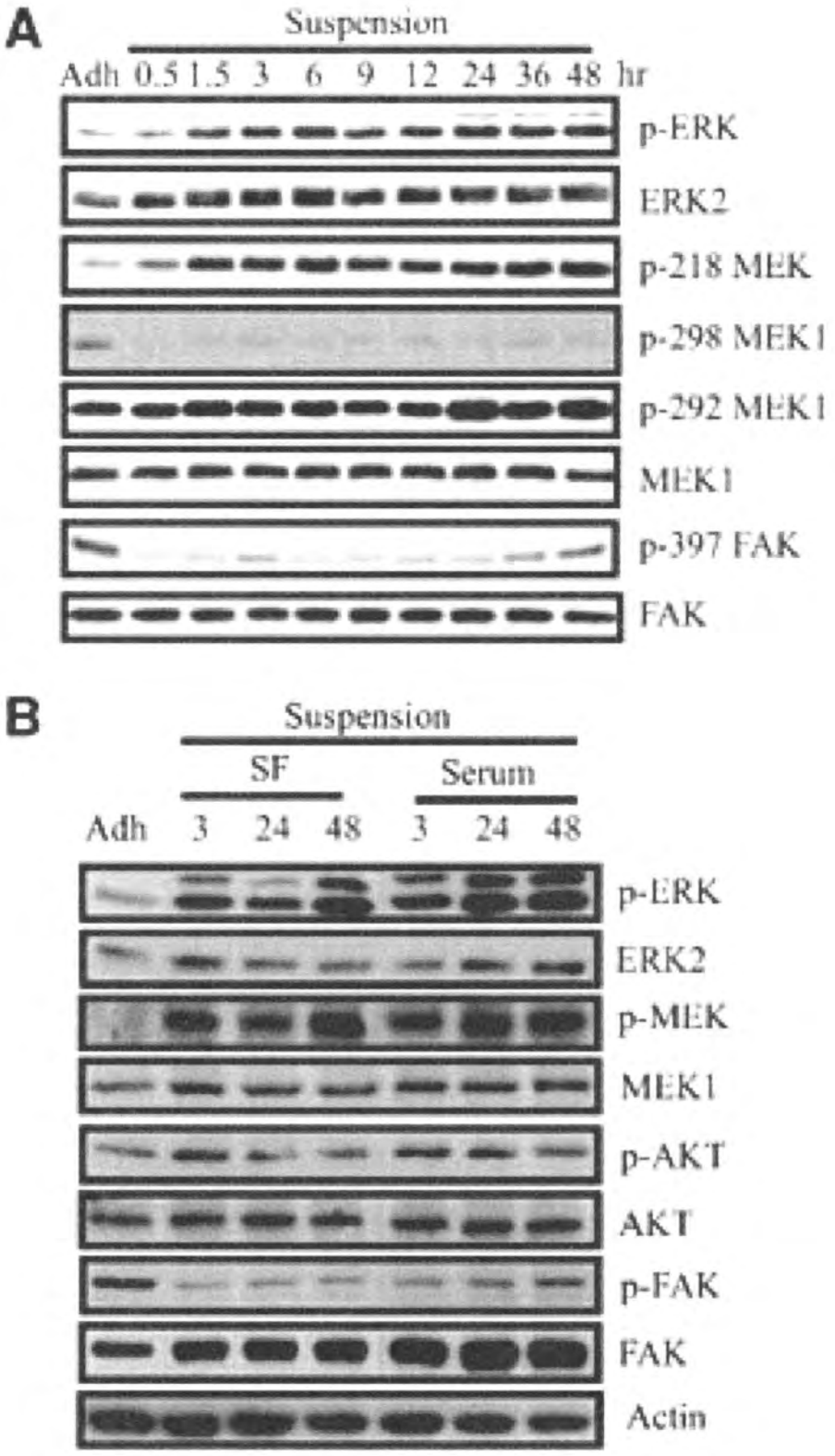

A
Suspension
Adh 0.5 1.5 3 6 9 12 24 36 48 hr
p-ERK
ERK2
p-218 MEK
p-298 MEK1
p-292 MEK1
MEK1
p-397 FAK
FAK
B
Suspension
SF
Serum
Adh 3 24 48 3 24 48
p-ERK
ERK2
p-MEK
MEK1
p-AKT
AKT
p-FAK
FAK
Actin

Figure 2

Figure 3

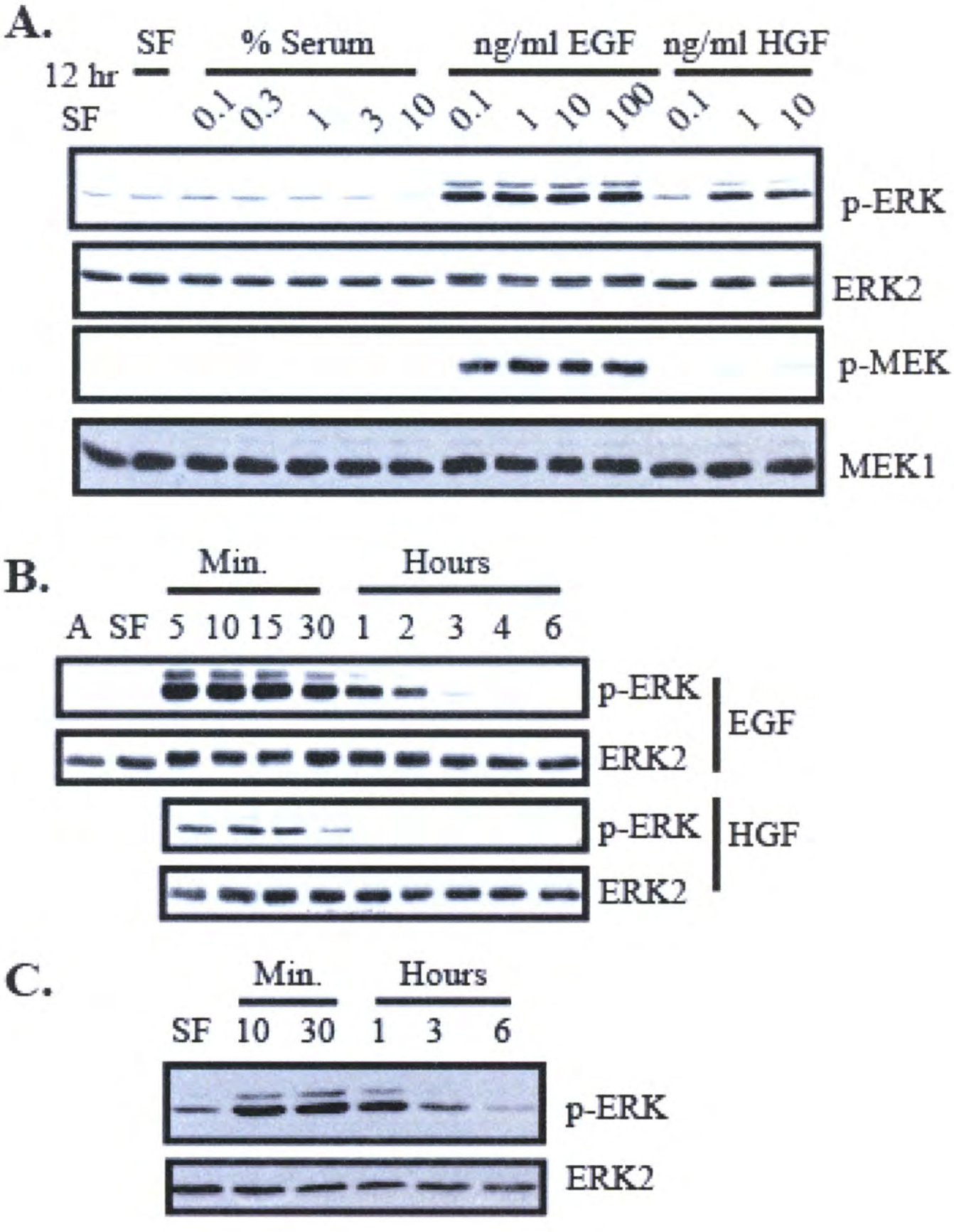

A.
12 hr SF
SF
% Serum
0.1 0.3 1 3 10
ng/ml EGF
0.1 1 10 100
ng/ml HGF
0.1 1 10
p-ERK
ERK2
p-MEK
MEK1
B.
Min.
Hours
A SF 5 10 15 30 1 2 3 4 6
p-ERK
ERK2
EGF
p-ERK
ERK2
HGF
C.
Min.
Hours
SF 10 30 1 3 6
p-ERK
ERK2

Figure 4

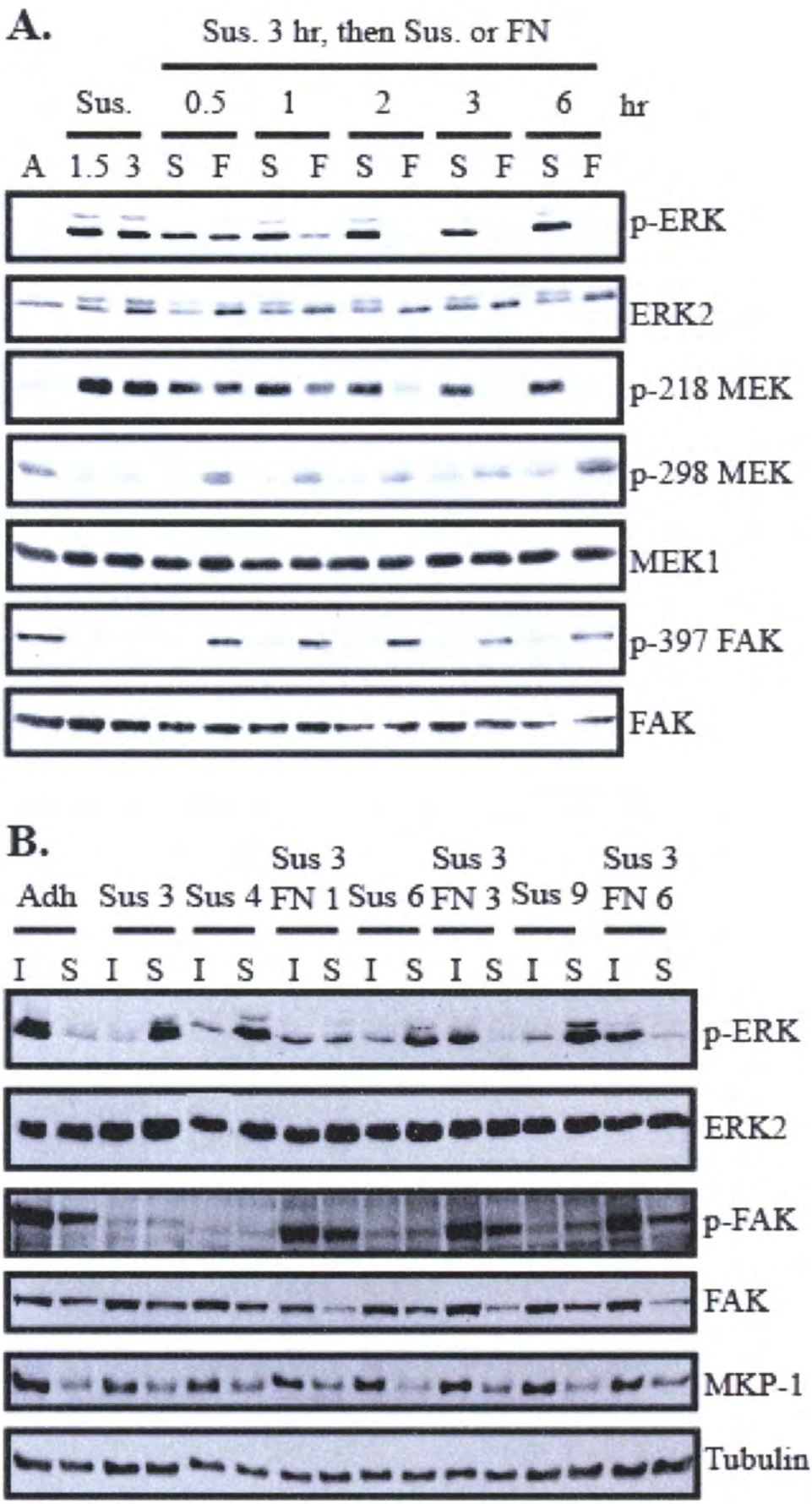

A.
Sus. 3 hr, then Sus. or FN
Sus.
0.5
1
2
3
6
hr
A 1.5 3 S F S F S F S F S F
p-ERK
ERK2
p-218 MEK
p-298 MEK
MEK1
p-397 FAK
FAK
B.
Adh
Sus 3
Sus 4
Sus 3 FN 1
Sus 6
Sus 3 FN 3
Sus 9
Sus 3 FN 6
I S I S I S I S I S I S I S I S I S
p-ERK
ERK2
p-FAK
FAK
MKP-1
Tubulin

Figure 5

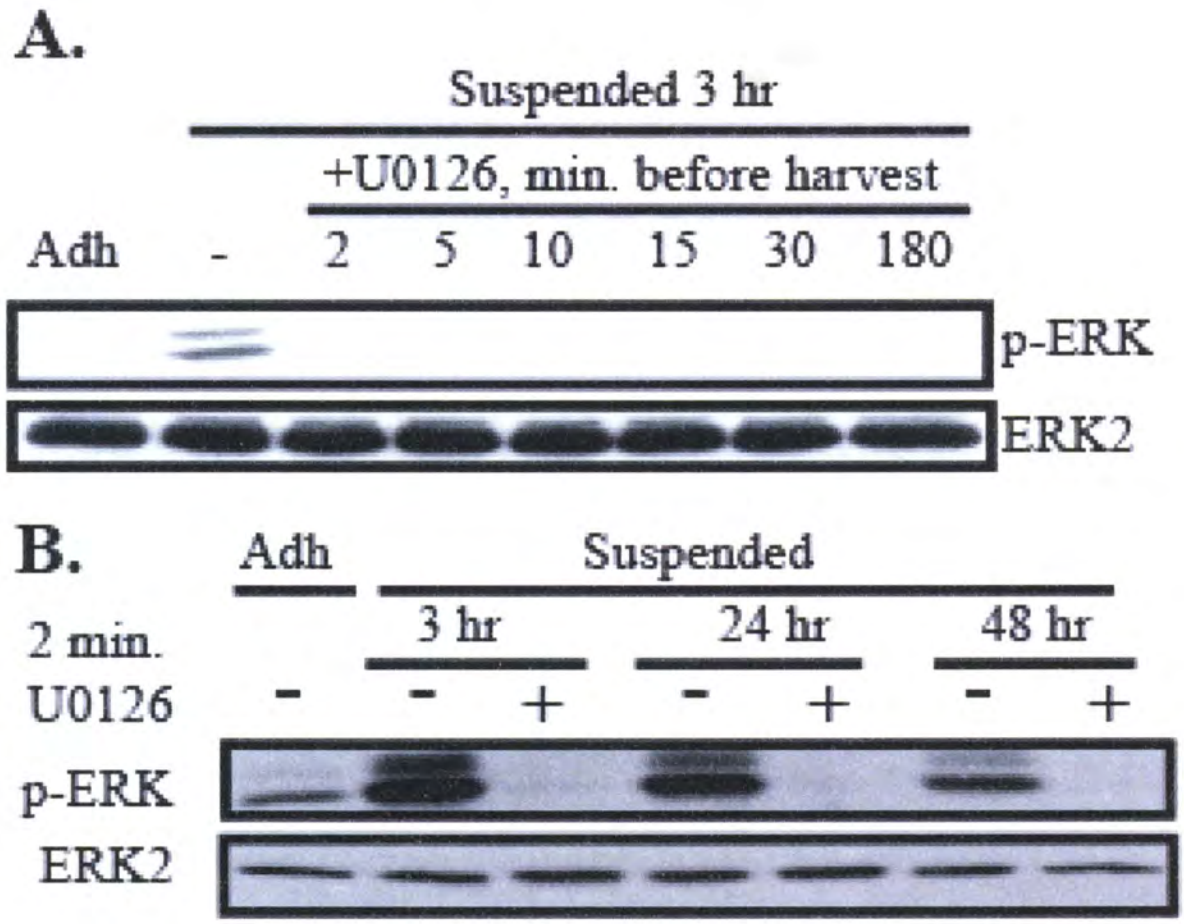

A.
Suspended 3 hr
+U0126, min. before harvest
Adh
-
2
5
10
15
30
180
p-ERK
ERK2
B.
Adh
Suspended
2 min.
3 hr
24 hr
48 hr
U0126
-
-
+
-
+
-
+
p-ERK
ERK2

Figure 6

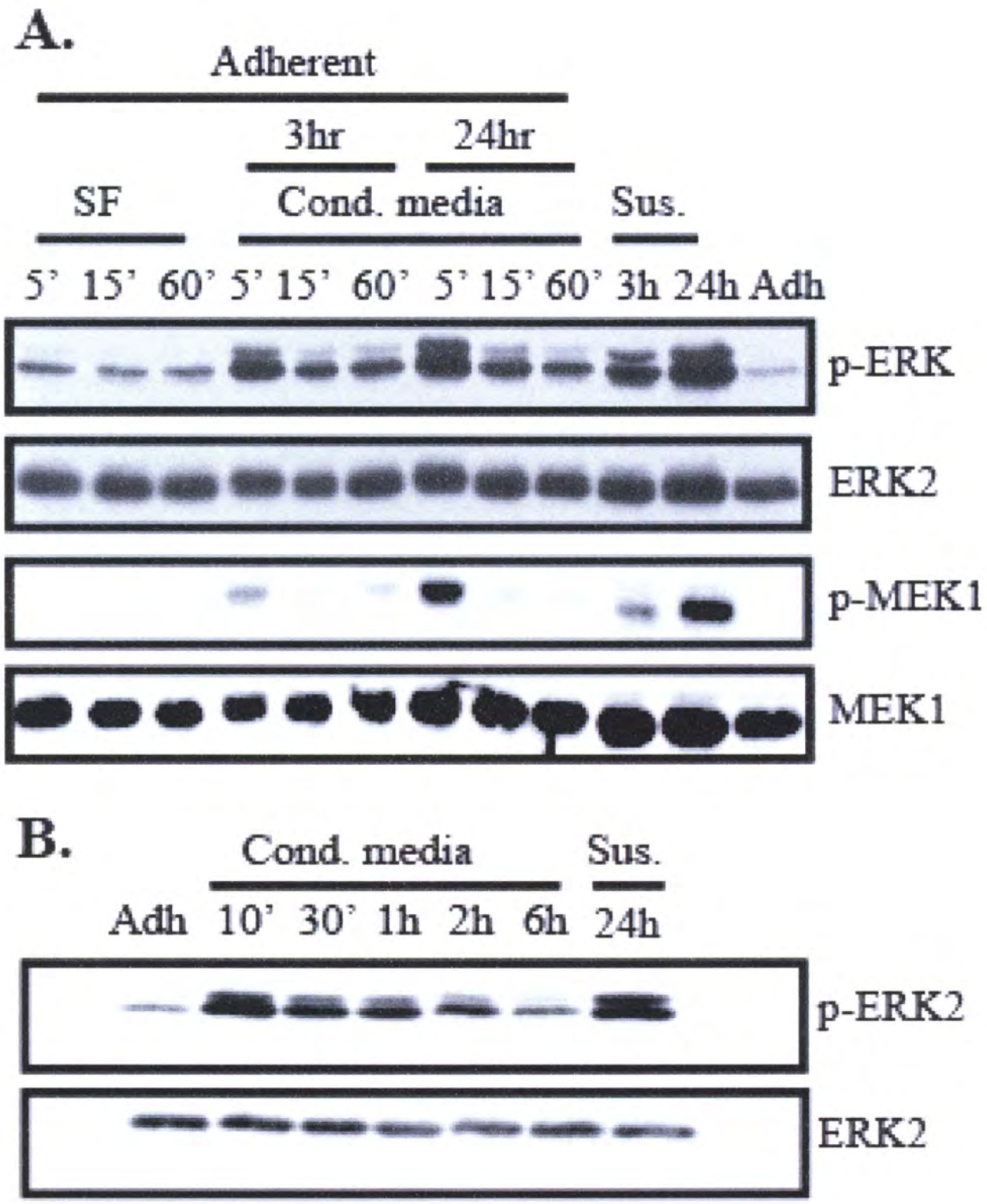

A.
Adherent
3hr
24hr
SF
Cond. media
Sus.
5' 15' 60' 5' 15' 60' 5' 15' 60' 3h 24h Adh
p-ERK
ERK2
p-MEK1
MEK1
B.
Cond. media
Sus.
Adh 10' 30' 1h 2h 6h 24h
p-ERK2
ERK2

Figure 7

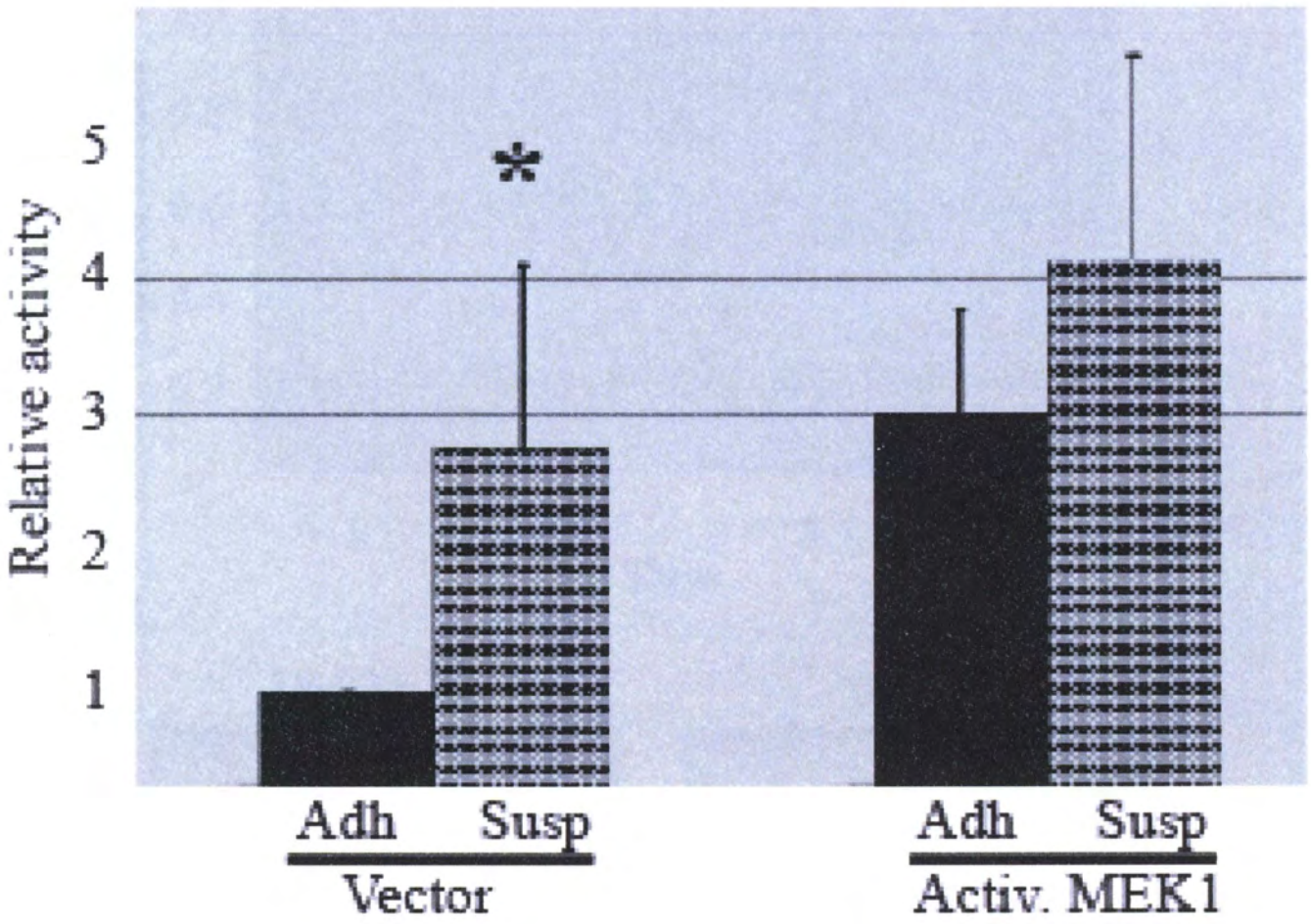
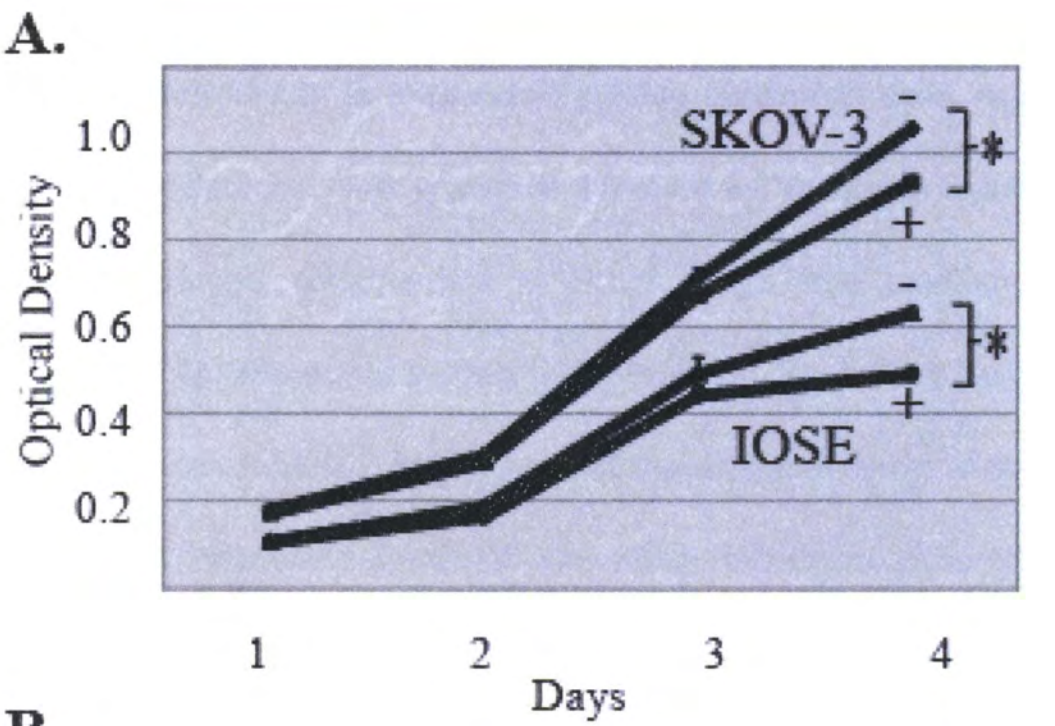
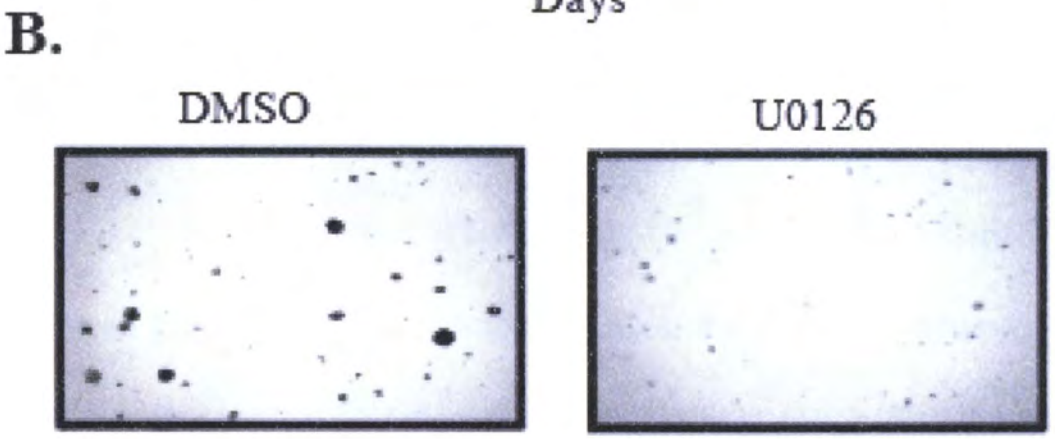

Relative activity
5
4
3
2
1
*
Adh
Susp
Vector
Adh
Susp
Activ. MEK1

Figure 8


A.
Optical Density
1.0
0.8
0.6
0.4
0.2
SKOV-3
IOSE
-
+
-
+
*
*
1
2
3
4
Days


B.
DMSO
U0126

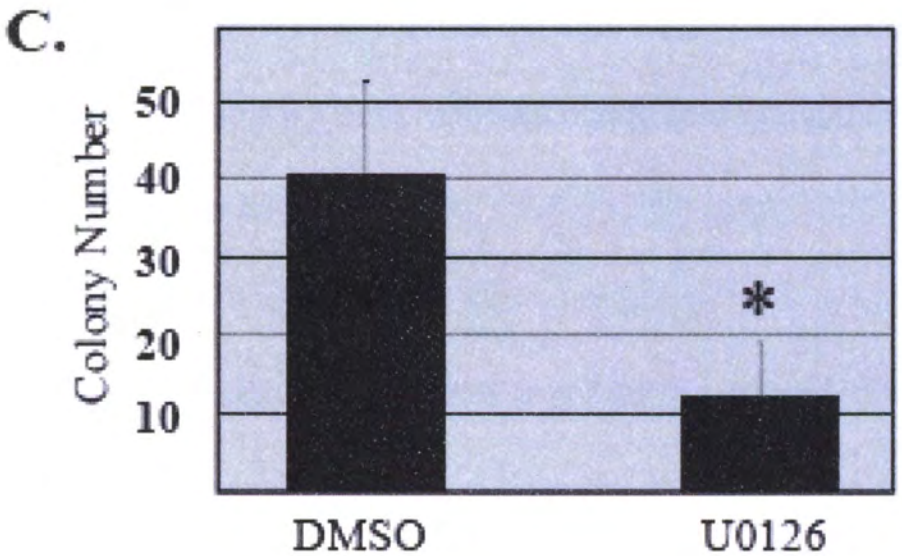

C.
Colony Number
50
40
30
20
10
*
DMSO
U0126

FIGURE LEGENDS

Figure 1. Activation of ERK in suspended ovarian carcinoma cells. A: Adherent (Adh) SKOV-3 cells were detached with trypsin and treated with soybean trypsin inhibitor. The cells were centrifuged, resuspended in either serum free medium (SF) or media containing 10 % FBS, and put in suspension culture for 3 hr before harvest. Cell lysates were immunoblotted for ERK2, MEK, FAK, and the activated forms of these proteins. **B:** Adherent SKOV-3 cells were detached with either trypsin or citric saline and put in suspension culture for 3 hr in serum-free media. **C** and **D:** Adherent SKOV-3 cells were detached with trypsin (**C**) or citric saline (**D**) and put in suspension culture in serum free media for the indicated time. **E**: Adherent (A) IOSE and SKOV-3 cells were detached with trypsin and put in suspension (S) for 3 hr in serum free media. Lysates were immunoblotted for ERK2 and active ERK. **F:** Six adherent (A) ovarian cancer cell lines were detached with trypsin and placed in suspension in serum-free media for 1 or 3 hr. **G:** Adherent (Adh) SKOV-3 cells detached with trypsin and put in suspension for 3 hr in either serum-free media (-), or serum-free media containing 0.5% bovine serum albumin (BSA), 4 mM EGTA, or BSA and EGTA.

Figure 2. Sustained activation of MEK and ERK in suspended cells. A: Adherent (Adh) SKOV-3 cells were trypsinized and suspended in serum-free media for the indicated time. Cell lysates were immunoblotted as indicated. The p-218 MEK antibody recognizes

the active form of the kinase, while the p-298 and p-292 antibodies recognize the PAK and ERK phosphorylation sites on MEK1, respectively . **B:** Adherent (Adh) SKOV-3 cells were put in suspension for 3, 24, or 48 hr in 10 % serum or serum-free media. Lysates were immunoblotted as indicated.

Figure 3. A: Adherent SKOV-3 cells were serum-starved for 12 hr and then treated with either fresh serum free media (SF) or increasing concentrations of serum, EGF, or HGF for 10 min. Cell lysates were immunoblotted for total and active ERK and MEK. **B**: Adherent (A) SKOV-3 cells were washed twice and incubated in serum-free (SF) media overnight. The cells were then stimulated for the indicated time with either 1 ng/ml EGF (top) or 5 ng/ml HGF (bottom). Cell lysates were immunoblotted for phosphorylated ERK and ERK2. **C:** Adherent cells were serum-starved overnight (SF) and then stimulated with 10 % serum for the indicated times. Cell lysates were immunoblotted for phosphorylated ERK and ERK2.

Figure 4. Re-attachment of SKOV-3 cells to an extracellular matrix restores down-regulation of ERK activity. A: Adherent (A) SKOV3 cells were detached with trypsin and suspended in serum-free media for 90 min or 3 hr. After 3 hr the cells were either left in suspension for the additional time indicated or replated onto fibronectin-coated dishes for the same amount of time. Cell lysates were prepared and immunoblotted for total and the activated forms of ERK, MEK, and FAK. **B:** Adherent (A) IOSE and SKOV-3 cells

were detached with trypsin and put in suspension for 3 hr. The cells were either kept in suspension for the number of hours indicated or replated onto fibronectin-coated dishes for the number of hours indicated. Cell lysates were immunoblotted for total and the activated forms of ERK, MEK, and FAK as well as MKP-1 and tubulin.

Figure 5. Sustained activation of ERK in suspended cells is not due to loss of phosphatase activity. A: Adherent (Adh) SKOV3 cells were trypsinized and put in suspension in serum-free media for 3 hr. The MEK inhibitor U0126 was added to the media at a final concentration of 10 □M for the indicated number of minutes prior to harvest of the suspended cells. Cell lysates were immunoblotted for phosphorylated ERK and ERK2. **B:** Adherent (Adh) SKOV-3 cells were put in suspension culture for 3 hr, 24 hr, or 48 hr. Two minutes before harvest the cells were treated with either DMSO or 10 □M U0126. Cell lysates were immunoblotted for phosphorylated ERK and ERK2.

Figure 6. Activation of ERK is suspended ovarian cells occurs through an autocrine mechanism. A: Adherent SKOV-3 cells (Adh) were put in suspension (Sus.) for 3 or 24 hr. The cells were harvested and conditioned media from these cells was collected. Either fresh serum-free media (SF) or conditioned media from the suspended cells was then added to serum-starved adherent SKOV-3 cells for 5, 15, or 60 minutes. Cells lysates for the suspended and adherent cells were prepared and immunoblotted for ERK, MEK, and the activated forms of these proteins. **B:** Adherent (Adh) cells were put in suspension for

24 hr (Sus.). Conditioned media from the suspended cells was collected and added to serum-starved adherent SKOV-3 cells from 10 min. to 6 hr. Cell lysates were immunoblotted with antibodies to ERK2 and phospho-ERK.

Figure 7. ERK nuclear signaling is enhanced in suspended ovarian cancer cells. SKOV-3 cells were co-transfected with GAL4-Elk1, 5X GAL4 luciferase, TK Renilla luciferase, and either empty vector or mutationally-activated MEK1. After a 5 hr transfection in serum-free media, the cells were incubated in 10% serum overnight. The following day, the cells were washed twice with PBS and either incubated in serum free media or trypsinized and incubated in serum-free media in suspension. After 24 hr all cells were harvested and both Renilla and firefly luciferase determined using a dual luciferase assay kit (Promega). Firefly luciferase activity was normalized to Renilla. Solid bars represent adherent cells and checked bars represent suspended cells. The results from the adherent cells transfected with the reporter construct, Gal4-Elk1, TK Renilla and empty vector (first column) was set at 100%. The results are an average of 5 experiments performed in duplicate. There was a significant difference (*, $p<0.05$) between normalized firefly luciferase activity between adherent and suspended cells (left columns). There was not a significant difference in normalized firefly luciferase activity between the adherent and suspended cells that were transfected with mutationally activated MEK1.

Figure 8. Inhibition of ERK activation impairs anchorage independent growth. A: SKOV-3 and IOSE cells were plated in triplicate at a density of 1 X 10^4 cells per well in 12 well dishes in 10% serum-containing media. The following day the cells were treated with either 10 □M U0126 or DMSO control. MTT reagent was added for 2 hrs before cell harvest for the next 4 days. MTT activity was measured after harvest. The data shown is a representative experiment that was performed 3 times. (*, $p < 0.05$). **B:** Adherent SKOV-3 cells were trypsinized and placed in 1ml of soft agar containing 10% serum at a density of 5000 cells per ml. The assay was performed 3 times in triplicate. Cells were treated every 3 days with either 10 □M U0126 or DMSO control. Four random fields were photographed using a 2.5 objective. A representative field from each treatment is shown. **C.** Colony count (>400 □m^2) and size was determined using Image J software. Quantitation of the total colony count from 4 photographed fields per dish were averaged and then compared within each group. There was a statistical significant difference (*, $p < 0.05$) between U0126 and the DMSO control.

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