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# The roles of PDK1 and SGK1 in colorectal cancer

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## Abbreviations

3-Phosphoinositide-dependent kinase (PDK)

Adenomatous polyposis coli protein (APC)

Apoptosis signal-regulating kinase (ASK)

Azoxymethane (AOM)

B-cell leukemia/lymphoma 2 (Bcl-2)

Bcl-2-associated death promoter (Bad)

Bcl-2–associated X protein (Bax)

cAMP response element binding (CREB)

Colorectal cancer (CRC)

Damage-inducible protein 45 (Gadd45a)

Dimethylhydrazine (DMH)

Dishevel (Dsh)

Familial adenomatous polyposis (FAP)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Human embryonic kidney (HEK)

Hereditary nonpolyposis colon cancer (HNPCC).

Insulin-like growth factor (IGF)

IκB kinase (IKK)

Lymphoid enhancing factor (LEF)

Mismatch repair (MMR)

Mitogen-and stress-activated protein kinase (MSK)

Multiple intestinal neoplasia (min)

PDK1-interacting fragment (PIF)

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN)

Phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$)

Phosphatidylinositol 4,5 bisphosphate ($PIP_2$)

Phosphoinositide 3-kinase (PI3K)

Pleckstrin Homology (PH)

Protein kinase A (PKA)

Protein kinase B (PKB/Akt)

Protein kinase C (PKC)

Protein kinase G (PKG)
Serine (S)
T-cell factor (TCF)
Threonine (T)
Tumor growth factor (TGF)
Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)
United States (U.S.)

# 1. Introduction

## 1.1. Colorectal cancer

### 1.1.1. Epidemiology of colorectal cancer

**Incidence and mortality of colorectal cancer**

Colorectal cancer (CRC) is the cancer of colon and rectum. It is a very common cancer disease worldwide and causes enormous deaths and huge economic burdens to the world. It was estimated in the report of GLOBOCAN 2002 that 1023252 new colorectal cancer cases occured over the world and caused 520980 deaths annually (Ferlay et al., 2004). The incidence of CRC varies from regions to regions (Khuhaprema et al. 2008; Malekzadeh et al., 2009). The incidence rates are higher in the developed areas, such as Europe, North America and Australia. It is the third most common form of cancer and the second leading cause of cancer-related death in the Western world (World Health Organization, 2006). In “Cancer Incidence of Europe 2006”, it was estimated that 217400 and 195400 new CRC cases occured annually in men and women in Europe, ranking third in the incidence in men and second in women. CRC is the second major cause of cancer death in Europe and it’s estimated to cause 207400 deaths annually. In addition, CRC is the most common form of cancer and the sencond major cause of death among cancer disease within Europe Union countries (Ferlay et al., 2007). It’s noticeable that the incidence of CRC used to be low in some eastern countries, such as Japan and China. However, in recent decades the incidence and mortality of CRC in these countries increased sharply, which is probably owing to their adoption of Western life style. The mortality of CRC in Japan increased 5.5-folds in the second half of the $20^{th}$ century (Honda et al., 1999). The incidence of CRC in China doubled in the past 30 years as well (Zhang et al., 2005a).

### Economic burdens resulted from CRC

The economic burdens resulted from CRC are a major challenge to public health care. The therapeutic cost for CRC increases substantially worldwide (Joubert et al., 2007; Augestad et al., 2008), particularly in Westernizing-lifestyle countries (Pickhardt et al., 2007). For example, in the U.S., during the period from 1991 to 1994, the mean number of admissions for colon cancer was 237,754 annually, the mean length of stay was 11.1 days per admission, mean total hospital charges were 4.57 billion US dollars annually. It was also predicted the annual number of hospital admission of colon cancer related disease would be doubled in 2050 (Searle et al., 1999).

### 1.1.2. Etiology of colorectal cancer

The risk factors of CRC include age older than 50, inflammatory bowel disease (IBD), high-fat low-vegetable diet, physical inactivity, smoking, alcohol and genetic predeposition (Benson, 2007; Jemal et al., 2007; Khuhaprema et al., 2008).

About 20% of CRC are familial, which means gene alterations may mediate the development of CRC (Benson, 2007). There are two key CRC related hereditary diseases, familial adenomatous polyposis (FAP) and hereditary nonpolyposis colon cancer (HNPCC). FAP is a rare autosomal dominant syndrome caused by an inherited mutation in the APC gene (Giardiello et al., 1997). It accounts for approximately 1% to 2% of all CRC cases. HNPCC or termed as Lynch syndrome (Lynch et al., 1985), an inherited autosomal dominant syndrome, is caused by inherited mutation in any one of five DNA mismatch repair (MMR) genes and microsatellite instability (Benson, 2007). HNPCC is predicted to account for 2% or less of all CRC cases (Aaltonen et al., 1998).

### 1.1.3. Therapy of colorectal cancer

**Surgical therapy of colorectal cancer**

Surgery is the most common treatment to potentially curable CRC. In most cases surgical treatment includes resection of primary tumor, regional lymph nodes and resectable metastatic lesion. Adjuvant radiation is currently a standard procedure for the treatment of rectum cancer (van der Voort van Zijp et al. 2008). When it comes to the CRC cases stage II, III and IV, adjuvant chemotherapy is recommended as adjuvant to surgery (American Joint Committee on Cancer, 2002)

**Gene therapy of colorectal cancer**

With the development of biomedicine, more and more genes have been identified to be responsible for carcinogenesis. As mutation and aberration of these genes cause CRC, correcting the defective genes or selectively overexpressing certain genes may prevent cancer development and exert therapeutic effect on tumors. At present, experiments and trials on gene therapies are underway worldwide to establish a safe, effective, and long lasting treatment to CRC. A study indicating that inhibition of phosphoinositide 3-kinase (PI3K) by RNA interference could sensitize resistant colon cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced cell death (Rychahou et al., 2005). Agents that selectively target the PI3K/PKB pathway may enhance the effects of chemotherapeutic agents and provide novel adjuvant treatment for selected colonrectal cancers.

### 1.1.4. Mouse models of colorectal cancer

To understand the mechanism beneath certain disease, and establish therapeutic strategies to it, it is essential to develop workable animal models that can simulate the disease in human beings. The mechanism underling the development of CRC has not been completely known yet. The studies on mouse models then provide us insight into the mechanism of CRC. The mouse

models of CRC can be classified into three types: the genetically engineered models, implantation models, and the chemical induced models (Heijstek et al., 2005).

### Genetically engineered models of colorectal cancer

FAP and HNPCC are the most common hereditary CRC forms (Giardiello et al., 1997; Aaltonen et al., 1998). The multistage carcinogenesis from local hyperplasia to adenoma to carcinoma requires the sequential mutation of various genes. In FAP patients, the dysfunction of tumor suppressor gene APC is usually found. Nonsense mutation at codon 850 of the mouse Apc gene is identified as mutant multiple intestinal neoplasia (*Min*; Su et al., 1992). The $Apc^{Min}$ heterozygous mice are expected to develop multiple small intestine adenomas and low penetrance of mammary adenosquamous carcinomas (Fodde et al., 2001). However, homozygosity for the *Min* mutation is embryonic lethal (Moser et al., 1995). Some other Apc gene modified models have also been reported, e.g. $Apc^{\Delta 716}$ (Oshima, M. et al., 1995), $Apc^{1638N}$ (Fodde et al., 1994), $Apc^{1638T}$ (Smits et al., 1999), $Apc^{\Delta 474}$ (Sasai et al., 2000), $Apc^{1309}$ (Quesada et al., 1998).

HNPCC is caused by the deactivation of DNA MMR genes, such as MLH-1, MSH-2 and MSH-6 et al. Single homozygous mutation in the DNA MMR genes rarely causes adenocarcinomas in mice (Taketo, 2006), but lymphoma is very common (Heijstek et al., 2005). It was noted that Apc gene inactivation coupled with Mlh-1 homozygously deleted lead to a 40-fold increase in the numbers of gastrointestinal tumors (Edelmann et al., 1999). Several other genes have also been modified artificially to generate mouse models of CRC, e.g. Transforming growth factor β1 (Engle et al., 1999), Smad3 (Zhu et al., 1998) and K-ras (Janssen et al., 2002).

### Implantation models of colorectal cancer

Tumor tissues and cells can be implanted into recipient mouse either at ectopic sites (mostly subcutaneously) or at orthotopic sites (e.g. cancer cells into the colon), to establish an implantation mouse model. The recipient mouse innate immune responses are destroyed to diminish graft rejection. Therefore, model mice are usually with severe combined immunodeficiency or thymus-less.

### Chemical induced models of colorectal cancer

Rodents have almost no spontaneous CRC (Corpet et al., 2003), but tumors can be reliably induced in rodents by repeated administrations of carcinogen, which is directly involved in the development of cancer. The incidence of CRC is contributed to the carcinogen used, the dosage, the duration and frequency of administration, as well as the routing and timing of administration (Heijstek et al., 2005).

### Cancerogenic character of DMH/AOM

Usually carcinogens cause malignancies in multiple organs, but some carcinogens predominantly induce cancer in the large intestine. The most commonly used carcinogen for CRC mouse model are dimethylhydrazine (DMH) and its metabolites azoxymethane (AOM). These reagents specially induce tumors in the descending colon and rectum. DMH/AOM induced tumors are very close to sporadic CRC in humans in morphology, histology and biological behavior. DMH can be administrated via oral, subcutaneous, intrarectal and intramuscular. Usually multiple treatment of DMH (20-40 mg/kg) and long-term experimental period (20-48 weeks) are employed to induce tumors in mouse colons (Balansky et al., 1999; Schmelz et al., 2000). The tumor incidence (40-94%) is inconsistent in different studies (Kobaek-Larsen et al., 2000). DMH and AOM are able to induce gene mutations, but require metabolic inactivation to form DNA reactive substance. First AOM is hydroyxlated to MAM. MAM then produces a methyldiazonium ion, which

alkylates macromolecules in liver and colon. For example, it transforms 2'-deoxyguanosin to $O^6$-Methyl-deoxyguanosin, and consequently promotes the DNA mismatch of $O^6$mG: T.

Various tumor-related genes are the targets of carcinogens in mouse models of CRC. There are some common genetic targets in both human and carcinogen-induced mouse CRC, such as K-Ras, Apc, p53, β-catenin (Rosenberg et al., 2009). High frequency of β-catenin mutations were detected in DMH/AOM-induced CRC mice, and these mutations were mostly G:C to A:T transitions (Takahashi et al., 2000; Koesters et al., 2001).

**DMH-DSS mouse model of colorectal cancer**

The risk of CRC increases with the extent and duration of IBD. A number of animal models have been reported to be applicable to the research on IBD. The most commonly used one is dextran sulfate sodium (DSS) induced chronic colitis model. In recent years, several colitis related CRC models have been established by administrating DMH/AOM-DSS. Wang et al. exposed the mice to a single, low dose of DMH followed by three repeated administrations of 30 g/L DSS in drinking water to induce CRC within 10 weeks (Wang et al., 2004). Tanaka et al. initiated the CRC mouse model treatment with AOM, then promoted with DSS. Mice developed tumors after a relatively short-term DSS exposure (as early as 12 weeks), compared with DSS alone treatment (Tanaka et al., 2003). Rosenberg et al. gave mice a single administration (10 mg/kg body weight) of AOM, followed by one-week oral exposure (2% in drinking water) of DSS. Within 20 weeks, numerous colonic neoplasms and dysplastic lesions were observed (Rosenberg et al., 2009).

In DMH/AOM alone models, the accumulation of β-catenin (Takahashi et al., 1998; Wang et al., 2004), cyclooxygenase 2 (Dong et al., 2003), and inducible nitric oxide synthase (Sengupta et al., 2005) were observed. β-Catenin gene mutations were detected in most DHM-DSS models as well (Wang et al., 2004; Kohno et al., 2005).

## 1.2. Pathways to colorectal cancer

Tumorigenesis is a multi-step process from normal cells to cancer cells in CRC. The molecular machinery seems to be complicated. However, several pathological pathways have been identified to be involved in the tumorigenesis of CRC. There is a vast body of documents showing that mutations and/or loss of some genes, e.g. PETEN, K-ras, APC, P53, β-catenin, TGF, BAX, MMR, lead to CRC (Samuels et al., 2006; Cheah, 2009).

### 1.2.1. The PI3K pathway

The PI3K pathway plays a pivotal role in tumorigenesis of CRC. PI3K is a dimeric enzyme, consisting of a catalytic and a regulatory subunit. The catalytic subunit has four isoforms, termed as p110α, p110β, p110γ and p110δ. The p110α, p110β and p110δ combine the regulatory subunit p85, whereas p110γ combines the regulatory subunit p101 (Zhao et al., 2008).

**Activation of PI3K pathway**

PI3K phosphorylates 3 position of the inositol ring of phosphatidylinositol 4,5 bisphosphate ($PIP_2$) to generate phosphatidylinositol 3,4,5-trisphosphate ($PIP_3$), a potent second-messenger required for cell survival, growth, proliferation and insulin action. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) reverses this reaction by hydrolyzing $PIP_3$ to $PIP_2$ (Myers et al., 1998). $PIP_3$, mainly exists on cell membrane, induces the translocation of protein kinase B (PKB) from the cytosol to the inner leaflet of the plasma membrane. 3-phosphoinositide-dependent kinase (PDK), binding to $PIP_3$ on the membrane by the virtue of its PH domain, phosphorylates and activates PKB (Vanhaesebroeck et al., 2000). Moreover, PDK can phosphorylate several other Ser/Thr protein kinases, which includes protein kinase A (PKA), protein kinase G (PKG), and protein kinase C (PKC) isoforms p70-S6Ks, p90-RSKs, serum- and glucocorticoid-inducible kinase (SGK) and

mitogen-and stress-activated protein kinase (MSK) (Vanhaesebroeck et al., 2000). These kinases activate a number of phosphorylation substrates, which consequently function physiologically and pathophysiologically through different ways. Their substrates, such as apoptosis signal-regulating kinase (ASK), Bcl-2-associated death promoter (Bad), cAMP response element binding (CREB), forkhead family proteins and IκB kinase (IKK), promote cell survival and inhibit apoptosis. GSK, mammalian target of rapamycin (mTOR), and p21 regulate the cell cycle (Nicholson et al., 2002; Fig. 1).

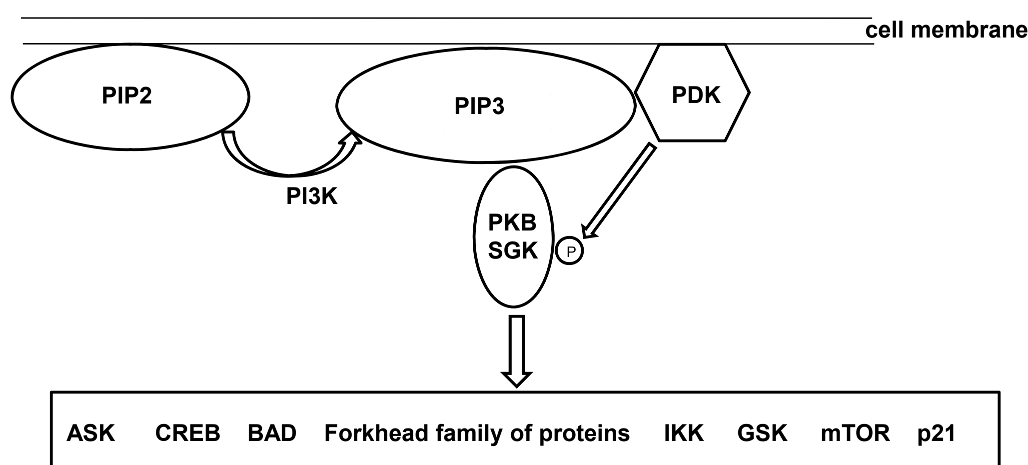



**Fig. 1 Overview of PI3K-PKB/SGK pathway**

## Role of the PI3K pathway in colorectal cancer

The involvement of the PI3K pathway in cancers has been verified by many studies. PIK3CA is the gene encoding p110α, and it is mutated frequently in cancers (Samuels et al., 2004; Samuels et al., 2006). The mutation of PIK3CA entitles PI3K the function in enzymatic and signaling activity and is oncogenic in cell culture and animal model systems (Ikenoue et al., 2005; Wang et al., 2005). Sasaki et al. reported the genetic inactivation of the p110γ catalytic subunit of PI3Kγ in mice with invasive colorectal adenocarcinomas and the loss of p110 protein expression in patients with primary CRC (Sasaki et al.,

2000). The high expression of PKB and phosphorylated PKB were detected in human CRC samples by immunohistochemistry, contrasting to normal colonic mucosa (Itoh et al., 2002). In colon cancer cell lines, the expression of phosphorylated PKB and phosphorylated p70S6 kinase were upregulated as well (Khaleghpour et al., 2004).

### 1.2.2. The Wnt pathway

Wnts are secreted proteins which mediates a series of developmental events, e.g. the formation of body axis, the development of brain, via a paracrine or autocrine manner (Smalley et al., 1999). Besides the effect on embryogenesis, the Wnt signaling also plays an important role in tumorigenesis. Under physiological condition, β-catenin is regulated by the multiprotein complex which is comprised of GSK3, Adenomatous Polyposis Coli (APC) protein and axin (Dale et al., 1998). The complex phosphorylates β-catenin, leading to subsequent ubiquitination and degradation of β-catenin (Liu et al., 2002). Wnt ligands bind to frizzled transmembrane receptors and low-density lipoprotein-receptor-related proteins co-receptors. As a result, the cytoplasmic protein Dishevel (Dsh) is phosphorylated. Then the phosphorylated Dsh binds to axin, leading to destruction of the complex. The intracellular β-catenin is thus stabilized and accumulated, resulting in its translocation into cell nuclei (Smalley et al., 1999). In nuclei, β-catenin associates the T-cell factor/lymphoid enhancing factor (TCF/LEF) to modulate the expression of a broad spectrum of target genes, which may affect cell proliferation and differentiation (Fig. 2).

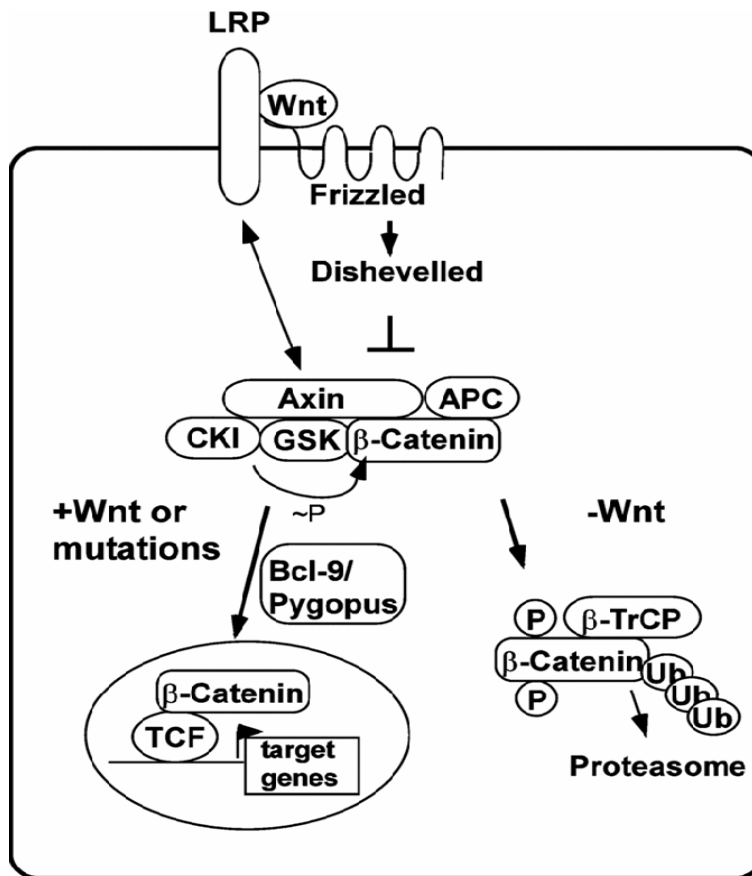


**Fig. 2 Overview of the Wnt pathway (Behrens, 2005)**

**Role of the Wnt pathway in colorectal cancer**

Mutations of components in the Wnt signaling pathway are involved in the tumorigenesis of CRC. The most common mutation of APC appears in the mutation cluster region (Behrens, 2005). That mutation makes a frameshift or stop codon, which generates truncated APC proteins. The truncated APC protein results in the destruction of the multiprotein β-catenin degradation complex. Polakis reported that the β-catenin mutation in sporadic colon cancers was up to 10% (Polakis, 2000). These mutations subsequently caused the stabilization of β-catenin and the subsequent activation of the Wnt signaling. A nonsense mutation in ANXIN2 gene, coding axin protein, was found in patients with hereditary CRC and oligodontia (Lammi et al., 2004). In addition, activation of the Wnt signaling pathway by autocrining of Wnt proteins is thought to be another mechanism of tumorigenesis in CRC. (Bafico et al., 2004)

### 1.2.3. PDK1

**Structure of PDK1**

PDK1 is a 63kD serine/threonine kinase. It activates PKB and SGK isoforms (Lang et al., 2006) by phosphorylation. As the activation of PKB (Nicholson et al., 2002; Roy et al., 2002) and SGK (Lang et al., 2006; Dehner et al., 2008) is involved in the development of CRC, PDK1 is critical to the research of mechanism of CRC. PDK1 possesses two regulatory domains. One is Pleckstrin Homology (PH) domain, which binds to the second messenger $PIP_3$. The other is the hydrophobic pocket, also termed as PDK1-interacting fragment (PIF)-pocket. It is a domain to interact with the substrates of PDK1 (Alessi et al., 1997).

**PDK1 activates other kinases**

PDK1 phosphorylates its substrates at their activation loops. The activated PI3K phosphorylates the $PIP_2$ on the 3-OH group, generating $PIP_3$. Afterwards $PIP_3$ recruits the PKB to the plasma membrane and alters its conformation to allow subsequent phosphorylation. Meanwhile PDK1 binds to $PIP_3$ on the plasma membrane via the PH domain. Therefore, these two kinases are co-localized (Nicholson et al., 2002). PKB is subsequently phosphorylated by PDK1 at T308 of the activation loop (Alessi et al., 1996). The full activation of PKB requires the phosphorylation at both T308 and S473 (Nicholson et al., 2002). The mechanism mediating S473 phosphorylation at hydrophobic motif remains controversial. The study of Balenderan et al. demonstrated that PDK1 was able to phosphorylate both T308 and S473 of PKB with the assist of fragment of a protein known as PRK-2 (Balendran et al., 1999). Whereas other evidence indicates that S473 is phosphrylated by PDK2 rather than PDK1. Williams et al. disclosed that insulin-like growth factor 1 (IGF1) induced PKB phosphorylation at S473 in PDK1 lacking mouse embryonic stem cells (Williams et al., 2000). Another study showed that phosphorylation at S473 of PKB was unaffected by the administration of staurosporine, an inhibitor of

PDK1 (Hill et al., 2001). Since members of AGC protein kinase family possess a highly conserved amino acid sequence in their catalytic domains, other members of this family, such as S6K isoforms, SGK isoforms, PKA, PKC are phosphorylated by PDK1 at their T-loop residue and their hydrophobic motif in a similar manner (Alessi, 2001).

**The regulation of PDK1 activity**

The mechanism of PDK1 activity regulation has not been well studied. Several studies indicated the activity of PDK1 may not be induced by insulin. It was reported that the PDK1 activity to PKB or S6K1 remained the same at the presence or absence of the stimulation of insulin (Pullen et al., 1998; Alessi et al., 1997). Casamayor and colleagues showed that the phosphorylation status was not changed by insulin or IGF-1 in HEK 293 cells (Casamayor et al., 1999). It is thus postulated that PDK1 is constitutively active in cells, rather than activated by the agonist. The substrates, rather than PDK1 itself, convert into a form suitable to be activated by PDK1.

### 1.2.4. SGK1

SGK1 was originally identified as an immediate early gene transcriptionally stimulated by serum and glucocorticoids in mammary tumor cells (Webster et al., 1993). Two other isoforms, SGK2 and SGK3, sharing 80% amino acid sequence identity in their catalytic domain with SGK1, have subsequently been identified (Kobayashi et al., 1999). In humans, the SGK1 expression has been detected in almost all tissues (Waldegger et al., 1997). In cells, the subcellular localization of SGK1 depends on the status of cells. The serum induced SGK1 activation was reported to result in SGK1 localization into nuclei (Maiyar et al., 2003). However, the activation by glucocorticoids enhances cytosolic localization (Firestone et al., 2003).

**The activation of SGK1**

The SGK family proteins exhibit structural similarity to PKB. They share 55% identity with the PKB kinase domain. They harbor a similar carboxy terminal HM as well as conserved residues, which are required for activation of kinases (Kobayashi et al., 1999). Similar to PKB, the activation of SGKs requires the phosphorylation via the signaling pathway including $PIP_3$, PDK1 and PDK2. The SGK1 is activated when phosphorylated at T256 in the activation loop by PDK1 and phosphorylated at S422 in the hydrophobic motif at its COOH terminus by PDK2 (Park et al., 1999). The phosphorylation at S422 contributes to the activation of SGK1 by promoting SGK1 binding to the PIF-binding pocket and phosphorylation at T256 by PDK1 (Biondi et al., 2001). More recently, $Na^+/H^+$ exchanger regulating factor 2 and with no lysine kinase 1 are also thought to be involved in SGK1 activation (Chun et al., 2002; Xu et al., 2005).

**The regulation of SGK1**

As its name implies, SGK1 is transcriptionally upregulated by both serum and glucocorticoids (Sheppard et al., 2002; Firestone et al., 2003). Some other hormones and cytokines have also been verified to induce the SGK1 transcription, such as mineralocorticoids (Gumz et al., 2003; Kellner et al., 2003), gonadotropins (Chu et al., 2002;), 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$; Akutsu et al., 2001), TGFβ (Waldegger et al., 1999), IL-6 (Meng et al., 2005), fibroblast and platelet derived growth factor (Mizuno et al., 2001), thrombin (Belaiba et al., 2006), and endothelin (Wolf et al., 2006). In addition, the SGK1 transcription is also upregulated by cell volume reduction (Waldegger et al., 1997), excessive glucose concentrations (Saad et al., 2005), heat shock, ultraviolet radiation, and oxidative stress (Leong et al., 2003).

### SGK1 promotes cell survival

Observations on roles of the SGK isoforms in cell survival are conflicting. Both SGK1 (Brunet et al., 2001; Shelly et al., 2002; Zhang et al., 2005b) and SGK3 (Liu et al. 2000; Xu et al., 2001) have been postulated to confer cell survival. SGK1 also appears to mediate IL-6-dependent survival of cholangiocarcinoma cells (Meng et al., 2005). Moreover, the transformation of intestinal epithelial cells by oncogenic β-catenin was followed by increased SGK1 expression (Naishiro et al., 2005). On the other hand, the downregulation of SGK1 has been observed in prostate cancer (Rauhala et al., 2005), ovarian tumors (Chu et al., 2002) and hepatocellular carcinoma (Chung et al., 2002). SGK1 and SGK3 interact with several signaling elements known to affect cell survival. SGK1 and SGK3 phosphorylate forkhead transcription factors such as Foxo3 (Brunet et al., 2001; Shelly et al., 2002; Liu et al., 2000; Xu et al., 2001; Dehner et al., 2008), and further inhibit its transcriptional function. Foxo-dependent expressions of certain pro-apoptotic genes are subsequently downregulated (Brunet et al., 2001; Dehner et al., 2008). In addtion, SGK3 phosphorylates and thus inactivates Bad (Liu et al., 2000; Xu et al., 2001). Phosphorylated Bad binds to the chaperone 14–3-3 and is thus prevented from traveling to the mitochondria where it triggers apoptosis (Lizcano et al., 2000). Moreover, SGK1 stimulates NF-κB (Zhang et al., 2005b; Vallon et al., 2006). SGK1 also phosphorylates and thus inhibits GSK3 (Sakoda et al., 2003), a kinase phosphorylating the transcription factor β-catenin and participating in the regulation of cell proliferation (Cohen et al., 2001).

### Other molecular and cellular functions of SGK1

Besides the regulation of cell proliferation and apoptosis, SGKs are responsible for regulations of many other molecular and cellular functions. SGK1 controls the activities of various ion channels, such as epithelial $Na^+$ channel ENaC (Wagner et al., 2001), renal epithelial $Ca^{2+}$ channel TRPV5 (Palmada et al., 2005), renal and inner ear $Cl^-$ channel ClC-Ka (Embark et al.,

2004), cystic fibrosis transmembrane conductance regulator $Cl^-$ channel CFTR (Wagner et al., 2001). It is also documented that SGK1 stimulates the activity of the $Na^+$ glucose cotransporter SGLT1 (Dieter et al., 2004), the facilitative glucose transporter GLUT1 (Palmada et al., 2006) and the glutamate transporters EAAT (Boehmer et al., 2006).

### 1.2.5. Foxo3a

**The forkhead family of transcription factors**

The forkhead box O transcription factors play a critical role in embryogenesis, tumorigenesis and maintenance of differentiated cell states. The forkhead, or winged-helix family of transcription factors are first discovered in drosophila gene (Kaufmann et al., 1996). Members of this family are characterized by a conserved 110 amino acid residues (also termed as FOX domain) encoding DNA binding motif of winged helix structure (Clark et al., 1993). The FOX genes are classified into 19 subfamilies, from FOXA to FOXS, by the homology within the FOX domain encoding region (Kaestner et al., 2000).

Foxo factors, or named Fox Other subfamily, belong to the Fox superfamily. They possess unique five amino-acid insertion immediately prior to helix H3 within the forkhead domain that is directly involved in sequence-specific interaction with DNA-binding sites (Fu et al., 2008). In human, the Foxo subfamily is comprised of Foxo1 (previously known as FKHR), Foxo3a (previously known as FKHRL1), Foxo4 (previously known as AFX) and Foxo6.

**Foxo3a regulates cell cycle**

Foxo proteins function predominantly as transcription factors in the nucleus and bind as monomers to their cognate DNA-targeting sequences. The consensus high-affinity DNA-binding sites for Foxo have been identified as Foxo recognition element (FRE; Biggs et al., 1999). Functional FRE sites have been identified in many promoters of Foxo target genes, such as Fas ligand,

IGF-binding protein 1, Bim, P21, Cyclin G2 (Accili et al., 2004; Greer et al., 2005).

Cell proliferation is controlled by factors that regulate the transition at the G1/S and the G2/M checkpoints. Foxos regulate these cell cycle factors at different stages. Foxo1, Foxo3a or Foxo4 transcriptional upregulates the cell cycle inhibitor $p27^{kip1}$ (Dijkers et al., 2000a), which can bind to the cyclin E/cdk2 complex and subsequently blocks the cell cycle transition from G1 phase to S phase. Moreover, Foxo1 and Foxo3a inhibit G1/S phase transition by downregulating cyclins D1 and D2 (Smith et al., 1996; Ramaswamy et al., 2002). Foxo3a also prevents G2/M cell cycle transition by induction of DNA damage-inducible protein 45 (Gadd45a), which interacts with cyclin/CDK complexes (Martinez-Gac et al., 2004). Foxo3a additionally induces a status of cellular quiescence (G0) by transcriptionally upregulating Rb family member p130, which suppresses the expression of genes required for re-entry into the cell cycle (Smith et al., 1996).

**Foxo3a regulates apoptosis**

The expression of Foxos in cell nucleus triggers cell death by upregulating pro-apoptosis proteins. Brunet et al. revealed that Foxo3a triggered apoptosis by inducing the expression of Fas ligands (Brunet et al., 1999). Modur and colleagues used microarrays to identify that the reduced expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in prostate carcinoma cells correlated directly with loss of Foxo1 and Foxo3a activities (Modur et al., 2002). Moreover, it was implied ttat active form of Foxo3a induced pro-apoptotic protein Bim; whereas inactivation of Foxo3a via a PI3K dependent manner decreased the Bim expression (Dijkers et al., 2000a; Stahl et al., 2002). The expression of active Foxos transcriptionally upregulates several genes involved in DNA repair (Ramaswamy et al., 2002; Tran et al., 2002), e.g. Gadd45a. Foxos also allow detoxification of reactive oxygen species by inducing antioxidative proteins (Nemoto et al., 2002; Ramaswamy

et al., 2002; Tran et al., 2002), such as manganese superoxide dismutase and catalase.

**The regulation of Foxo3a**

One prerequisite for Foxos to function as transcription factor is their nuclear localization. Accumulating evidence indicates Foxo1, Foxo3a can be phosphorylated on isoform-specific phosphorylation sites, which leads to their nuclear exclusion and a subsequent loss of their transcriptional function (Greer et al., 2005; Fu et al., 2008). The activation of PI3K-PKB/SGK pathway, which is usually at present in tumorigenesis, inhibits Foxos by direct phosphorylation (Brunet et al., 1999; Shelly et al., 2002; Dehner et al., 2008). PKB phosphorylates mammalian Foxo1 at sites Thr24, S256 and S319 (Biggs et al., 1999), and Foxo3a at T32, S256 and S319 (Kashii et al., 2000). SGK isoforms preferentially phosphorylate Foxo factors at the site S315 and T32 (Shelly et al., 2002; Dehner et al., 2008). The inhibitory phosphorylation by PKB/SGK relocalizes Foxos from cell nucleus to cytoplasm. The phosphorylation exposes the biding sites for chaperone molecules 14-3-3 proteins and promotes the interaction between Foxos and 14-3-3 proteins in cell nucleus. Thus, the Foxo-DNA binding is disrupted and Foxo is releasesd from nuclear DNA anchor (Brunet et al., 2002). Meanwhile the association of Foxos with 14-3-3 proteins induces the comformational change of Foxos, resulting in its coupling with exportins, i.e. Crm1 and Ran, and finally enables the shift of Foxos into cytoplasm (Brunet et al., 2002). In cytoplasma, the phosphorylated Foxo factors are degraded in a ubiquitin–proteasome dependent manner (Plas et al., 2003; Aoki et al., 2004).

### 1.2.6. Bad and Bim

**The Bcl-2 family of proteins**

Bcl-2 is the first protein to be identified as the apoptotic regulator (Cory et al., 2002). It is discovered in human follicular lymphoma as the oncoprotein

activated via chromosome translocation (Vaux et al., 1988). Since then more than thirty relative proteins have been identified, which compose the Bcl-2 family (Reed, 2006; Willis et al., 2005). Members of Bcl2 family of proteins share at least one Bcl-2 homology (BH) region homology, namely BH1-4 domains. In humans and mice, the Bcl-2 family are classified into anti-apoptotic multi-domain proteins (prototypes: Bcl-2 and Bcl-xl), which contain four BH domains (numbered BH1 to BH4), pro-apoptotic multi-domain proteins (prototypes: Bax and Bak), which contain three BH domains (BH1, BH2 and BH3), and the pro-apoptotic BH3-only protein family (which has more than a dozen members, e.g. Bad, Bim, Bid).

The Bcl-2 family of proteins constitute a critical intracellular checkpoint of apoptosis within a distal common cell death pathway (Chao et al., 1998). As well known the impaired apoptosis is a crucial step in tumorigenesis, the Bcl-2 family of proteins related apoptosis pathway is currently under tremendous studies in cancer research.

Bcl-2 and its closest homologues, Bcl-xl and Bcl-w, potently inhibit apoptosis in response to many cytotoxic insults. Bcl-2 and its pro-apoptosis relatives locate mainly on the outer mitochondrial membrane, the endoplasmic reticulum and the nuclear envelope (Janiak et al., 1994). They share the very conserved core three dimension structure (Muchmore et al., 1996; Sattler et al., 1997), which comprises a globular bundle of five amphipathic α-helices that surround two central hydrophobic α-helices. There is a hydrophobic groove in Bcl-2, which can bind the BH3 α-helix of an interacting BH3-only relative (Petros et al., 2001).

The pro-apoptosis relatives of Bcl-2 family induce the apoptosis, associating with caspase family of proteins. Bax and Bak can form homo-oligomers within the mitochondrial membrane and release cytochrome c. Cytochrome c subsequently activates Apaf1, allowing it to activate caspase-9 (Knudson et al., 2008). Bcl-2 is thought to sustain cell survival by sequestering Apaf1 and preventing cytochrome c release (Zou et al., 1997). In addition,

Bcl-2 may control the activation of several initiator caspases. The Bcl-2 can inhibit the apoptosis via inactivation of caspase-12, in the absence of Apaf1 or cytochrome (Rao et al., 2002).

**Bad promotes apoptosis**

Bad, the member of BH3-only subfamily, is the first pro-apoptotic regulator to be identified in Bcl-2 family of proteins (Zha et al., 1996). The endogenous Bad is dephosphorylated and localized on the outer mitochondria membrane. It promotes apoptosis by binding to and antagonising the functions of survival members of the Bcl-2 family, e.g. Bcl-2 and Bcl-xl, on mitochondria membrane (Datta et al., 2000). The structure biology study has disclosed the BH3 domain of Bad interacts with the hydrophobic groove of the survival proteins in Bcl-2 family (Lama et al., 2008).

**The inhibitory phosphorylation of Bad**

There is a huge body of evidence indicating that PKB regulates Bad directly. Studies disclosed that Bad and PKB coimmunoprecipitated when overexpressed and interacted in GST-pulldown experiment (Datta et al., 1997; Blume-Jensen et al., 1998). In addition, PKB and Bad colocalization was detected by means of immunofluorescence microscopy within cells (Blume-Jensen et al., 1998).

PKB phosphorylates Bad at the site of S112 and S36, which is deleterious to the pro-apoptotic function of Bad. del Peso et al. reported the active forms of PKB phosphorylated Bad both in vivo and in vitro at S112 and S136 in response to IL-3, which was an agonist of PKB via PI3K. Moreover, the phosphorylation of Bad was abrogated by the inhibitor of PI3K (del Peso et al., 1997). Correspondently, transfection of cells with dominant–negative PKB alleles blocks phosphorylation of transfected Bad at S136 (Wang et al., 1999). It’s noticeable that constitutively active PKB alleles were not able to promote survival of cells when cotransfected with Bad molecules, in which the S136

had been alterd to alanine (Datta et al., 1999). This result may suggest the biological importance of the phosphorylation at S136 by survival kinases.

Phosphorylation of Bad disrupts its ability to bind to and inactivate Bcl-xl (Wang et al., 1999). The phosphorylation at S136 recruits the 14-3-3 proteins (molecular chaperones binding to a variety of signaling molecules in a phosphorylation-dependent manner) to Bad/Bcl-xl complex (Yaffe et al., 1997). The 14-3-3 proteins only weakly binds to the Bad/Bcl-xl complex but remarkably increase the accessibility of S155 to survival kinases, such as PKA and PKB. The subsequent phosphorylation at S155 disrupts the interaction between the hydrophobic face of the Bad BH3 domain and the Bcl-xl hydrophobic groove. Thus, Bad is disassociated with Bcl-xl and translocated from mitochondrial membrane to cytoplasma (Datta et al., 2000).

**The discovery of Bim**

Bim belongs to BH-3 only subfamily of Bcl-2 family of proteins. It was originally identified as a Bcl-2-interacting protein by screening a bacteriophage cDNA expression library constructed from a mouse thymic lymphoma (O'Connor et al., 1998). There are three splice forms of Bim, i.e. Bim-S, Bim-L, and Bim-EL. They all possess the same BH3 domains, although variation in regions towards the N-terminus (Herman et al., 2008). Bim localizes on intracytoplasmic membranes, i.e. mitochondrial membrane, the endoplasmic reticulum and the nuclear envelope (O'Connor et al., 1998).

**The role of Bim in apoptosis**

As its relatives in pro-apoptosis subfamily, the BH-3 domains of Bim can interact with the hydrophobic groove of the survival proteins in Bcl-2 family (Petros et al., 2001). The interaction subsequently suppresses the function of survival proteins, and triggers the apoptosis pathway by releasing cytochrome c, Apaf-1, and activating caspases (Wakeyama et al., 2007). The apoptosis was observed in HEK 293 cells that overexpressed Bim, and the extent of cell

death was related to the expression level of Bim (O'Connor et al., 1998). The Bim involved cell death could be antagonized by caspases inhibitor (O'Connor et al., 1998). O'Reilliy et al. demonstrated Bim deficient (Bim-/-) mice were resistant to the Bcl-2 inhibitable apoptotic stimuli (O'Reilly et al., 2000). Moreover, Bim plays an important role in embryogenesis, leukocyte homeostasis, and autoimmunity (Bouillet et al., 2000).

**The regulation of Bim**

There is accumulating evidence indicating Bim is regulated in both transcriptional and posttranscriptional level (Wakeyama et al., 2007). The transcription factor Foxo, upregulates the expression of Bim by binding as monomers to the Foxo recognition element (FRE) of BIM promotor (Fu et al., 2008). Dijkers et al. showed the expression of Bim was elevated both in mRNA and protein levels after transfection with inducible FOXO3a vector (Dijkers et al., 2000b). Essafi et al. revealed that Bim expression and apoptosis can be induced by activation of Foxo3a, and abrogated by silencing of Foxo3a in human hematopoietic cells (Essafi et al., 2005).

The posttranslational regulation of Bim includes phosphorylation and ubiquitination (Wakeyama et al., 2007). Extracellular-regulated kinase (Ley et al., 2004; Ley et al., 2005), Jun N-terminal kinase (Putcha et al., 2003) and PKB (Qi et al., 2006) phosphorylate Bim, consequently affect the expression level or the pro-apoptotic function of Bim. Other studies disclosed Bim could be regulated via the ubiquitin–proteasome degradation process (Styles et al., 2005; Meller et al., 2006).

### 1.2.7. β-Catenin

β-Catenin is a 90 kD cytosolic protein, which is first identified due to its binding to the cell-adhesion protein, E-cadherin. β-Catenin and its relative γ-catenin directly interact with the cytoplasmic tail of E-cadherin catenins, serving as a link to connect E-cadherin and the actin cytoskeleton (Behrens, 2000).

## The structure of β-catenin

β-Catenin belongs to the armadillo family of proteins, which are characterized by a central domain of 12 repeats of approximate 40 amino acids, also termed as arm-repeats (Peifer et al., 1994). X-ray cristallography illustrated the structure of the central domain of β-catenin as arm-repeats form a superhelix of helices (Huber et al., 1997). A positively charged groove within the superhelix appears to harbor the binding site for most of the interaction partners of β-catenin (Behrens, 2000).

## β-Catenin acts as the transcription factor

In addition to cadherins, β-catenin associates with other partners, such as tumor suppressor APC, TCF (T-cell factor)/LEF (lymphoid enhancer factor) family of transcription factors. β-Catenin binds to TCF/LEF factors through its arm-repeats. The coupling of β-catenin provides transactivation domains to TCF/LEF factors (Hatsell et al., 2003), which are able to recognize specific DNA motifs within the regulatory regions of the target genes. The β-catenin/TCF transcription factor regulates the expression of numerous target genes which play important roles in physiological and pathological processes. c-Myc and cyclin D1 have been identified as target genes of β-catenin/TCF (Kolligs et al., 2002). c-MYC is an oncogene that has been known to be overexpressed in the mRNA and protein levels in CRC (He et al., 1998; Liao et al., 2007). He et.al. reported expression of c-Myc was activated by β-catenin in colon cancer cells, further they found dominant-negative TCF-4 reduced the endogenous levels of c-MYC, suggesting these effects were mediated through TCF-4 binding sites in the c-MYC promoter (He et al., 1998). Cyclin D1, a major regulator of the progression into the proliferative stage of the cell cycle, is critical for tumorigenesis (Liao et al., 2007). Shtutman et.al. demonstrated overexpression of β-catenin activated cyclin D1 promoter and increased cyclin D1 in the protein level in colon cancer cells, whereas wild type APC and cadherin cytoplasmic domain deactivated cyclin D1 promoter (Shtutman et.al.,

1999).

### The degradation of β-catenin

In normal cells, cytosolic β-catenin is rapidly eliminated by proteasomes (Behrens, 2000). The degradation of β-catenin is initiated by the formation of the complex, comprised of APC, GSK3β and axin (Dale et al., 1998; Liu et al., 2002). In the complex, GSK3β phosphorylats β-catenin at specific serine and threonine residues in its N-terminus, leading to the ubiquitination and degradation of β-catenin (Li et al., 2002; Fuchs et al., 2005). In tumor cells, β-catenin is stabilized by escaping from phosphorylation-dependent ubiquitination and degradation. APC truncation (Fodde et al., 2001; Morin et al., 1998), mutations at phosphor-acceptor sites of β-catenin (Morin et al., 1998), or inhibition of GSK3β activity (Liu et al., 2002) can destroy the β-catenin degradation complex. The accumulation of stabilized cytoplasmic β-catenin leads to its translocation into cell nucleus. There it carries out a pivotal role in tumorigenesis by associating with TCF/LEF to regulate the transcription target genes.

## 1.3. Aims of the studies

The aims of the studies are to identify the role of PDK1 and SGK1 in the colorectal tumorigenesis in vivo, and further investigate the mechanism involved in the PDK1/SGK1-dependent tumor growth. Because of the low incidence of spontaneous CRC in mice, I chemically induced CRC with DMH-DSS treatment in gene targeted mice and their wild type littermates.

As described above, PDK1 plays a pivotal role in tumorigenesis by phosphorylating its substrates (Nicholson et al., 2002; Lang et al., 2006). To explore the role of PDK1 in CRC development, the number of tumors in the large intestine, the survival span of PDK1 hypomorphic mice ($pdk1^{hm}$) and their wild type littermates ($pdk1^{wt}$) were observed. To elucidate the signaling pathway underlying, the phosphorylation of Bad was measured by Western blot and immunofluorescence in the large intestine of $pdk1^{hm}$ mice with the comparison to that of $pdk1^{wt}$ mice. SGK1 has been disclosed to promote the tumor in previous studies (Dehner et al., 2008). The number of colorectal tumors of mice lacking functional SGK1 ($sgk1^{-/-}$) and their wild type littermates ($sgk1^{+/+}$) was counted to identify the role of SGK1 in tumorigenesis. Then the Western blot and immunofluorescence were employed to detect the expression of transcription factor Foxo3a and pro-apoptotic regulator Bim in $sgk1^{-/-}$ mice and $sgk1^{+/+}$ mice. A further series of experiments was performed in HEK 293 cells, in which SGK1 was knocked down by siRNA silencing. The expression of Foxo3a, Bim and the apoptosis were measured to elucidate the SGK1 dependent signaling pathway in vitro. SGK1 may favor tumorigenesis in other ways (Brunet et al., 2001; Shelly et al., 2002; Lang et al., 2006), more than the downregulation of Foxo3a and Bim. As previous studies showed, SGK1 promoted the stabilization and nuclear translocation of β-catenin in vitro (Sakoda et al., 2003; Cohen et al., 2001). To confirm the involvement of SGK1 in the upregulation of β-catenin, Western blot and immunofluorescence were again employed to compare the expression of β-catenin in the large intestine

of *sgk1*$^{-/-}$ mice and *sgk1*$^{+/+}$ mice. Further, β-catenin abundance was detected by Western blot in HEK 293 cells treated with dexamethasone to activate SGK1.

# 2. Material and Method

## 2.1. Material

### 2.1.1. Chemical and biological reagents

Alexa 488 anti-rabbit (Molecular Probes, Eugene, US)
Anti-Bad antibody (Santa Cruz, Santa Cruz, US)
Anti-Bim antibody (Cell signaling, Beverly, US)
Antifade reagent (Invitrogen, Carlsbad, US)
Anti-Foxo3a antibody (Cell signaling, Beverly, US)
Anti-GAPDH antibody (Cell signaling, Beverly, US)
Anti-lamin B antibody (Santa Cruz, Santa Cruz, US)
Anti-mouse IgG antibody (Amersham, Louisville, UK)
Antimycin A (Sigma-Aldrich, St. Louis, US)
Anti-phospho-Bad antibody (Santa Cruz, Santa Cruz,US)
Anti-rabbit IgG antibody (Cell signaling, Beverly, US)
Anti-β-catenin antibody (Cell signaling, Beverly, US)
APOPercentage Apoptosis Assay kit (Biocolor,Carrickfergus, UK)
APS (Merck, Darmstadt, Germany)
Biomax X flim (Kodak, Rochester, US)
Bovine serum albumin (Roth, Karlsruhe, Germany)
Bradford solution (Biorad, Hercules, US)
cDNA synthesis kit (Roche, Basel, Switzerland)
Cryomatrix (Sakura, Zoeterwoude, Netherlands)
Developing solution (Kodak, Rochester, US)
Dexamethasone (Sigma-Aldrich, St. Louis, US)
DMEM medium (Gibco, Carlsbad, US)
DMH (Sigma-Aldrich, St. Louis, US)
DRAQ-5 dye (Biostatus, Leicestershire, UK)
DSS (Wako Pure, Led, Japan)
ECL detection reagent (Amersham, Louisville, UK)
EDTA (Sigma-Aldrich, St. Louis, US)

Ethanol (Roth, Karlsruhe, Germany)
Fetal calf serum (Gibco, Carlsbad, US)
Filter paper (Whatman, Maidstone, UK)
Fixing solution (Kodak, Rochester, US)
Formaldehyde (Roth, Karlsruhe, Germany)
Goat serum (Invitrogen, Carlsbad, US)
Light cycler master SYBR green I kit (Roche, Basel, Switzerland)
β-Mercaptoethanol (Roth, Karlsruhe, Germany)
Methanol (Roth, Karlsruhe, Germany)
$Na_3VO_4$ (Sigma-Aldrich, St. Louis, US)
NaCl (Roth, Karlsruhe, Germany)
NaF (Sigma-Aldrich, St. Louis, US)
Nitrocerullous blot membrane (Whatman, Maidstone, UK)
Nonfat milk (Roth, Karlsruhe, Germany)
Nuclear extraction kit (Imgenex, San Diego, US)
PBS solution (Gibco, Carlsbad, US)
Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, US)
PVDF blot membrane (Millipore, Billerica, US)
RT-PCR primer for GAPDH (Search LC, Heidelberg, Germany)
RT-PCR primer for SGK1(Search LC, Heidelberg, Germany)
SDS (Roth, Karlsruhe, Germany)
SDS loading buffer (Roth, Karlsruhe, Germany)
siPORT Amine transfection agent (Ambion, Austin, US)
Sodium citrate (Roth, Karlsruhe, Germany)
Standard mouse diet (C1310, Altromin, Langen, Germany)
Stripping buffer (Roth, Karlsruhe, Germany)
TEMED (Roth, Karlsruhe, Germany)
Total RNA isolation kit (Peqlab, Erlangen, Germany)
Tris (Roth, Karlsruhe, Germany)
Triton X-100 (Roth, Karlsruhe, Germany)
Tween 20 (Roth, Karlsruhe, Germany)
Validated siRNA for SGK1 (Ambion, Austin, US)
Xylene (Sigma-Aldrich, St. Louis, US)

### 2.1.2. Equipment

-20°C refrigerator (Liebherr, Lindau, Germany)
-80°C refrigerator (Sanyo, Osaka, Japan)
4°C refrigerator (Heraeus, Massachusets, US)
Balence (Sartorius, Goetingen, Germany)
BioPhotometer Eppendorf (Eppendorf, Hamburg, Germany)
Biorad ChemiDoc XRS (Biorad, Hercules, US)
Cell culture hood (Thermo, Waltham, US)
Cell incubator (Heraeus, Massachusets, US)
Centrifuge 22R (Heraeus, Waltham, US)
Centrifuge 5417 R (Eppendorf, Hamburg, Germany)
Confocal Laser Scanning Microscope (Carl Zeiss, Jena, Germany)
Cyrostat (Thermo, Waltham, US)
Electronic hematology particle counter (Medical Diagnostics Marx, Butzbach, Germany)
Electrophoresis cell (Biorad, Hercules, US)
Electrophoresis power supply (Biorad, Hercules, US)
Electrophoretic transfer cell (Biorad, Hercules, US)
Folie bag sealer (Roth, Karlsruhe, Germany)
Heator (Schutron, Pocklington, UK)
Homogenize rotor (Roth, Karlsruhe, Germany)
LightCycler System (Roche, Basel, Switzerland)
Magnetic stirrer (Roth, Karlsruhe, Germany)
Micorwave (Roth, Karlsruhe, Germany)
Pippets (ABimed, Langenfeld, Germany)
Shaker (Roth, Karlsruhe, Germany)
Spectronic GENESYS 6 UV-Vis Spectrophotometer (Thermo, Waltham, US)
Vortex (Peqlab,Erlangen, Germany)
Waterbath (Labortechnik, Seelbach, Germany)

## 2.2. Method

### 2.2.1. Relative resistance of PDK1 hypomorphic mice against colorectal tumor induced by chemical cancerogenesis

**Mice**

The mice have kindly been provided by Dario Alessi, (Department of Biochemistry, University of Dundee, United Kingdom). The blood was collected in PBS containing 2 mM EDTA. Generation and basic properties of those mice have been described previously (Lawlor et al., 2002). Genotyping was made by PCR on tail DNA using PDK1-and neo-R-specific primers. Mice had free access to standard mouse diet and tap water. Animal experiments were conducted according to the guidelines of the American Physiological Society and the German law for the care and welfare of animals and were approved by local authorities.

**Induction and evaluation of colorectal tumors**

Colorectal tumors were generated as described previously (Wang et al., 2004). At the age of 8 weeks the animals were treated with intraperitoneal 20 mg/kg DMH and subsequently by three cycles of alternating administration of $dH_2O$ containing 30 g/L synthetic DSS for 7 days followed by $dH_2O$ for subsequent 14 days. All mice were anesthetized with ether and sacrificed at the age of 20 weeks. After death, the entire large intestine from the colocecal junction to the anal verge was examined. The large intestine was opened longitudinally, washed with PBS, divided into three portions (proximal, middle and distal). The number of colorectal tumors was counted under macroscopic inspection.

**Western blot**

For Western blot, mice were sacrificed by cervical dislocation under ether anesthesia, and the abdomen was opened. The large intestine was then longitudinally cut, and the lumen was cleaned with PBS. A piece of 0.5 g of large intestine containing tumor tissue was added to 1ml lysis buffer (50 mM

Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1mM NaF, 1mM $Na_3VO_4$, 0.4% β-mercaptoethanol) containing protease inhibitor cocktail. The tissue was then homogenized for 30 min on ice. Samples were centrifuged at 17,000 rpm for 20 min, and supernatants were collected. After measurement of the total protein concentration (Bradford assay), 48 µg of protein were solubilized in Laemmli sample buffer at 95ºC for 5 min and resolved by 10%SDS-PAGE. Then proteins were electro-transferred onto a PVDF membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 at room temperature for 60 min Then the membrane was incubated with affinity purified rabbit anti-phospho-Bad antibody (1:200; 23 kD) at 4°C overnight. After washing (TBST) and subsequent blocking, the blots were incubated with secondary goat anti rabbit IgG antibody (1:2000) for 1 h at room temperature. After washing antibody binding was detected with the ECL detection reagent. For loading control the blot was stripped in stripping buffer at 56°C for 30 min. After washing with TBST, the blot was blocked with TBST + 5% milk for 1 h at room temperature. Then, the blot was incubated with anti-Bad antibody (1:200, 23 kD) at 4°C overnight. After washing with TBST and incubation with anti-rabbit IgG secondary antibody (1:2000), antibody binding was detected. Bands were quantified with Quantity One Software.

**Frozen section preparation**

Mice were sacrificed by cervical dislocation under ether anesthesia, and the abdomen was opened. The large intestine was then longitudinally cut, and the lumen was cleaned with PBS. A piece of colon containing tumor tissue was fixed with 4% parafomaldehyde overnight at room temperature. Afterwards tissue was washed with PBS, embed with cryomatrix, and frozen at -80°C. For sectioning frozen tissue block was attached on the cryostat chuck. Routine section were cut at 8 microns and picked up onto slides. The section were dried at room temperature till the sections are firmly adhered to the slide, then stored at -80°C until use.

### Immunofluorescence

To reduce nonspecific background staining, slides were incubated with 5% normal goat serum/1x PBS/0.3% Triton for 1 hour at room temperature and then washed twice in PBS for 3 min. Then, slides were incubated overnight at 4°C with rabbit anti- phospho-Bad (1:200) antibody. After three rinses in 1 x PBS for 10 min each section was incubated with the secondary antibody FITC goat anti-rabbit (1:100) for 1.5 hours at room temperature. Nuclei were stained with DRAQ-5 dye (1:1000) for 5 min at 37ºC. The slides were mounted with an aqueous mountant. Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope with a water immersion Plan-Neofluar 40/1.3 NA DIC.

### Statistical analysis

Data are provided as means ± SEM, *n* represents the number of independent experiments. Data were tested for significance using paired, unpaired student's t-test, ANOVA as appropriate. Differences were considered statistically significant if *p*-values were $< 0.05$

### 2.2.2. The SGK1-dependent upregulation of Foxo3a and Bim promotes colorectal tumor growth

**Silencing of SGK1**

HEK 293 cells were grown in DMEM medium containing 10% fetal calf serum under standard culture conditions (37°C, 5% $CO_2$). $2\times10^5$ cells were seeded in 6 well plates and cultivated with fresh culture medium for 8 h to reach 50-80% confluency. 3 µl of siPORT transfection reagent was diluted with 100 µl of DMEM medium, incubated at room temperature for 10 min. 75 pmol of validated siRNA for SGK1 or 12.5 pmol of select valid non-sense siRNA was diluted with 100 µl of DMEM medium, mixed with diluted siPORT transfection reagent,and incubated at room temperature for 10 min to allow the transfection complex formation. 200 µl of newly formed transfection complex was dispensed to the HEK 293 cells in each well, with gently rocking the cell culture plate back and forth to evenly distribute the complexes.

**Total RNA isolation**

Total RNA was isolated from HEK 293 cells using the total RNA isolation kit. The cells were scraped from cell culture plates. The cell suspension was centrifuged at 800 g for 5 min, and the cell pellet was lysated with 400 µl of TRK lysis buffer. Then the lysate was transferred into shredder column, centrifuged at 12000 g for 1 min at room temperature. The flow through was mixed with 1 volume of 70% ethanol by votexing, and transferred to the Hibind RNA spin column. The mixture was centrifuged at 10000g for 1 min, then RNA was washed with wash buffer I and II by centrifugation at 10000g for 15 sec respectively. After the blank wash, the RNA was eluted in 50 µl of RNase free $H_2O$. The concentration of RNA was measured with Biophotometer. The samples were kept at -80°C till use.

**cDNA synthesis**

cDNA was generated from total RNA of HEK 293 cells using the cDNA synthesis kit. 1 µg of total RNA was mixed with 2 µl of random primer and PCR grade $H_2O$ to the final volume of 11.4 µl. The primer-template mixture was denatured at 65°C for 10 min in a thermal block cycler. Afterwards, 4 µl of 5×reaction buffer, 0.5 µl of RNase inhibitor, 2 µl of dNTP mix, 1 µl of DTT and 1.1 µl of reverse transcriptase were add, mixed with the denatured primer-template mixture, followed by incubation at 48°C for 30 min, and 85°C for 5 min. The concentration of cDNA was measured with Biophotometer. The samples were kept at -20°C till use.

**Quantitative RT-PCR**

2 µl of Mix SYBR Green I was mixed with 14 µl of PCR grade $H_2O$, 2 µl of primer mix for SGK1 or GAPDH, and 2 µl of sample cDNA from either SGK1 siRNA transfected HEK 293 cells or non-sense siRNA transfected HEK 293 cells. The mixture was loaded into capillary tubes and the quantitative real time RT-PCR was performed in a light cycler. The settings are as follows.

**Denaturation**

| Parameter | Value |
|---|---|
| Cycles | 1 |
| Type | Regular |
| Temp. Targets | Segment 1 |
| Target Temperature | 95 |
| Incubation time (h:min:s) | 10:00 |
| Temp. Transition Rate (°C/s) | 20 |
| Secondary Target Temp. | 0 |
| Step Size | 0 |
| Step Delay | 0 |
| Aquisition Mode | None |

## Amplification

| Parameter | Value | | |
|---|---|---|---|
| Cycles | 35 | | |
| Type | Quantification | | |
| Temp. Targets | Seg.1 | Seg.2 | Seg.3 |
| Target Temperature | 95 | 68 | 72 |
| Incubation time (h:min:s) | 10 | 10 | 16 |
| Temp. Transition Rate (°C/s) | 20 | 20 | 20 |
| Secondary Target Temp. | 0 | 58 | 0 |
| Step Size | 0 | 0.5 | 0 |
| Step Delay | 0 | 1 | 0 |
| Aquisition Mode | None | None | Single |
| Gains | F1 = 5 | | |

## Melting Curve Analysis

| Parameter | Value | | |
|---|---|---|---|
| Cycles | 1 | | |
| Type | Melting Curve | | |
| Temp. Targets | Seg.1 | Seg. 2 | Seg.3 |
| Target Temperature | 95 | 58 | 95 |
| Incubation time (h:min:s) | 0 | 10 | 0 |
| Temp. Transition Rate (°C/s) | 20 | 20 | 0.1 |
| Secondary Target Temp. | 0 | 0 | 0 |
| Step Size | 0 | 0 | 0 |
| Step Delay | 0 | 0 | 0 |
| Aquisition Mode | None | None | Cont. |

## Cooling

| Parameter | Value |
|---|---|
| Cycles | 1 |
| Type | Regular |
| Temp. Targets | Segment 1 |
| Target Temperature | 40 |
| Incubation time (h:min:s) | 30 |
| Temp. Transition Rate (°C/s) | 20 |
| Secondary Target Temp. | 0 |
| Step Size | 0 |
| Step Delay | 0 |
| Aquisition Mode | None |

The efficiency of silencing was checked by quantitative RT-PCR 48 h after transfection. The copies of PCR product were caculated with the instrument's

software (Light cycler 3.0). Approximately 24% reduction of the concentration of SGK1 mRNA.was detected.

### Antimycin A induced apoptosis of HEK 293 cells

HEK 293 cells were cultivated at the density of $5\times10^4$/well in 24 well plates. siRNA silencing was performed as described in the previous experiment. After 48-hour-incubation with siRNA complex, culture medium was replaced by fresh culture medium with 50 µM of AMA, and incubated for another 24 h.

### Measure the apoptosis of HEK 293 cells with the APOPercentage

The extent of apoptosis was measured with the APOPercentage kit. 30 min before the 24 h AMA incubation was reached, 10 µl/well of APOPercentage dye was add into the cell culture medium. The HEK 293 cells were consecutively incubated with APOPercentage dye for the remaining 30 min. at 37°C. Then the APOPercentage dye and cultured medium mixture were aspirated. Cells were washed with PBS twice to remove any unbound dye. 300 µl/well APOPercentage dye release reagent was added to the cells, with gently shaking the cell culture plate for 10 min at room temperature. Finally the absorbance of cell bound dye recovered solution at 550 nm was measured with biophotometer.

### Mice

Experiments were carried out on gene targeted mice lacking functional SGK1 ($sgk1^{-/-}$) and their wild type littermates ($sgk1^{+/+}$) as previously described (Wulff et al., 2002). The animals were housed under controlled environmental conditions (22-24°C, 50-70% humidity and a 12 h light/dark cycle). Throughout the study mice had free access to standard pelleted food and tap water as indicated. All animal experiments were conducted according to the guidelines of the American Physiological Society and the German law for the care and welfare of animals and were approved by local authorities.

**Induction and evaluation of colorectal tumors**

Colorectal tumors were generated as described previously (Wang et al., 2004). At the age of 8 weeks the animals were treated with intraperitoneal 20 mg/kg DMH and subsequently by three cycles of alternating administration of distilled water containing 30 g/L synthetic DSS for 7 days followed by $dH_2O$ for subsequent 14 days. All mice were anesthetized with ether and sacrificed at the age of 20 weeks. After death, the entire large intestine from the colocecal junction to the anal verge was examined. The large intestine length was measured. Then, the large intestine was opened longitudinally, washed with PBS, divided into three portions (proximal, middle and distal). The number of colorectal tumor was counted under macroscopic inspection.

**Blood count**

For blood count 10 µl of blood was withdrawn from the tail. Erythrocyte number, packed cell volume, and blood hemoglobin concentration were determined using an electronic hematology particle counter equipped with a photometric unit for hemoglobin determination. The gating was adjusted for the application on mouse erythrocytes. All measurements were done according to the manufacturer’s instructions.

**Nuclear protein extraction**

For the analysis of Foxo3a a nuclear extraction was performed using the Nuclear extraction kit according to the instruction manual. The details are as follows. Mice were sacrificed by cervical dislocation under ether anesthesia, and the abdomen was opened. The colon was then longitudinally cut, and the lumen was cleaned with PBS. A piece of 0.5 g of colon containing tumor tissue was lysated with hypertonic buffer supplemented with 1 mM DTT and 1% detergent solution, and homogenized on ice for 30 min. Then tissue lysate was centrifuged at 10000 g for 10 min at 4°C, and the pellet was resuspended in 200 µl of nuclear lysis buffer. After incubation at 4°C for 30 min, the suspension was centrifµged at 16000 g for

10 min at 4°C. The supernatant was collected and stored at -20°C for later use.

**Western blot**

Mice were sacrificed by cervical dislocation under ether anesthesia, and the abdomen was opened. The colon was then longitudinally cut, and the lumen was cleaned with PBS. A piece of 0.5 g of large intestine containing tumor tissue was added to 1 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1mM NaF, 1mM $Na_3VO_4$, 0.4% β-mercaptoethanol) containing protease inhibitor cocktail. For the analysis of Foxo3a, a nuclear protein extraction was performed (as described above). The tissue was then homogenized for 30 min on ice. Samples were centrifuged at 17,000 rpm for 20 min, and supernatants were collected. After measurement of the total protein concentration (Bradford assay), 70 µg (Foxo3a) or 80 µg (Bim) of protein were solubilized in Laemmli sample buffer at 95ºC for 5 min and resolved by 8% (Foxo3a) or 10% (Bim) SDS-PAGE. For immunoblotting proteins were electro-transferred onto a PVDF membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 at room temperature for 1 h (Foxo3a) or 2 h (Bim). Then, the membrane was incubated with anti-Foxo3a antibody (1:500; 90 kD) or anti-Bim antibody (1:1000, 23 kD) at 4°C overnight. After washing (TBST) and subsequent blocking, the blots were incubated with anti-rabbit IgG secondary antibody (1:2000) for 1 h at room temperature. After washing antibody binding was detected with the ECL detection reagent. For loading control the blot was stripped in stripping buffer (Roth, Karlsruhe, Germany) at 56°C for 30 min. After washing with PBST (Foxo3a) or TBST (Bim) the blot was blocked with PBST + 5% milk (Foxo3a) or TBST + 5% milk (Bim) for 1 h at room temperature. Then, the blot was incubated with an anti-lamin B antibody (Foxo3a, 1:100, 67 kD) for 2 h at room temperature or with anti-GAPDH antibody (Bim, 1:1000, 37 kD) at 4°C overnight. After washing with PBST or TBST and incubation with anti-goat IgG secondary antibody (lamin B; 1:5000) or anti-rabbit IgG secondary antibody (GAPDH, 1:2000),

antibody binding was detected. Bands were quantified with Quantity One Software.

### Paraffinized section preparation

Mice were sacrificed by cervical dislocation under ether anesthesia, and the abdomen was opened. The large intestine was then longitudinally cut, and the lumen was cleaned with PBS. A piece of large intestine containing tumor tissue was fixed with 10% formalin overnight at room temperature. After fixation, the tissue was embed with 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol for 1 h each, then Xylene, Paraffin wax for 3 h each. The paraffin block was attached on the cryostat chuck. Routine section were cut at 8 microns and picked up onto slides. The section were dried at room temperature till the sections are firmly adhered to the slide, then stored at -20°C until use.

### Immunofluorescence

The cover slips with HEK 293 cells grown on and paraffinized tissue section were used for immunofluorescence stain. The tissue sections were deparaffinized by two xylene rinses of 5 minutes each followed by two rinses with decreasing concentrations of ethanol for 2 minutes each and two rinses in $dH_2O$ for 5 minutes each. Antigen retrieval was performed by boiling the slides in a pressure cooker in 10 mM sodium citrate buffer at pH 6.0 for 10 minutes. Sections were cooled 30 min on bench top and briefly rinsed in $dH_2O$ and 1x PBS for 5 minutes each. To reduce nonspecific background staining, slides were incubated with 5% normal goat serum/1x PBS/0.3% Triton for 1 hour at room temperature and then washed twice in PBS for 3 min. HEK293 cells were similarly blocked. Then, slides were incubated overnight at 4°C with rabbit anti-Foxo3a (1:200) or rabbit anti-Bim (1:200) antibody. After three rinses in 1x PBS for 10 minutes each section was incubated with the secondary antibody Alexa 488 goat anti-rabbit (1:100) for 1.5 hours at room temperature. Nuclei were stained with DRAQ-5 dye (1:500) for 5 min at 37ºC. The slides were mounted with an aqueous mountant. Images were taken on a Zeiss

LSM 5 EXCITER Confocal Laser Scanning Microscope with a water immersion Plan-Neofluar 40_/1.3 NA DIC.

### Statistical analysis

Data are provided as means ± SEM. *n* represents the number of independent experiments. Data were tested for significance using paired, unpaired student's t-test, ANOVA as appropriate. Differences were considered statistically significant if *p*-values were $< 0.05$

### 2.2.3. The regulation of β-Catenin by SGK1 in mice with chemically induced colorectal cancer

**Dexamethasone induced upregµlation of β-catenin**

HEK 293 cells were grown in DMEM medium containing 10% fetal calf serum under standard culture conditions (37°C, 5% $CO_2$). 3 million cells were incubated for 72 hours in the presence or absence of 1 µM dexamethasone.

**Mice**

Experiments were carried out on gene targeted mice lacking functional SGK1 (*sgk1*$^{-/-}$) and their wild type littermates (*sgk1*$^{+/+}$) as previously described (Wulff et al., 2002). The animals were housed under controlled environmental conditions (22-24°C, 50-70% humidity and a 12 h light/dark cycle). Throughout the study mice had free access to standard pelleted food and tap water as indicated. All animal experiments were conducted according to the guidelines of the American Physiological Society and the German law for the care and welfare of animals and were approved by local authorities.

**Induction of colorectal tumors**

Colorectal tumors were generated as described previously (Wang et al., 2004). At the age of 8 weeks the animals were treated with intraperitoneal 20 mg/kg DMH and subsequently by three cycles of alternating administration of distilled water containing 30 g/L synthetic DSS for 7 days followed by distilled water for subsequent 14 days. All mice were anesthetized with ether and sacrificed at the age of 20 weeks.

**Western blot**

Mice were sacrificed by cervical dislocation under ether anesthesia, and the abdomen was opened. The large intestine was then longitudinally cut, and the lumen was cleaned with PBS. Cells were scraped from culture dishes, and

collected by centrifugation at 800 g for 5 min. A piece of 0.5 g of large intestine containing tumor tissue was added to 1ml lysis buffer or $10^6$ HEK 293 cells was added to 100 μl lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1mM NaF, 1mM $Na_3VO_4$, 0.4% β-mercaptoethanol) containing protease inhibitor cocktail (Sigma). The tissue was then homogenized for 30 min on ice. Samples were centrifμged at 17,000 rpm for 20 min, and supernatants were collected. After measurement of the total protein concentration (Bradford assay), 80 μg (colon tissue sample) or 40 μg (HEK 293 cell sample) of protein were solubilized in Laemmli sample buffer at 95ºC for 5 min and resolved by 10% SDS-PAGE. For immunoblotting, proteins were electro-transferred onto a PVDF membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 at room temperature for 2 h. Then, the membrane was incubated with affinity purified rabbit anti-β-catenin antibody (1:1000, 92 kD) at 4°C overnight. After washing (TBST) and subsequent blocking the blots were incubated with secondary anti rabbit antibody (1:2000) for 1 h at room temperature. After washing antibody binding was detected with the ECL detection reagent. For loading control the blot was stripped in stripping buffer at 56°C for 30 min. After washing with TBST, the blot was blocked with TBST + 5% milk for 1 h at room temperature. Then, the blot was incubated with anti-GAPDH antibody (1:1000, 37 kD) at 4°C overnight. After washing with TBST and incubation with anti-rabbit IgG secondary antibody (1:2000), antibody binding was detected. Bands were quantified with Quantity One Software.

**Frozen section preparation**

Mice were sacrificed by cervical dislocation under ether anesthesia, and the abdomen was opened. The large intestine was then longitudinally cut, and the lumen was cleaned with PBS. A piece of colon containing tumor tissue was fixed with 4% parafomaldehyde overnight at room temperature. Afterwards, tissue was washed with PBS, embed with frozen tissue matrix, and frozen at -80°C. For

sectioning, frozen tissue block was attached on the cryostat chuck. Routine section were cut at 8 microns and picked up onto slides. The section were dried at room temperature till the sections are firmly adhered to the slide, then stored in a -80°C freezer until use.

**Immunofluorescence**

To localize β-catenin protein expression, 5-μm-thick frozen tissue sections from the colon were incubated with 5% normal goat serum/1x PBS/0.3% Triton for 1 hour at room temperature. After washing twice with PBS the slides were incubated overnight at 4°C with rabbit anti-β-catenin (1:200). The slides were rinsed three times with PBS and incubated with secondary FITC goat anti-rabbit antibody (1:400) for 1.5 h at room temperature. After three washing steps the nuclei were stained with DRAQ-5 dye (1:1000) for 10 min at room temperature. The slides were washed again three times and then mounted with ProLong Gold antifade reagent. Images were taken on a Zeiss LSM 5 EXCITER Confocal Laser Scanning Microscope with a water immersion (Plan-Neofluar 40_/1.3 NA DIC).

**Statistical analysis**

Data are provided as means ± SEM, *n* represents the number of independent experiments. Data were tested for significance using paired, unpaired student's t-test as appropriate. Differences were considered statistically significant if *p*-values were $< 0.05$

# 3. Results

## 3.1. Relative resistance of PDK1 hypomorphic mice against colorectal tumorinduced by chemical cancerogenesis

To induce chemical cancerogenesis, mice were were treated by intraperitoneal injection of the carcinogenic drug DMH followed by 3 cycles of DSS in the drinking water. The carcinogenic treatment was followed by the development of multiple colorectal tumors (Fig. 3). The number of tumors was significantly smaller in $pdk1^{hm}$ than in $pdk1^{wt}$ mice (Fig. 3).

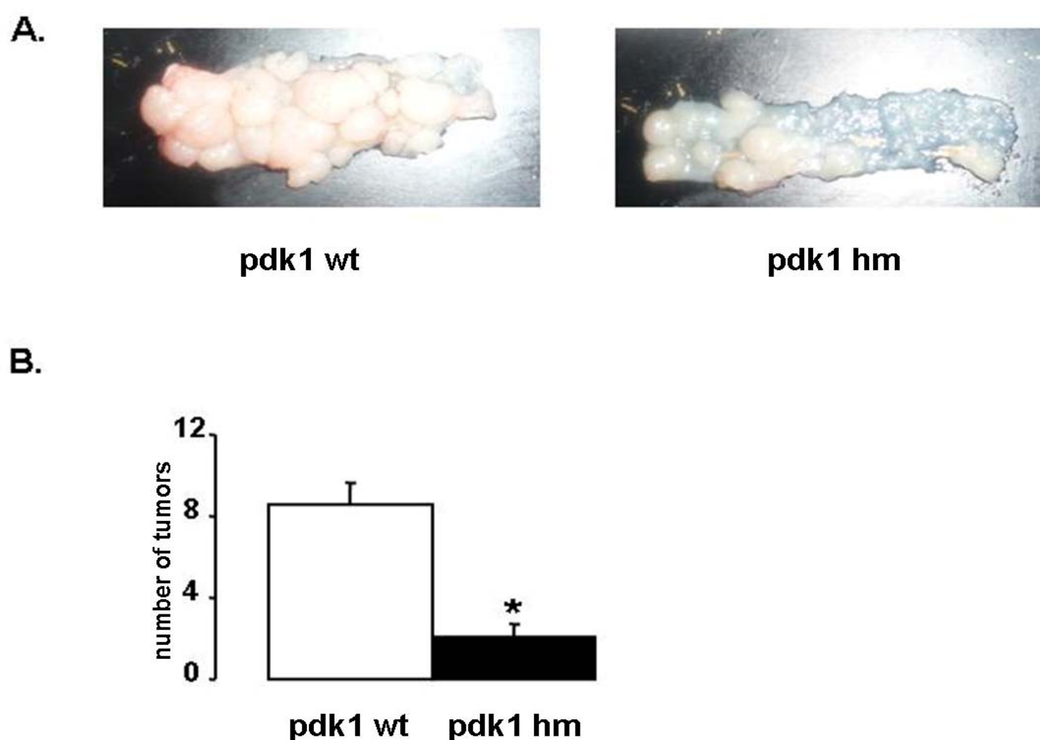



**Fig. 3 Effect of chemical cancerogenesis on tumor incidence in $pdk1^{hm}$ and $pdk1^{wt}$ mice.** **A.** Photograph of colorectal tissue from PDK1 hypomorphic mice ($pdk1^{hm}$) and their wild type littermates ($pdk1^{wt}$) 12 weeks after beginning of treatment with the carcinogenic drug DMH followed by DSS. **B.** Arithmetic means ± SEM (n = 4) of colorectal tumor incidence in $pdk1^{wt}$ (white bar) and $pdk1^{hm}$ (black bar) mice 12 weeks following treatment with the carcinogenic drug DMH followed by DSS. * indicates significant difference from $pdk1^{wt}$ mice ($p<0.05$).

According to Weastern blot phosphorylation of Bad was significantly less pronounced in *pdk1*$^{hm}$ than in *pdk1*$^{wt}$ mice (Fig. 4A). The immunofluorescence was employed to illustrate the further test for PDK1-dependent phosphorylated Bad expression. As illustrated in Fig. 4C, the phosphorylated Bad abundance was indeed higher in *pdk1* $^{wt}$ than in *pdk1* $^{hm}$ mice.

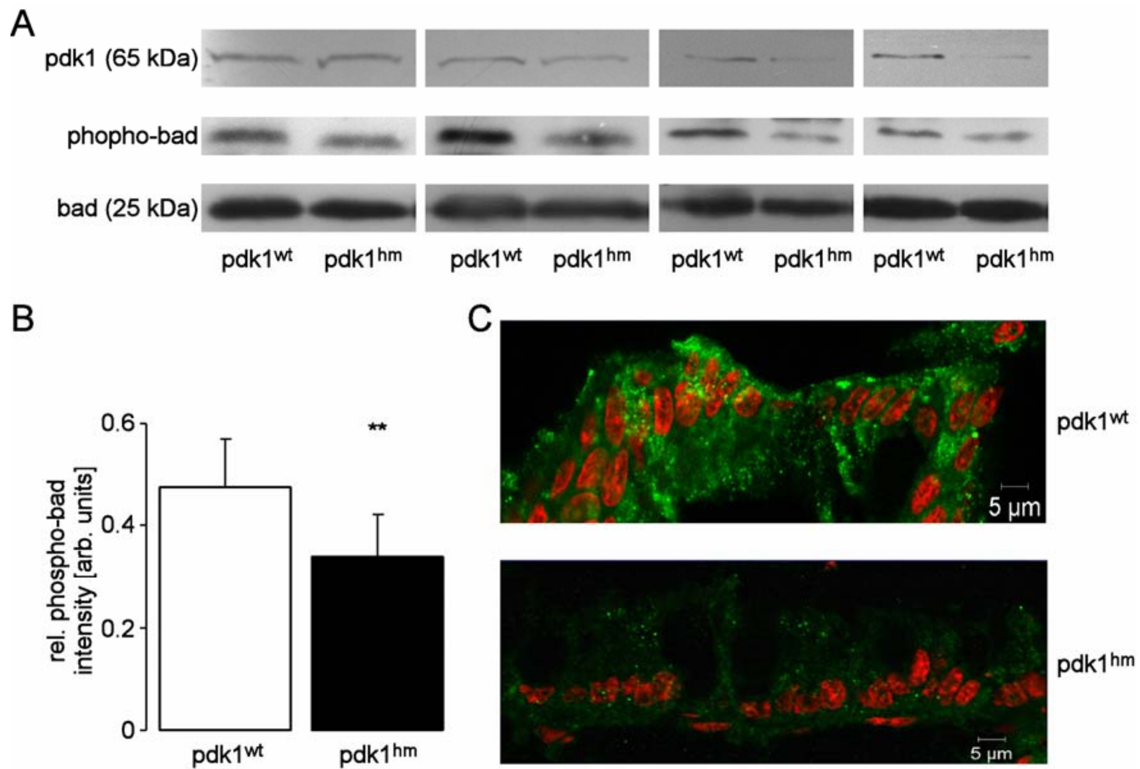



**Fig. 4 Phosphorylation of Bad following chemical cancerogenesis on tumor incidence in *pdk1*$^{hm}$ and *pdk1*$^{wt}$ mice.**

**A.** Original Western Blot illustrating Bad phosphorylation in tumors from PDK1 hypomorphic mice (*pdk1*$^{hm}$) and their wild type littermates (*pdk1*$^{wt}$). **B.** Arithmetic means ± SEM (n =5) of relative densities of Bad and phosphorylated Bad in tumors from PDK1 hypomorphic mice (*pdk1*$^{hm}$) and their wild type littermates (*pdk1*$^{wt}$). * indicates significant difference from and *pdk1*$^{wt}$ mice ($p<0.05$). **C.** Immunofluorescence of Bad phosphorylation (green color: phospho-Bad; red color: nuclear staining) in tumor tissue from PDK1 hypomorphic mice (*pdk1*$^{hm}$, lower panel) and their wild type littermates (*pdk1*$^{wt}$, upper panel).

The accelerated development of tumors led to early death of the animals. A Kaplan–Maier plot reveals that the treatment was survived significantly longer by *pdk1*$^{hm}$ than by *pdk1*$^{wt}$ mice (Fig. 5).

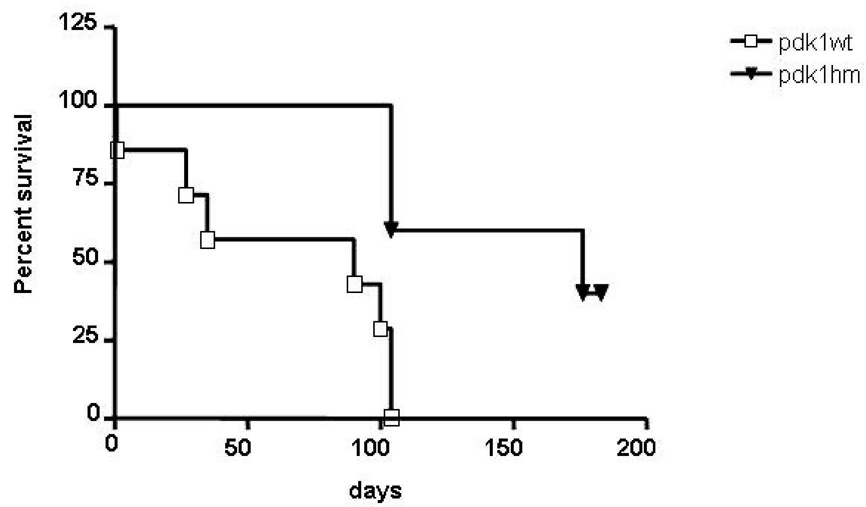



**Fig. 5 Survival of *pdk1$^{hm}$* and *pdk1$^{wt}$* mice following chemical cancerogenesis.**
Kaplan-Maier plot of surviving PDK1 hypomorphic mice (*pdk1$^{hm}$*) and their wild type littermates (*pdk1$^{wt}$*) following chemical cancerogenesis.

### 3.2. The SGK1-dependent upregulation of Foxo3a and Bim promotes colorectal tumor growth

To determine the role of SGK1 in the regulation of Bim expression, HEK293 cells were transfected with siRNA specific for the mRNA of SGK1. As shown in Fig. 6A, 6B, silencing of SGK1 resulted in a significant upregulation of the expression of Bim. To further characterize the cellular effects of silencing of SGK1 immunofluorescence was employed. Fig. 6C illustrates that silencing of SGK1 (right panels) does not only result in upregulation of Bim but also in upregulation of the transcription factor Foxo3a.

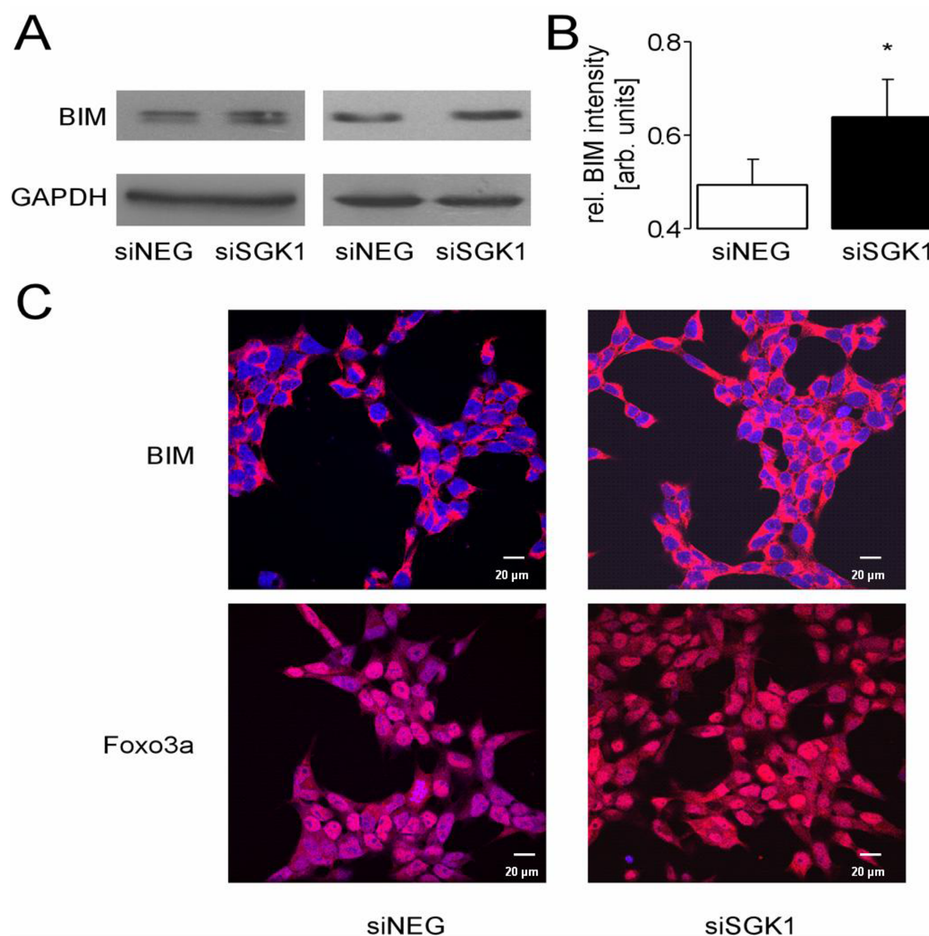



**Fig. 6 Effect of SGK1 silencing on Foxo3a and Bim protein abundance in HEK293 cells.** A. Original Western Blots illustrating the Bim (upper panels) and GAPDH (lower panels) protein abundance in HEK293 cells transfected with nonsense siRNA (siNEG) or with siRNA specific for SGK1 (siSGK1). B. Arithmetic means ± SEM (n = 4) of relative densities of Bim protein expression in HEK293 cells transfected with nonsense siRNA (siNEG) or with siRNA specific for SGK1 (siSGK1). * indicates significant difference ($p<0.05$). C. Immunofluorescence of Bim (upper panels; red color: Bim; blue color: nuclear staining) and Foxo3a (lower panels;

red color: Foxo3a; blue color: nuclear staining) in HEK293 cells transfected with nonsense siRNA (left panels) or with siRNA specific for SGK1 (right panels).

Further experiments were performed to elucidate the significance of SGK1 for apoptosis of HEK293 cells. To this end, apoptosis with or without silenced SGK1 was induced by incubation with the inhibitor of the respiratory chain antimycin A. As shown in Fig. 7 silencing of SGK1 significantly enhanced antimycin A-induced apoptosis, pointing to a protective effect of SGK1 from apoptosis.

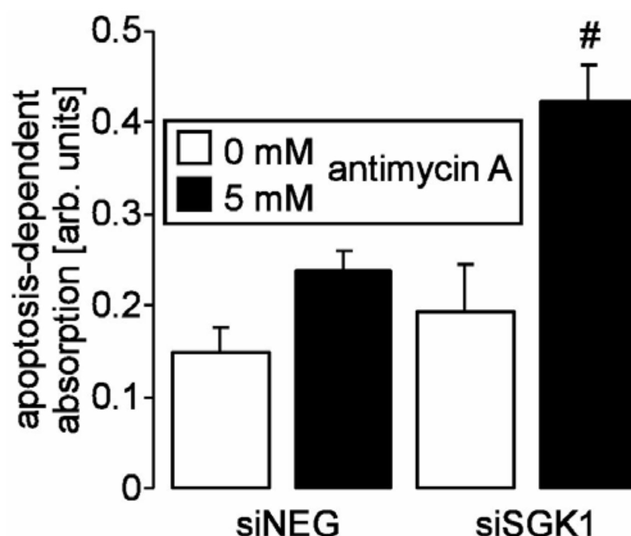



**Fig. 7 Absence of SGK1 activity promotes apoptotic cell death of hypoxic HEK293 cells.** Arithmetic means ± SEM (n = 4-5) of the apoptosis-dependent APOPercentage absorption of HEK 293 cells transfected with nonsense siRNA (siNEG) or with siRNA specific for SGK1 (SGK1) and further incuated for 24 hours in the presence (black bars) or absence (white bars) of 50 µM inhibitor of the respiratory chain antimycin A. # indicates significant difference from siSGK1 (ANOVA).

To disclose a possible role of SGK1 in the development of tumors *in vivo*, gene targeted mice lacking functional SGK1 (*sgk1*$^{-/-}$) and their wild type littermates (*sgk1*$^{+/+}$) were exposed to chemical cancerogenesis by intraperitoneal injection of the carcinogenic drug DHM followed by 3 cycles of DSS in the drinking water. As listed in Table 1, body weight was not significantly different between *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice following the treatment. In addition, the large intestine was significantly longer in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ mice.

The carcinogenic treatment was followed by development of anemia, characterized by a decrease of red blood cell number, hemoglobin concentration, and packed cell volume (Table 1).

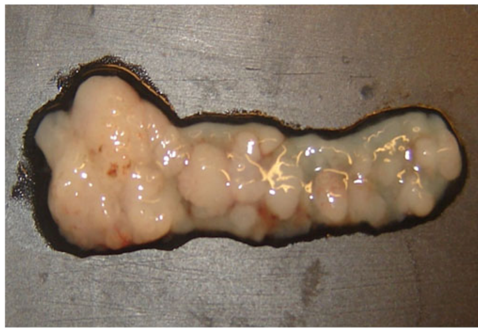
| | *Sgk1*$^{+/+}$ | | *Sgk1*$^{-/-}$ | |
|---|---|---|---|---|
| | Start | End | Start | End |
| Body weight [g] | 24.0 ± 0.7 | 25.7 ± 0.7 | 25.7 ± 0.9 | 27.0 ± 0.7 |
| Hemoglobin [g/l] | 17.3 ± 0.6 | 15.4 ± 0.5 * | 16.4 ± 0.8 | 13.6 ± 0.7 * # |
| RBC [Mio/µl] | 9.0 ± 0.1 | 8.0 ± 0.2 * | 8.3 ± 0.2 | 7.3 ± 0.3 * |
| Hematocrit [%] | 42.1 ± 0 .6 | 36.6 ± 1.2 * | 37.7 ± 1.1 # | 32.9 ± 1.6 * |
| Colon length [cm] | | 8.4 ± 0.3 | | 9.2 ± 0.2 # |

**Table 1. Effect of chemical cancerogenesis on body weight, blood count and colon length**

Body weight, hemoglobin concentration, red blood cell count (RBC), packed cell volume (hematocrit), and colon length of gene targeted mice lacking functional SGK1 (*sgk1*$^{-/-}$) and their wild type littermates (*sgk1*$^{+/+}$) prior to (Start) and 12 weeks after treatment with the carcinogenic drug DMH followed by DSS (End). * indicates statistically significant difference between the respective value prior to and 12 weeks after the beginning of the DMH+DSS treatment; # indicates statistically significant difference between *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice.

The carcinogenic treatment triggered the development of multiple colorectal tumors in each of the *sgk1*$^{+/+}$ mice (Fig. 8). The number of tumors was significantly smaller in *sgk1*$^{-/-}$ mice as compared to *sgk1*$^{+/+}$ mice (Fig. 8).

A.



Sgk1 wt    Sgk1 ko

B.

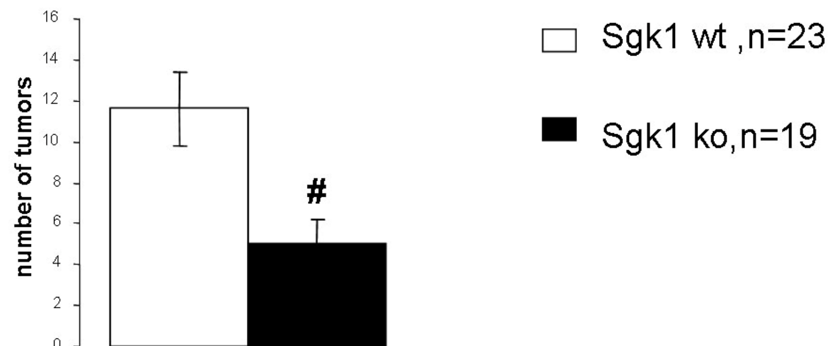



**Fig. 8 Effect of chemical cancerogenesis on tumor incidence in SGK1 knockout mice and their wild type littermates**

A. Photograph of colorectal tissue from gene targeted mice lacking functional SGK1 ($sgk1^{-/-}$) and their wild type littermates ($sgk1^{+/+}$) 12 weeks after beginning of treatment with the carcinogenic drug DMH followed by DSS. B. Arithmetic means ± SEM (n = 19-23) of the number of colorectal tumors in 23 $sgk1^{+/+}$ (white bar) and 19 $sgk1^{-/-}$ mice (black bar) 12 weeks after treatment with the carcinogenic drug DMH followed by DSS. # indicates significant difference from $sgk1^{+/+}$ mice.

Western blot was employed to elucidate the molecular mechanisms accounting for or contributing to the role of SGK1 in tumor growth. Specifically, experiments explored the putative influence of SGK1 on the protein abundance of the pro-apoptotic transcription factor Foxo3a. As illustrated in Fig. 9 Western blot indeed revealed that the Foxo3a protein abundance was significantly higher in $sgk1^{-/-}$ mice as compared to $sgk1^{+/+}$ mice. Foxo3a-dependent genes include Bim. Western blot indeed disclosed a significantly higher Bim abundance in $sgk1^{-/-}$ than in $sgk1^{+/+}$ mice (Fig. 9).

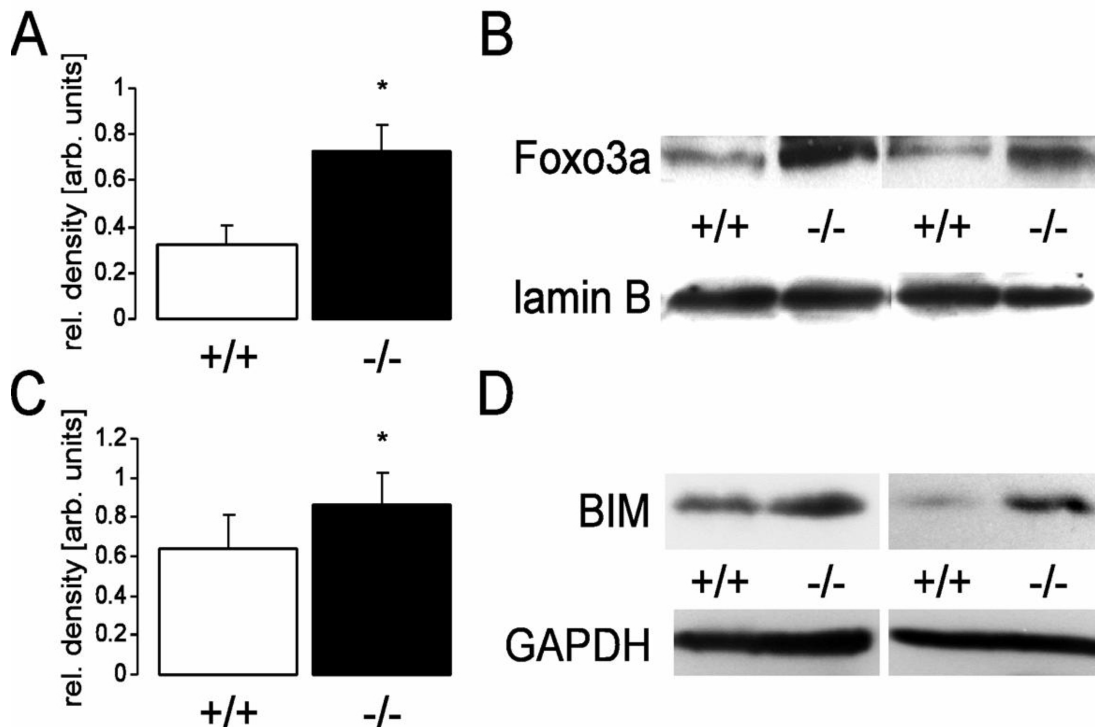



**Fig. 9 Expression of Foxo3a and Bim in SGK1 knockout mice and wild type littermates**
A. Arithmetic means ± SEM (n = 4) of relative densities of Foxo3a protein expression in tumors from sgk1+/+ mice (open bars) and sgk1-/- mice (closed bars). * indicates significant difference from sgk1+/+ mice ($p<0.05$). B. Original Western Blot illustrating the Foxo3a (upper panel) and lamin B (lower panel) protein abundance in tumors from gene targeted mice lacking functional SGK1 (sgk1-/-) and their wild type littermates (sgk1+/+). C. Arithmetic means ± SEM (n = 4) of relative densities of Bim protein expression in tumors from sgk1+/+ mice (open bars) and sgk1-/- mice (closed bars). * indicates significant difference from sgk1+/+ mice ($p<0.05$). D. Original Western Blot illustrating the Bim (upper panel) and GAPDH (lower panel) protein abundance in tumors from gene targeted mice lacking functional SGK1 (sgk1-/-) and their wild type littermates (sgk1+/+).

In a next series of experiments Immunofluorescence was employed to further test for SGK1-dependent Foxo3a and Bim expression. As illustrated in Fig. 10 the Foxo3a abundance was indeed higher in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ mice. Moreover, Bim abundance was again clearly higher in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ mice (Fig. 11).

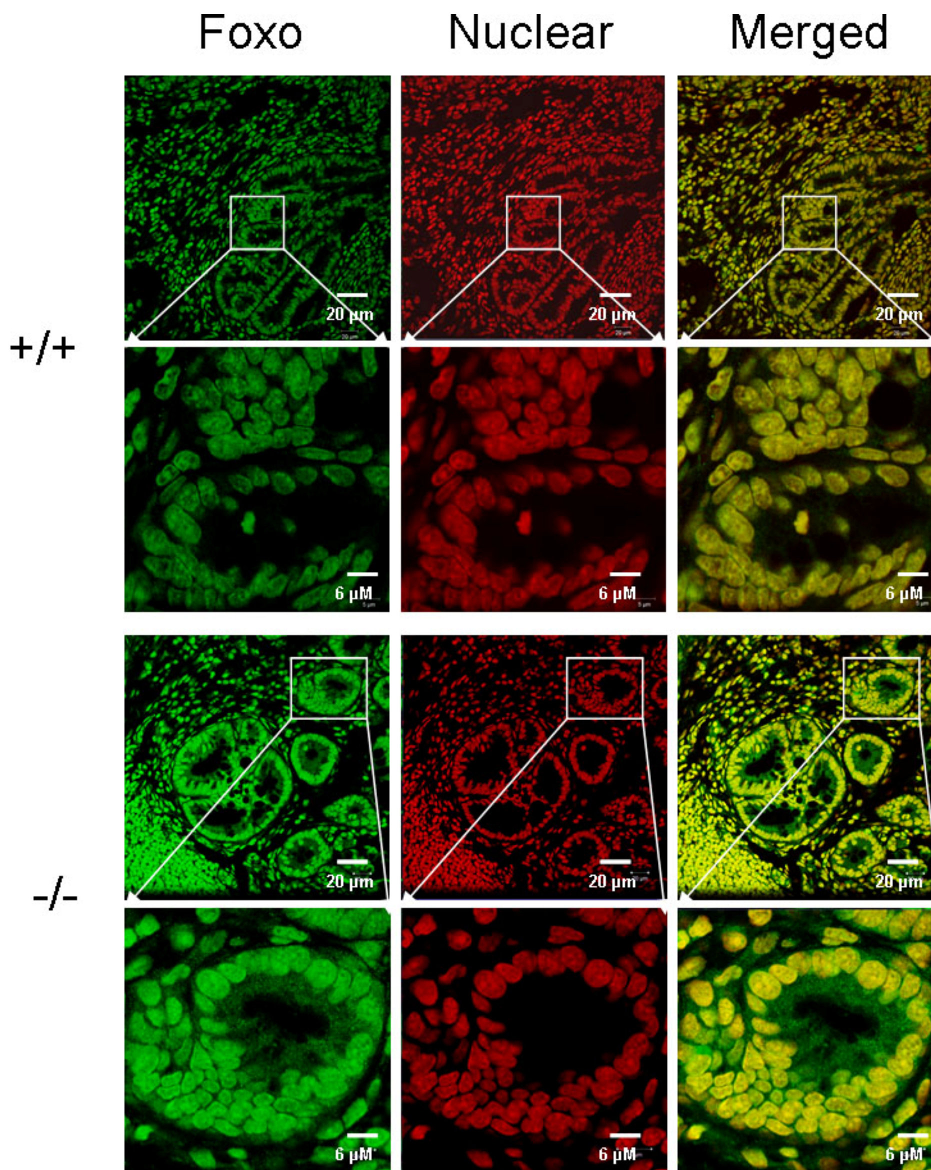



**Fig. 10 Immunofluorescence of Foxo3a abundance in SGK1 knockout mice and wild type littermates**

Immunofluorescence of Foxo3a in tumor tissue from sgk1+/+ mice (upper panels) and sgk1-/- mice (lower panels). The left panels show Foxo3a-dependent fluorescence (green), the middle panels show DNA-dependent DRAQ5 fluorescence (nuclear staining, red) and the right panels show merged red and green fluorescence in sections of mice colon tumors.

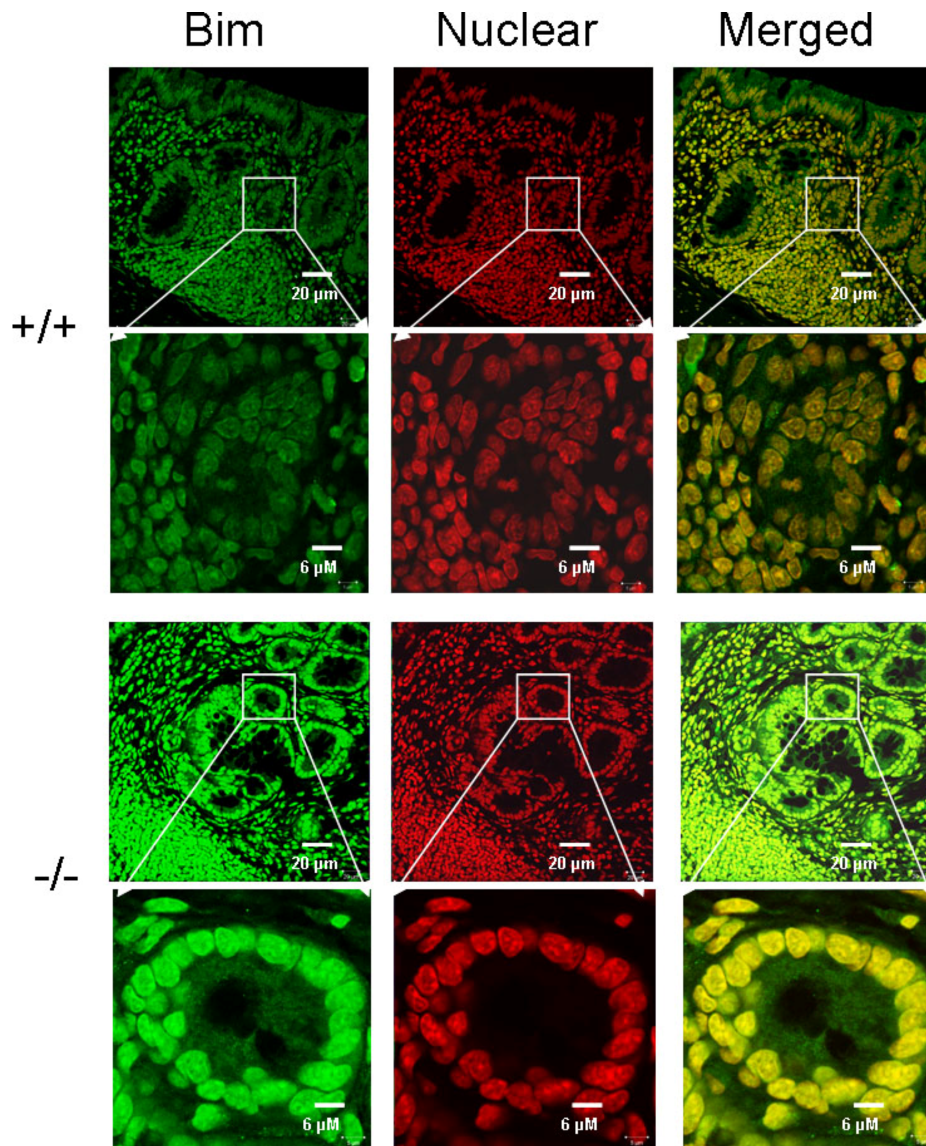



**Fig. 11 Immunofluorescence of Bim abundance in SGK1 knockout mice and wild type littermates**

Immunofluorescence of Bim in tumor tissue from *sgk1*$^{+/+}$ mice (upper panels) and *sgk1*$^{-/-}$ mice (lower panels). The left panels show Bim-dependent fluorescence (green), the middle panels show DNA-dependent DRAQ5 fluorescence (nuclear staining, red) and the right panels show merged red and green fluorescence in sections of mice colon tumors.

### 3.3. The regulation of β-Catenin by SGK1 in mice with chemically induced colorectal cancer

Western Blot was performed to determine the β-catenin abundance in chemically-induced colon tumor tissue from *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ mice. As illustrated in Fig. 12 colorectal tissue from *sgk1*$^{-/-}$ mice expressed significantly less β-catenin than colorectal tissue from *sgk1*$^{+/+}$ mice.

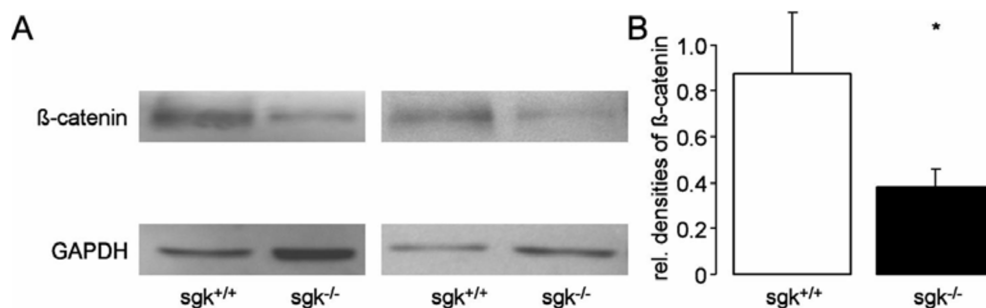



**Fig. 12 Western Blot of β-catenin expression in colon tumors from SGK1 knockout and SGK1-expressing mice**

**A.** Original Western Blots illustrating the β-catenin (upper panel) and GAPDH (lower panel) protein abundance in chemically-induced colorectal tumors from SGK knockout (sgk$^{-/-}$) and wild type (sgk$^{+/+}$) mice. **B.** Arithmetic means ± SEM (n = 5) of the relative densities of β-catenin protein expression in chemically-induced colorectal tumors from SGK knockout (sgk$^{-/-}$) and wild type (sgk$^{+/+}$) mice; * indicates significant difference between the genotypes ($p<0.05$).

The results from Western blot were confirmed by immunofluorescence (Fig. 13). Again, β-catenin protein abundance was significantly reduced in chemically-induced colon tumor tissue from *sgk1*$^{-/-}$ compared to *sgk1*$^{+/+}$ mice.

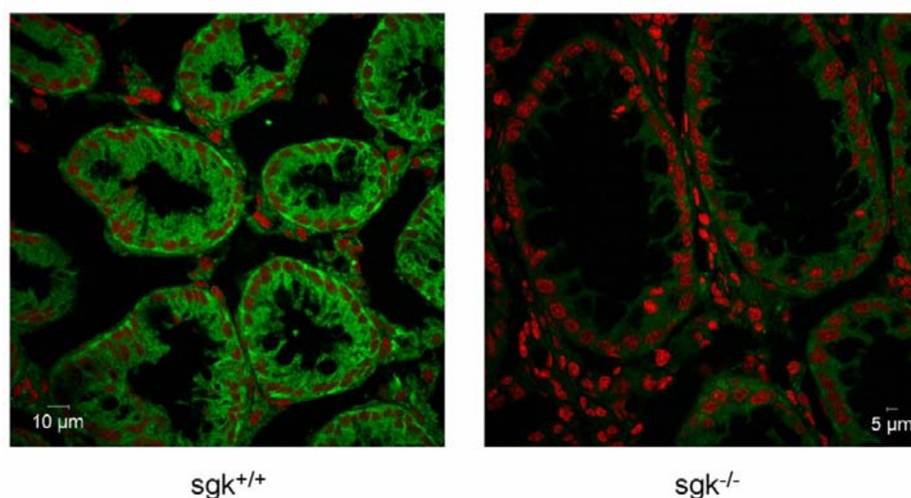


**Fig. 13: Immunofluorescence of β-catenin expression in colorectal tumors from SGK1 knockout and SGK1-expressing mice**

Immunofluorescence of β-catenin expression (green) and nuclear staining (red) in colorectal tumor tissue from SGK knockout ($sgk^{-/-}$) and wild type ($sgk^{+/+}$) mice.

An additional series of experiments was performed to elucidate whether stimulation of SGK1 expression in HEK 293 cells by dexamethasone was followed by increased β-catenin expression. As shown in Fig. 14, the treatment of HEK 293 cells with dexamethasone indeed significantly increased the β-catenin protein abundance.

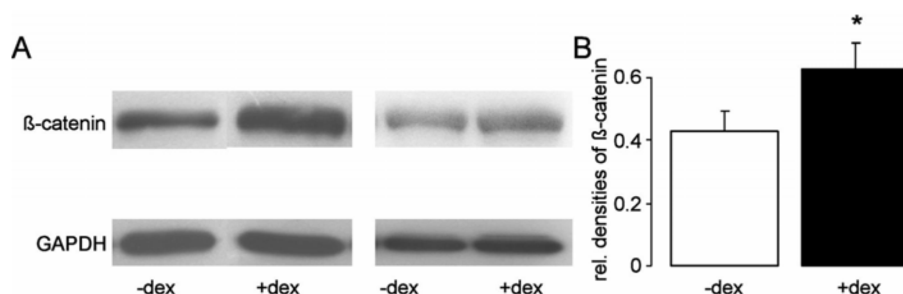



**Fig. 14: Western Blot of β-catenin expression in HEK 293 cells incubated in the presence or absence of dexamethasone**

**A.** Original Western Blots illustrating the β-catenin (upper panel) and GAPDH (lower panel) protein abundance in HEK 293 cells treated for 72 h with (+dex) or without (-dex) 1 µM dexamethasone to stimulate SGK1 activation. **B.** Arithmetic means ± SEM (n = 3) of relative densities of β-catenin protein expression in HEK 293 cells treated for 72 h with (+dex) or without (-dex) 1 µM dexamethasone to upregulate SGK1 expression. * indicates significant difference from absence of dexamethasone ($p<0.05$).

# 4. Discussion

## 4.1. Relative resistance of PDK1 hypomorphic mice against colorectal tumor induced by chemical cancerogenesis

The present study reveals that partial PDK1 deficiency confers some protection against the development of colorectal tumors following chemical cancerogenesis. The colorectal tumors were significantly less frequent in PDK1 hypomorphic mice ($pdk1^{hm}$) than in their wild type littermates ($pdk1^{wt}$). However, without cancerogenesis, the hypomorphic mutation of PDK1 in PTEN heterozygous mice ($PTEN^{+/-}$) was indicated to significantly suppress the development of a wide range of tumors, including lymphoma, endometrial carcinoma, prostate carcinoma, breast adenocarcinoma, rather than CRC (Bayascas et al., 2005). That may be due to the low incidence of spontaneous CRC in mice.

The phosphorylation of Bad was significantly less pronounced in $pdk1^{hm}$ than in $pdk1^{wt}$ mice. The activatory phosphorylation of PDK1 substrates may be involved in the the Bad phosphorylation. PDK1 phosphorylates and activates its substrates, such as PKB and SGK isoforms (Vanhaesebroeck et al., 2000). The active forms of PKB (Brunet et al., 1999; Peruzzi et al., 1999; Russo et al., 2006; Yano et al., 1998) and SGK3 (Liu et al., 2000; Xu et al., 2001) have been known to phosphorylate Bad, which leads to binding of this pro-apoptotic protein to 14–3-3 thus preventing the insertion into the mitochondria and the subsequent stimulation of apoptosis (Lizcano et al., 2000).

Further mechanisms may participate in the PDK1 dependent stimulation of tumor growth. For instance, the other studies of my research indicated that SGK1 downregulated pro-apoptotic transcription factor Foxo3a, pro-apoptotic regulator Bim, and upregulated oncogenic protein β-catenin. Moreover the

PKB and SGK isoforms may affect tumor cell survival by influencing nutrient transport. PKB stimulates cellular glucose uptake (Ishiki et al., 2005; Plas et al., 2005; Whiteman et al., 2002) and the SGK isoforms upregulate several nutrient transporters (Lang et al., 2006) and may thus support the excessive demand of proliferating cells for nutrient delivery.

In conclusion, PDK1 is critically important for colorectal tumor growth. PDK1 is at least partially effective through stimulation of Bad phosphorylation.

## 4.2. The SGK1-dependent upregulation of Foxo3a and Bim promotes colorectal tumor growth

The present study discloses for the first time a critical role of SGK1 in the in vivo development of colorectal tumors following chemical cancerogenesis. Lack of SGK1 leads to the significant inhibition of tumor growth.

Mechanisms involved in SGK1-dependent tumor growth apparently include Foxo3a and Bim. SGK1 is known to phosphorylate and thus to inhibit forkhead transcription factors such as Foxo3 (Liu et al., 2000; Xu et al., 2001; Shelly et al., 2002; Dehner et al., 2008). Downregulation of Foxo3a fosters cell survival and therefore stimulates cell growth (Kandel et al., 1999; Mikosz et al., 2001; You et al., 2004).

The pro-apoptotic effect of Foxo3a is in part due to stimulation of the expression of the Bcl2-interacting mediator Bim, which stimulates apoptosis and inhibits cell proliferation (Dijkers et al., 2000a; Dijkers et al., 2000b). The enhanced expression of Bim in tissue of SGK1-deficient mice provides an explanation for the reduced tumor growth.

However, the role of SGK1 is probably not confined to downregulation of Foxo3a and Bim, but may involve further mechanisms. For instance, SGK1 may affect cell survival by influencing nutrient transport. SGK1 upregulates a wide variety of nutrient transporters (Lang et al., 2006) and may thus support the demand of proliferating cells for enhanced nutrient delivery. Moreover SGK1 inhibits the expression of FAS and FASL, thus affecting the doxorubicin dependent caspase8 activation in kidney cancer cells (Amato et al., 2007).

SGK1 may be further important in vascularization of tumor tissue. SGK1 is upregulated by ischemia (Feng et al., 2006; Nishida et al., 2004) and may well participate in the stimulation of vascularization. According to the unpublished observations of my colleagues, angiogenin transcript levels were 47 ± 7 % lower in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ mice.

SGK1 has further been invoked to participate in the glucocorticoid- or colony stimulating factor 1-induced stimulation of invasiveness, motility and adhesiveness (Tangir et al., 2004) and may thus support tumor metastasis. SGK1 may further contribute to the resistance of tumor cells to therapy, as transcription of SGK1 is upregulated by ultraviolet radiation, oxidative stress and heat shock and inhibition of SGK1 expression by silencing RNA increases the toxicity of chemotherapeutic drugs (Meng et al., 2005). Moreover, overexpression of SGK1 by glucocorticoids inhibits chemotherapy-induced apoptosis of breast cancer cells (Wu et al., 2004). In addition, SGK1 transcription was stimulated following radiation of tumor cells resistant to radiation but not in tumor cells sensitive to radiation (Simon et al., 2007).

The present observations are in seeming contrast to the observation that SGK1 expression may be downregulated in some tumors, such as prostate cancer (Rauhala et al., 2005), ovarian tumors (Chu et al., 2002) and hepatocellular carcinoma (Chung et al., 2002). It should be kept in mind, though, that most SGK1-dependent functions are shared by the other SGK isoforms and the PKB isoforms, which are, similar to SGK1, activated by the PI kinase pathway (Lang et al., 2006). Thus, SGK-sensitive functions are expected to be upregulated in cells with enhanced activation of the PI3K/PKB isoforms irrespective of SGK1 expression (Cheng et al., 2008; Salmena et al., 2008; Steelman et al., 2008). For instance, loss-of-function mutation of the phosphatase and tensin homolog PTEN, which usually disrupts PI3 kinase signaling by degradation of $PIP_3$ (Lian et al., 2005; Oudit et al., 2004; Sulis et al., 2003), lead to excessive activation of PKB (Salmena et al., 2008). In cells carrying those mutations SGK1-sensitive functions may be upregulated by excessive activation of PKB isoforms and SGK3 despite downregulation of SGK1. As a matter of fact, the downregulation of SGK1 could in those cells result from the influence of a negative feedback. Clearly, not all tumors require enhanced SGK1 expression for survival. However, at least in the tumor model analyzed here, upregulation of SGK1 significantly modifies tumor growth. In

those tumors SGK1 may be considered an attractive pharmacological target for cytostatic therapy.

In conclusion, SGK1 is critically important for colorectal tumor growth. SGK1 is at least partially effective through downregulation of Foxo3a and Bim.

## 4.3. The regulation of β-catenin by SGK1 in mice with chemically induced colorectal cancer

In parallel experiments, I have observed the partial resistance of SGK1 deficient mice to chemical cancerogenesis. In this study, evidence has been obtained for SGK1 sensitive expression of the transcription factor Foxo3a and the Bcl2-interacting mediator Bim. SGK1 has previously been shown to phosphorylate and thus to inhibit Foxo3. (Liu et al., 2000; Xu et al., 2001; Shelly et al., 2002; Dehner et al., 2008), which counteracts cell survival and cell growth (Kandel et al., 1999; Mikosz et al., 2001; You et al., 2004) at least partially by stimulating the expression of the proapoptotic Bim (Dijkers et al., 2000a; Dijkers et al., 2000b).

There are various substrates of SGK, associated with cell division, cell proliferation and apoptosis. The present study reveals that SGK1 impacts on the abundance of β-catenin in vivo. Several studies have shown similar results in vitro. In mammary epithelial tumor cells, previous study demonstrated the glucocorticoid induced SGK overexpression resulted in production of a nonphosphorylated β-catenin that localizes in both the nucleus and the cell periphery, and the dominant-negative forms of SGK could blunt the effect of glucocorticoid on β-catenin dynamics (Failor et al., 2007). It was also reported that SGK3 increased the nuclear expression of β-catenin in mouse hair bulb keratinocytes (McCormick et al., 2004). As shown earlier, SGK1 phosphorylates GSK3 (Sakoda et al., 2003). The phosphorylation of GSK inhibits its function in the β-catenin degradation complex, and leads to the impairment of β-catenin degradation (Liu et al., 2002). The accumulation of cytoplasmic β-catenin leads to its nuclear translocation, where it functions as the transcriptional factor to regulate its transcriptional target genes. c-Myc and cyclin D1 have been identified as target genes of β-catenin/TCF transcription complex. The upregulation of c-Myc and cyclin D1 by β-catenin could be the possible mechanism of tumorigenesis (Kolligs et al., 2002).

There was a study in colon cancer cells with stabilizing mutations in β-catenin indicating SGK1 was the target gene of β-catenin (Dehner et al., 2008). In that study, DNA microarray identified SGK1 was one of the most up-regulated genes following APC knockdown. Further, the upregulation of SGK1 in response to APC knockdown was inhibited by concomitant knockdown of β-catenin. Taken together with our results, the stablization of β-catenin by SGK1 may be positively fed back by the transcriptional upregulation of SGK1. The SGK1 upregulation then influences tumor growth by mechanisms other than β-catenin stablization. As I showed previously, SGK1 phosphorylated and deactivates the transcription factor Foxo3a, resulting in the transcriptional downregulation of pro-apoptotic protein Bim. Moreover, SGK1 upregulates a wide variety of nutrient transporters (Lang et al., 2006) and may thus contribute to the enhanced nutrient delivery required by fast proliferating cells. SGK1 further inhibits the expression of FAS and FASL, thus affecting the doxorubicin dependent caspase8 activation in kidney cancer cells (Amato et al., 2007). The interactive regulation of SGK1 and β-catenin thus results in tumorigenesis.

In conclusion, SGK1 may play a role in the development of CRC via the regulation of β-catenin.

# 5. Summary

Colorectal cancer is a common cancer disease and major death cause worldwide. The economic burdens resulted from CRC are a major challenge to public health care. The molecular machinery beneath the colorectal tumorigenesis seems to be complicated. Several pathological pathways have been identified to be involved in the tumorigenesis of CRC, including PI3K pathway, Wnt pathway et al. PDK1 activates the protein kinase PKB and other ACG family kinases, which may in turn favor cell survival and thus the development of tumors. The kinases are in part effective through the phosphorylation of Bad, which counteracts its apoptotic activity. SGK1 was found to be upregulated in a variety of tumors, but downregulated in several distinct tumors. Thus, evidence for a role of SGK1 in tumor growth remained conflicting. According to *in vitro* observations, SGK1 promoted the stabilization and nuclear translocation of oncogene β-catenin and negatively regulates the transcription factor Foxo3a, which in turn stimulates transcription of the pro-apoptotic regulator Bim. To this end, PDK1 hypomorphic mice ($pdk1^{hm}$), SGK1 knockout mice ($sgk1^{-/-}$) and their wild type littermates ($pdk1^{wt}$ or $sgk1^{+/+}$) were subjected to chemical cancerogenesis (intraperitoneal injection of 20 mg/kg DMH followed by three cycles of 30 g/L synthetic DSS for 7 days). Abundance of phosphorylated Bad was determined by Western blot and immunofluorescence. As a result, following chemical cancerogenesis, $pdk1^{hm}$ mice developed significantly less colorectal tumors than $pdk1^{wt}$ mice. Accordingly, following chemical cancerogenesis $pdk1^{hm}$ mice lived significantly longer than $pdk1^{wt}$ mice. As evident from Western blot and immunofluorescence, PDK1 deficiency decreased the abundance of phosphorylated Bad. In $sgk1^{-/-}$mice significantly less colorectal tumors than $sgk1^{+/+}$mice were observed as well. According to Western blot and

immunofluorescence, SGK1 deficiency enhances the expression of Foxo3a and Bim, but decrease the abundance of β-catenin. Futhermore, SGK1 was silenced in HEK293 cells. SGK1 knock down consequently enhanced apoptosis. Western blot and immunofluorescence were employed to detect a reduced expression of Foxo3a and Bim in SGK1 knock down cells. An additional experiment was performed to in HEK 293 cells treated with dexamethasone to activate SGK1 by Western blot. The abundance of β-catenin was significantly increased after dexamethasone treatment. In conclusion, PDK1 favors development of CRC following chemical cancerogenesis, an effect at least partially due to the increased phosphorylation of Bad, and SGK1 expression favors the development of CRC, which may be effective at least in part through the downregulation of Foxo3a, Bim and the upregulation of β-catenin.

# 6. Reference

Aaltonen, L.A., Salovaara, R., Kristo, P., Canzian, F., Hemminki, A., Peltomaki, P., Chadwick, R.B., Kääriäinen, H., Eskelinen, M., Järvinen, H., Mecklin, J.P., de la Chapelle, A. (1998). Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. *N Engl J Med*. **338**, 1481–7.

Accili, D., Arden, K.C. (2004). FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. *Cell*. **117**, 421-6.

Akutsu, N., Lin, R., Bastien, Y., Bestawros, A., Enepekides, D.J., Black, M.J., White, J.H. (2001). Regulation of gene expression by 1 alpha 25-dihydroxyvitamin D3 and its analog EB1089 under growth-inhibitory conditions in squamous carcinoma cells. *Mol Endocrinol*. **15**, 1127–1139.

Alessi, D.R. (2001). Discovery of PDK1, one of the missing links in insulin signal transduction. Colworth Medal Lecture. *Biochem Soc Trans*. **29**, 1-14.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., Hemmings, B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J*. **15**, 6541– 51.

Alessi, D.R., Deak, M., Casamayor, A., Caudwell, F.B., Morrice, N., Norman, D.G., Gaffney, P., Reese, C.B., MacDougall, C.N., Harbison, D., Ashworth, A., Bownes, M. (1997). 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol*. **7**, 776-89.

Alves, A., Vibert, E., Trajcevski, S., Solly, S., Fabre, M., Soubrane, O., Qian, C., Prieto, J., Klatzmann, D., Panis, Y. (2004). Adjuvant interleukin-12 gene therapy for the management of colorectal liver metastases. *Cancer Gene Ther*. **11**, 782-789.

Amato, R., Menniti, M., Agosti, V., Boito, R., Costa, N., Bond, H.M. Barbieri, V., Tagliaferri, P., Venuta, S., Perrotti, N. (2007). IL-2 signals through Sgk1 and inhibits proliferation and apoptosis in kidney cancer cells. *J Mol Med*. **85**, 707-721.

American Joint Committee on Cancer. (2002). AJCC *Cancer Staging Handbook. 6th ed*. New York: Springer-Verlag.

Aoki, M., Jiang, H., Vogt, P.K. (2004). Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. *Proc Natl Acad Sci U S A*. **101**, 13613-7.

Augestad, K.M., Vonen, B., Aspevik, R., Nestvold, T., Ringberg, U., Johnsen, R., Norum, J., Lindsetmo, R.O. (2008). Should the surgeon or the general practitioner (GP) follow up patients after surgery for colon cancer? A randomized controlled trial protocol focusing on quality of life, cost-effectiveness and serious clinical events. *BMC Health Serv Res*. **8**,137.

Bafico, A., Liu, G., Goldin, L., Harris, V., Aaronson, S.A. (2004). An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. *Cancer Cell*. **6**, 497-506.

Balansky, R., Gyosheva, B., Ganchev, G., Mircheva, Z., Minkova, S., Georgiev, G. (1999). Inhibitory effects of freeze-dried milk fermented by selected Lactobacillus bulgaricus strains on carcinogenesis induced by 1, 2-dimethylhydrazine in rats and by diethylnitrosamine in hamsters. *Cancer Lett*. **147**, 125-137.

Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C.P., Alessi, D.R. (1999). PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr Biol*. **9**, 393-404.

Bayascas, J.R., Leslie, N.R., Parsons, R., Fleming, S., Alessi, D.R. (2005). Hypomorphic mutation of PDK1 suppresses tumorigenesis in PTEN(+/-) mice. Curr Biol. **15**, 1839-46.

Behrens J. (2005). The role of the Wnt signalling pathway in colorectal tumorigenesis. *Biochem Soc Trans*. **33**, 672-5.

Behrens, J. (2000). Control of beta-catenin signaling in tumor development. *Ann N Y Acad Sci*. **910**, 21-33.

Belaiba, R.S., Djordjevic, T., Bonello, S., Artunc, F., Lang, F., Hess, J., Gorlach, A. (2006). The serum- and glucocorticoid-inducible kinase Sgk-1 is involved in pulmonary vascular remodeling. Role in redoxsensitive regulation of tissue factor by thrombin. *Circ Res*. **98**, 828–836.

Benson AB 3rd. (2007). Epidemiology, disease progression, and economic burden of colorectal cancer. *J Manag Care Pharm*. 13, S5-18.

Biggs, W.H. 3rd, Meisenhelder, J., Hunter, T., Cavenee, W.K., Arden, K.C. (1999). Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A*. **96**, 7421-6.

Biondi, R.M., Kieloch, A., Currie, R.A., Deak, M., Alessi, D.R. (2001). The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. *EMBO J*. **20**, 4380–4390.

Blume-Jensen, P., Janknecht, R., Hunter, T. (1998). The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. *Curr Biol*. **8**, 779-82.

Boehmer, C., Rajamanickam, J., Schiepp, R., Amara, S., Palmada, M., Lang, F. (2006). Posttranslational regulation of EAAT2 function by coexpressed ubiquitin ligase Nedd4–2 is impacted by SGK kinases. *J Neurochem*. **97**, 911–921.

Bouillet, P., Huang, D.C., O'reilly, L.A., Puthalakath, H., O'Connor, L., Cory, S., Adams, J.M., Strasser, A. (2000). The role of the pro-apoptotic Bcl-2 family member Bim in physiological cell death. *Ann N Y Acad Sci*. **926**, 83-9.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*. **96**, 857-68.

Brunet, A., Kanai, F., Stehn, J., Xu, J., Sarbassova, D., Frangioni, J.V., Dalal, S.N., DeCaprio, J.A., Greenberg, M.E., Yaffe, M.B. (2002). 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. *J Cell Biol*. **156**, 817-28.

Brunet, A., Park, J., Tran, H., Hu, L.S., Hemmings, B.A., Greenberg, M.E. (2001). Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol*. **21**, 952-965.

Casamayor, A., Morrice, N.A., Alessi, D.R. (1999). Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo. *Biochem J*. **342**, 287-92.

Chao, D.T., Korsmeyer, S.J. (1998). BCL-2 family: regulators of cell death. *Annu Rev Immunol*. **16**, 395-419.

Cheah, P.Y. (2009). Recent advances in colorectal cancer genetics and diagnostics. *Crit Rev Oncol Hematol*. **69**, 45-55.

Cheng, G.Z., Park, S., Shu, S., He, L., Kong, W., Zhang, W., Yuan, Z., Wang, L.H., Cheng, J.Q. (2008). Advances of AKT pathway in human oncogenesis and as a target for anti-cancer drug discovery. *Curr Cancer Drug Targets*. **8**, 2-6.

Chu, S., Rushdi, S., Zumpe, E.T., Mamers, P., Healy, D.L., Jobling, T., Burger, H.G., Fuller, P.J. (2002). FSH-regulated gene expression profiles in ovarian tumours and normal ovaries. *Mol Hum Reprod*. **8**, 426–433.

Chun, J., Kwon, T., Lee, E., Suh, P.G., Choi, E.J., Sun, Kang, S. (2002). The Na(+)/H(+) exchanger regulatory factor 2 mediates phosphorylation of serum- and glucocorticoid-induced protein kinase 1 by 3-phosphoinositide-dependent protein kinase 1. *Biochem Biophys Res Commun*. **298**, 207-15.

Chung, E.J., Sung, Y.K., Farooq, M., Kim, Y., Im, S., Tak, W.Y., Hwang, Y.J., Kim, Y.I., Han, H.S., Kim, J.C., Kim, M.K. (2002). Gene expression profile analysis in human hepatocellular carcinoma by cDNA microarray. *Mol Cells*. **14**, 382-387.

Clark, K.L., Halay, E.D., Lai, E., Burley, S.K. (1993). Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. *Nature*. **364**, 412-20.

Cohen, P., Frame, S. (2001). The renaissance of GSK3. *Nat Rev Mol Cell Biol*. **2**, 769-776.

Corpet, D.E., Pierre, F. (2003). Point: From animal models to prevention of colon cancer. Systematic review of chemoprevention in min mice and choice of the model system. *Cancer Epidemiol Biomarkers Prev*. **12**, 391-400.

Cory, S., Adams, J.M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer*. **2**, 647-56.

Dale, T. C. (1998). Signal transduction by the Wnt family of ligands. *Biochem J*. **329**, 209–223.

Datta, S.R., Brunet, A., Greenberg, M.E. (1999). Cellular survival: a play in three Akts. *Genes Dev*. **13**, 2905-27.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*. **91**, 231-41.

Datta, S.R., Katsov, A., Hu, L., Petros, A., Fesik, S.W., Yaffe, M.B., Greenberg, M.E. (2000). 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol Cell*. **6**, 41-51.

Dehner, M., Hadjihannas, M., Weiske, J., Huber, O., Behrens, J. (2008). Wnt signaling inhibits Forkhead box O3a-induced transcription and apoptosis through up-regulation of serum- and glucocorticoid-inducible kinase 1. *J Biol Chem*. **283**, 19201-10.

del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., Nunez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science*. **278**, 687-9.

Dieter, M., Palmada, M., Rajamanickam, J., Aydin, A., Busjahn, A., Boehmer, C., Luft, F.C., Lang, F. (2004). Regulation of glucose transporter SGLT1 by ubiquitin ligase Nedd4–2 and kinases SGK1, SGK3, and PKB. *Obes Res*. **12**, 862–870.

Dijkers, P.F., Medema, R.H., Pals, C., Banerji, L., Thomas, N.S., Lam, E.W., Burgering, B.M., Raaijmakers, J.A., Lammers, J.W., Koenderman, L. (2000a). Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27 (KIP1). *Molecular and Cellular Biology*. **20**, 9138–9148.

Dijkers, P.F., Medema, R.H., Lammers, J.W., Koenderman, L., Coffer, P.J. (2000b). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol*. **10**, 1201-4.

Dong, M., Guda, K., Nambiar, P.R., Rezaie, A., Belinsky, G.S., Lambeau, G., Giardina, C., Rosenberg, D.W. (2003). Inverse association between phospholipase A2 and COX-2 expression during mouse colon tumorigenesis. *Carcinogenesis*. **24**, 307–315.

Edelmann, W., Yang, K., Kuraguchi, M., Heyer, J., Lia, M., Kneitz, B., Fan, K., Brown, A.M., Lipkin, M., Kucherlapati, R. (1999).Tumorigenesis in Mlh1 and Mlh1/Apc1638N mutant mice. *Cancer Res*. **59**, 1301–1307.

Embark, H.M., Bohmer, C., Palmada, M., Rajamanickam, J., Wyatt, A.W., Wallisch, S., Capasso, G., Waldegger, P., Seyberth, H.W., Waldegger, S., Lang, F. (2004). Regulation of CLC-Ka/barttin by the ubiquitin ligase Nedd4–2 and the serum and glucocorticoid-dependent kinases. *Kidney Int*. **66**, 1918–1925.

Engle, S.J., Hoying, J.B., Boivin, G.P., Ormsby, I., Gartside, P.S., Doetschman, T. (1999). Transforming growth factor β1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res*. **59**, 3379–86.

Essafi, A., Fernandez de Mattos, S., Hassen, Y.A., Soeiro, I., Mufti, G.J., Thomas, N.S., Medema, R.H., Lam, E.W. (2005). Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. *Oncogene*. **24**, 2317-29.

Failor, K.L., Desyatnikov, Y., Finger, L.A., Firestone, G.L. (2007). Glucocorticoid-induced degradation of glycogen synthase kinase-3 protein is triggered by serum- and glucocorticoid-induced protein kinase and Akt signaling and controls beta-catenin dynamics and tight junction

formation in mammary epithelial tumor cells. *Mol Endocrinol*. **21**, 2403-15.

Feng, Y., Wang, Y., Xiong, J., Liu, Z., Yard, B., Lang, F. (2006). Enhanced expression of serum and glucocorticoid-inducible kinase-1 in kidneys of L-NAME-treated rats. *Kidney Blood Press Res*. **29**, 94-99.

Ferlay, J., Bray, F., Pisani, P., Parkin, D.M. (2004). GLOBOCAN 2002: Cancer incidence, mortality and prevalence worldwide. *IARC cancer Base No.5, Version 2.0*. Lyon: International Agency for Research on Cancer.

Ferlay, J., Autier, P., Boniol, M., Heanue, M., Colombet, M., Boyle, P. (2007). Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol*. **18**, 581-92.

Firestone, G.L., Giampaolo, J.R., O'Keeffe, B.A. (2003). Stimulus dependent regulation of the serum and glucocorticoid inducible protein kinase (Sgk) transcription, subcellular localization and enzymatic activity. *Cell Physiol Biochem*. **13**, 1–12.

Fodde, R., Edelmann, W., Yang, K., van Leeuwen, C., Carlson, C., Renault, B., Breukel, C., Alt, E., Lipkin, M., Khan, P.M.. (1994). A targeted chain–termination mutation in the mouse Apc gene results in multiple intestinal tumors. *Proc Natl Acad Sci U S A*. **91**, 8969–73.

Fodde, R., Smits, R. (2001). Disease model: familial adenomatous polyposis. *Trends Mol Med*. **7**, 369-73.

Fu, Z., Tindall, D.J. (2008). FOXOs, cancer and regulation of apoptosis. *Oncogene*. **27**, 2312-9.

Fuchs, S.Y., Ougolkov, A.V., Spiegelman, V.S., Minamoto, T. (2005). Oncogenic beta- catenin signaling networks in colorectal cancer. *Cell Cycle*. **4**, 1522-39.

Giardiello, F.M., Brensinger, J.D., Petersen, G.M., Luce, M.C., Hylind, L.M., Bacon, J.A., Booker, S.V., Parker, R.D., Hamilton, S.R. (1997). The use and interpretation of commercial APC gene testing for familial adenomatous polyposis. *N Engl J Med*. **336**, 823-27.

Greer, E.L., Brunet, A. (2005). FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene*. **24**, 7410-25.

Gumz, M.L., Popp, M.P., Wingo, C.S., Cain, B.D. (2003). Early transcriptional effects of aldosterone in a mouse inner medullary collecting duct cell line. *Am J Physiol Renal Physiol*. **285**, F664–F673.

Hatsell, S., Rowlands, T., Hiremath, M., Cowin, P. (2003). Beta-catenin and

Tcfs in mammary development and cancer. *J Mammary Gland Biol*. Neoplasia. **8**,145-58.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., Kinzler, K.W. (1998). Identification of c-MYC as a target of the APC pathway. *Science*. **281**, 1509-12.

Heijstek, M.W., Kranenburg, O., Borel Rinkes, I.H.M. (2005). Mouse models of colorectal cancer and liver metastases. *Dig Surg*. **22**, 16-25.

Herman, M.D., Nyman, T., Welin, M., Lehtio, L., Flodin, S., Tresaugues, L., Kotenyova, T., Flores, A., Nordlund, P. (2008). Completing the family portrait of the anti-apoptotic Bcl-2 proteins: crystal structure of human Bfl-1 in complex with Bim. *FEBS Lett*. **582**, 3590-4.

Hill, M.M., Andjelkovic, M., Brazil, D.P., Ferrari, S., Fabbro, D., Hemmings, B.A. (2001). Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase. *J Biol Chem*. **276**, 25643-6.

Honda, T., Kai, I., Ohi, G. (1999). Fat and dietary fiber intake and colon cancer mortality: a chronological comparison between Japan and the United States. *Nutr Cancer*. **33**, 95-9.

Huber, A.H., Nelson, W.J., Weis, W.I. (1997). Three-dimensional structure of the armadillo repeat region of beta-catenin. *Cell*. **90**, 871–882.

Ikenoue, T., Kanai, F., Hikiba, Y., Obata, T., Tanaka, Y., Imamura, J., Ohta, M., Jazag, A., Guleng, B., Tateishi, K., Asaoka, Y., Matsumura, M., Kawabe, T., Omata, M. (2005). Functional analysis of PIK3CA gene mutations in human colorectal cancer. *Cancer Res*. **65**, 4562–4567.

Ishiki, M., Klip, A. (2005). Minireview: recent developments in the regulation of glucose transporter-4 traffic: new signals, locations, and partners. *Endocrinology*. **146,** 5071-5078.

Itoh, N., Semba, S., Ito, M., Takeda, H., Kawata, S., Yamakawa, M. (2002). Phosphorylation of Akt/PKB is required for suppression of cancer cell apoptosis and tumor progression in human colorectal carcinoma. *Cancer*. **94**, 3127-34.

Janiak, F., Leber, B., Andrews, D.W. (1994). Assembly of Bcl-2 into microsomal and outer mitochondrial membranes. *J Biol Chem*. **269**, 9842–9849.

Janssen, K.P., El Marjou, F., Pinto, D., Sastre, X., Rouillard, D., Fouquet, C., Soussi, T., Louvard, D., Robine, S. (2002). Targeted expression of oncogenic K-ras in intestinal epithelium causes spontaneous tumorigenesis in mice. *Gastroenterol*. **123**, 492–504.

Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Thun, MJ. (2007). Cancer statistics, *CA Cancer J Clin*. 57, 43-66.

Joubert, J., Norman, R., Bradshaw, D., Goedecke, J.H., Steyn, N.P., Puoane, T., South African Comparative Risk Assessment Collaborating Group. (2007). Estimating the burden of disease attributable to excess body weight in South Africa in 2000. *S Afr Med J*. **97**, 683-90.

Kaestner, K.H., Knochel, W., Martinez, D.E. (2000). Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev*. **14**, 142–146.

Kandel, E.S., Hay, N. (1999). The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp. *Cell Res*. **253**, 210-229.

Kashii, Y., Uchida, M., Kirito, K., Tanaka, M., Nishijima, K., Toshima, M., Ando, T., Koizumi, K., Endoh, T., Sawada, K., Momoi, M., Miura, Y., Ozawa, K., Komatsu, N. (2000). A member of Forkhead family transcription factor, FKHRL1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. *Blood*. **96**, 941-9.

Kaufmann, E., Knöchel, W. (1996). Five years on the wings of fork head. *Mech Dev*. **57**, 3-20.

Kellner, M., Peiter, A., Hafner, M., Feuring, M., Christ, M., Wehling, M., Falkenstein, E., Losel, R. (2003). Early aldosterone up-regulated genes: new pathways for renal disease? *Kidney Int*. **64**, 1199–1207.

Khaleghpour, K., Li, Y., Banville, D., Yu, Z., Shen, S.H. (2004). Involvement of the PI 3-kinase signaling pathway in progression of colon adenocarcinoma. *Carcinogenesis*. **25**, 241-8.

Khuhaprema, T., Srivatanakul, P. (2008). Colon and Rectum Cancer in Thailand: An Overview. *Jpn J Clin Oncol*. **38**, 237–243.

Knudson, C.M., Brown, N.M. (2008). Mitochondria potential, bax "activation," and programmed cell death. *Methods Mol Biol*. **414**, 95-108.

Kobaek-Larsen, M., Thorup, I., Diederichsen, A., Fenger, C., Hoitinga, M.R. (2000). Review of colorectal cancer and its metastases in rodent models: Comparative aspects with those in humans. *Comp Med*. **50**, 16–26.

Kobayashi, T., Deak, M., Morrice, N., Cohen, P. (1999). Characterization of the structure and regulation of two novel isoforms of serumand

glucocorticoid-induced protein kinase. *Biochem J*. **344**, 189–197.

Koesters, R., Hans, M.A., Benner, A., Prosst, R., Boehm, J., Gahlen, J., Doeberitz, M.K. (2001). Predominant mutation of codon 41 of the beta-catenin proto-oncogene in rat colon tumors induced by 1,2-dimethylhydrazine using a complete carcinogenic protocol. *Carcinogenesis*. **22**, 1885-90.

Kohno, H., Suzuki, R., Sugie, S., Tanaka, T. (2005). Beta-Catenin mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate. *Cancer Sci*. **96**, 69-76.

Kolligs, F.T., Bommer, G., Goeke, B. (2002). Wnt/beta-catenin/tcf signaling: a critical pathway in gastrointestinal tumorigenesis. *Digestion*. **66**, 131-44.

Lama, D., Sankararamakrishnan, R. (2008). Anti-apoptotic Bcl-XL protein in complex with BH3 peptides of pro-apoptotic Bak, Bad, and Bim proteins: comparative molecular dynamics simulations. *Proteins*. **73**, 492-514.

Lammi, L., Arte, S., Somer, M., Jarvinen, H., Lahermo, P., Thesleff, I., Pirinen, S., Nieminen, P. (2004). Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. *Am J Hum Genet*. **74**, 1043-50.

Lang, F., Bohmer, C., Palmada, M., Seebohm, G., Strutz-Seebohm, N., Vallon, V. (2006). (Patho)physiological significance of the serum- and glucocorticoid-inducible kinase isoforms. *Physiol Rev*. **86**, 1151-1178.

Lawlor, M.A., Mora, A., Ashby, P.R., Williams, M.R., Murray-Tait, V., Malone, L., Prescott, A.R., Lucocq, J.M., Alessi, D.R. (2002). Essential role of PDK1 in regulating cell size and development in mice. *EMBO J*. **21**, 3728-38.

Leong, M.L., Maiyar, A.C., Kim, B., O'Keeffe, B.A., Firestone, G.L. (2003). Expression of the serum- and glucocorticoid-inducible protein kinase, Sgk, is a cell survival response to multiple types of environmental stress stimuli in mammary epithelial cells. *J Biol Chem*. **278**, 5871–5882.

Ley, R., Ewings, K.E., Hadfield, K., Cook, S.J. (2005). Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. *Cell Death Differ*. **12**, 1008–1014.

Ley, R., Ewings, K.E., Hadfield, K., Howes, E., Balmanno, K., Cook, S.J. (2004). Extracellular signal-regulated kinases 1/2 are serum-stimulated "Bim (EL) kinases" that bind to the BH3-only protein Bim (EL) causing its phosphorylation and turnover. *J Biol Chem*. **279**, 8837–8847.

Li, H., Pamukcu, R., Thompson, W.J. (2002). Beta-Catenin signaling: therapeutic strategies in oncology. *Cancer Biol Ther*. **1**, 621-5.

Lian, Z., Di, Cristofano, A. (2005). Class reunion: PTEN joins the nuclear crew. *Oncogene*. **24**, 7394-7400.

Liao, D.J., Thakur, A., Wu, J., Biliran, H., Sarkar, F.H. (2007). Perspectives on c-Myc, Cyclin D1, and their interaction in cancer formation, progression, and response to chemotherapy. *Crit Rev Oncog*. **13**, 93-158.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G.H., Tan, Y., Zhang, Z., Lin, X., He, X. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell*. **108**, 837–847.

Liu, D., Yang, X., Songyang, Z. (2000). Identification of CISK, a new member of the SGK kinase family that promotes IL-3-dependent survival. *Curr Biol*. **10**, 1233-1236.

Lizcano, J.M., Morrice, N., Cohen, P. (2000). Regulation of BAD by cAMP-dependent protein kinase is mediated via phosphorylation of a novel site, Ser155. *Biochem J*. **349**, 547-557.

Lynch, P.M., Lynch, H.T. (1985). *Colon cancer genetics*. New York (NY). Van Nostrand Rheinhold.

Maiyar, A.C., Leong, M.L., Firestone, G.L. (2003). Importin-alpha mediates the regulated nuclear targeting of serum- and glucocorticoidinducible protein kinase (Sgk) by recognition of a nuclear localization signal in the kinase central domain. *Mol Biol Cell*. **14**, 1221– 239.

Malekzadeh, R., Bishehsari, F., Mahdavinia, M., Ansari, R. (2009). Epidemiology and molecular genetics of colorectal cancer in iran: a review. *Arch Iran Med*. **12**, 161-9.

Martinez-Gac, L., Marques, M., Garcia, Z., Campanero, M.R., Carrera, A.C. (2004). Control of cyclin G2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead. *Molecular and Cellular Biology*. **24**, 2181–2189.

McCormick, J.A., Feng, Y., Dawson, K., Behne, M.J., Yu, B., Wang, J., Wyatt, A.W., Henke, G., Grahammer, F., Mauro, T.M., Lang, F., Pearce, D. (2004). Targeted disruption of the protein kinase SGK3/CISK impairs postnatal hair follicle development. *Mol Biol Cell*. **15**, 4278-88.

Meller, R. Cameron, J.A., Torrey, D.J., Clayton, C.E., Ordonez, A.N., Henshall, D.C., Minami, M., Schindler, C.K., Saugstad, J.A., Simon, R.P. (2006). Rapid degradation of Bim by the ubiquitin-proteasome pathway mediates short-term ischemic tolerance in cultured neurons. *J Biol*

*Chem*. **281**, 7429–7436.

Meng, F., Yamagiwa, Y., Taffetani, S., Han, J., Patel, T. (2005). IL-6 activates serum and glucocorticoid kinase via p38alpha mitogenactivated protein kinase pathway. *Am J Physiol Cell Physiol*. **289**, C971–C981.

Mikosz, C.A., Brickley, D.R., Sharkey, M.S., Moran, T.W., Conzen, S.D. (2001). Glucocorticoid receptor-mediated protection from apoptosis is associated with induction of the serine/threonine survival kinase gene, sgk-1. *J Biol Chem*. **276**, 16649-16654.

Mizuno, H., Nishida, E. (2001). The ERK MAP kinase pathway mediates induction of SGK (serum- and glucocorticoid-inducible kinase) by growth factors. *Genes Cells*. **6**, 261–268.

Modur, V., Nagarajan, R., Evers, B.M., Milbrandt, J. (2002). FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. *J Biol Chem*. **2002**. 277, 47928-37.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., Kinzler, K.W. (1998). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science*. **275**, 1787-90.

Moser, A.R., Shoemaker, A.R., Connelly, C.S., Clipson, L., Gould, K.A., Luongo, C., Dove, W.F., Siggers, P.H., Gardner, R.L. (1995). Homozygosity for the Min allele of Apc results in disruption of mouse development prior to gastrulation. *Dev Dyn*. **203**, 422–433.

Muchmore, S.W., Sattler, M., Liang, H., Meadows, R.P., Harlan, J.E., Yoon, H.S., Nettesheim, D., Chang, B.S., Thompson, C.B., Wong, S.L., Ng, S.L., Fesik, S.W. (1996). X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature*. **381**, 335–341.

Myers, M.P., Pass, I., Batty, I.H., Van der Kaay, J., Stolarov, J.P., Hemmings, B.A., Wigler, M.H., Downes, C.P., Tonks, N.K. (1998). The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc Natl Acad Sci USA*. **95**, 13513–13518.

Naishiro, Y., Yamada, T., Idogawa, M., Honda, K., Takada, M., Kondo, T., Imai, K., Hirohashi, S. (2005). Morphological and transcriptional responses of untransformed intestinal epithelial cells to an oncogenic beta-catenin protein. *Oncogene*. **24**, 3141-3153.

Nemoto, S., Finkel, T. (2002). Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. *Science*. **295**, 2450–2452.

Nicholson, K.M., Anderson, N.G. (2002). The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal*. **14**, 381-95.

Nishida, Y., Nagata, T., Takahashi, Y., Sugahara-Kobayashi, M., Murata, A., Asai, S. (2004). Alteration of serum/glucocorticoid regulated kinase-1 (sgk-1) gene expression in rat hippocampus after transient global ischemia. *Brain Res Mol Brain Res*. **123**, 121-125.

O'Connor, L. Strasser, A., O'Reilly, L.A., Hausmann, G., Adams, J.M., Cory, S., Huang, D.C. (1998). Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J*. **17**, 384-395.

O'Reilly, L.A., Cullen, L., Visvader, J., Lindeman, G.J., Print, C., Bath, M.L., Huang, D.C., Strasser, A. (2000). The proapoptotic BH3-only protein bim is expressed in hematopoietic, epithelial, neuronal, and germ cells. *Am J Pathol*. **157**, 449-61.

Oshima, M., Oshima, H., Kitagawa, K., Kobayashi, M., Itakura, C., Taketo, M. (1995). Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. *Proc Natl Acad Sci U S A*. **92**, 4482–86.

Oudit, G.Y., Sun, H., Kerfant, B.G., Crackower, M.A., Penninger, J.M., Backx, P.H. (2004). The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. *J Mol Cell Cardiol*. **37**, 449-471.

Palmada, M., Boehmer, C., Akel, A., Rajamanickam, J., Jeyaraj, S., Keller, K., Lang, F. (2006). SGK1 kinase upregulates glut1 activity and plasma membrane expression. *Diabetes*. **55**, 421–427.

Palmada, M., Poppendieck, S., Embark, H.M., van de Graaf, S.F., Boehmer, C., Bindels, R.J., Lang, F. (2005). Requirement of PDZ domains for the stimulation of the epithelial Ca2+ channel TRPV5 by the NHE regulating factor NHERF2 and the serum and glucocorticoid inducible kinase SGK1. *Cell Physiol Biochem*. **15**, 175–182.

Park, J., Leong, M.L., Buse, P., Maiyar, A.C., Firestone, G.L., Hemmings, B.A. (1999). Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway. *EMBO J*. **18**, 3024–3033.

Peifer, M., Berg, S., Reynolds, A.B., (1994). A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell*. **76**, 789–791.

Peruzzi, F., Prisco, M., Dews, M., Salomoni, P., Grassilli, E., Romano, G., Calabretta, B., Baserga, R. (1999). Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. *Mol Cell Biol*. **19**, 7203-7215.

Petros, A.M., Medek, A., Nettesheim, D.G., Kim, D.H., Yoon, H.S., Swift, K., Matayoshi, E.D., Oltersdorf, T., Fesik, S.W. (2001). Solution structure of

the antiapoptotic protein Bcl-2. *Proc Natl Acad Sci USA*. **98**, 3012–3017.

Pickhardt, P.J., Hassan, C., Laghi, A., Zullo, A., Kim, D.H., Morini, S. (2007). Cost-effectiveness of colorectal cancer screening with computed tomography colonography: the impact of not reporting diminutive lesions. *Cancer*. **109**, 2213-21.

Plas, D.R., Thompson, C.B. (2003). Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. *J Biol Chem*. **278**, 12361-6.

Plas, D.R., Thompson, C.B. (2005). Akt-dependent transformation: there is more to growth than just surviving. *Oncogene*. **24**, 7435-7442.

Polakis, P. (2000). Wnt signaling and cancer. Genes Dev. **14**, 1837-51.

Pullen, N., Dennis, P.B., Andjelkovic, M., Dufner, A., Kozma, S.C., Hemmings, B.A., Thomas, G. (1998). Phosphorylation and activation of p70s6k by PDK1. *Science*. **279**, 707-10.

Putcha, G.V., Le, S., Frank, S., Besirli, C.G., Clark, K., Chu, B., Alix, S., Youle, R.J., LaMarche, A., Maroney, A.C., Johnson, E.M. Jr. (2003). JNK-mediated BIM phosphorylation potentiates BAX dependent apoptosis. *Neuron*. **38**, 899–914.

Qi, X.J., Wildey, G.M., Howe, P.H. (2006). Evidence that Ser87 of BimEL is phosphorylated by Akt and regulates BimEL apoptotic function. *J Biol Chem*. **281**, 813–823.

Quesada, C.F., Kimata, H., Mori, M., Nishimura, M., Tsuneyoshi, T., Baba, S. (1998). Piroxicam and acarbose as chemopreventive agents for spontaneous intestinal adenomas in APC gene 1309 knockout mice. *Jpn J Cancer Res*. **89**, 392–396.

Ramaswamy, S., Nakamura, N., Sansal, I., Bergeron, L. Sellers, W.R. (2002). A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. *Cancer Cell*. **2**, 81–91.

Rao, R.V., Castro-Obregon, S., Frankowski, H., Schuler, M., Stoka, V., del Rio, G., Bredesen, D.E., Ellerby, H.M. (2002). Coupling endoplasmic reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. *J Biol Chem*. **277**, 21836-42.

Rauhala, H.E., Porkka, K.P., Tolonen, T.T., Martikainen, P.M., Tammela, T.L., Visakorpi, T. D. (2005). Dual-specificity phosphatase 1 and serum glucocorticoid regulated kinase are downregulated in prostate cancer. *Int J Cancer*.**117**, 738-45.

Reed, J.C. (2006). Pro-apoptotic multidomain Bcl-2/Bax-family proteins: mechanisms, physiological roles, and therapeutic opportunities. *Cell*

*Death Differ*. **13**, 1378–1386.

Rosenberg, D.W., Giardina, C., Tanaka, T. (2009). Mouse models for the study of colon carcinogenesis. *Carcinogenesis*. **30**, 183-96.

Russo, P., Catassi, A., Cesario, A., Servent, D. (2006). Development of novel therapeutic strategies for lung cancer: targeting the cholinergic system. *Curr Med Chem*. **13**, 3493-3512.

Rychahou, P.G., Murillo, C.A., Evers, B.M. (2005). Targeted RNA interference of PI3K pathway components sensitizes colon cancer cells to TNF related apoptosis-inducing ligand. *Surgery*. **138**, 391-397.

Saad, S., Stevens, V.A., Wassef, L., Poronnik, P., Kelly, D.J., Gilbert, R.E., Pollock, C.A. (2005). High glucose transactivates the EGF receptor and up-regulates serum glucocorticoid kinase in the proximal tubule. *Kidney Int*. **68**, 985–997.

Sakoda, H., Gotoh, Y., Katagiri, H., Kurokawa, M., Ono, H., Onishi, Y., Anai, M., Ogihara, T., Fujishiro, M., Fukushima, Y., Abe, M., Shojima, N., Kikuchi, M., Oka, Y., Hirai, H., Asano, T. (2003). Differing roles of Akt and serum- and glucocorticoid-regulated kinase in glucose metabolism, DNA synthesis, and oncogenic activity. *J Biol Chem*. **278**, 25802-25807.

Salmena, L., Carracedo, A., Pandolfi, P.P. (2008). Tenets of PTEN tumor suppression. *Cell*. **133**, 403-414.

Samuels, Y., Ericson, K. (2006). Oncogenic PI3K and its role in cancer. *Curr Opin Oncol*. **18**, 77–82.

Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S.M., Riggins, G.J., Willson, J.K., Markowitz, S., Kinzler, K.W., Vogelstein, B., Velculescu, V.E. (2004). High frequency of mutations of the PIK3CA gene in human cancers. *Science*. **304**, 554.

Sasai, H., Masaki, M., Wakitani, K. (2000). Suppression of polypogenesis in a new mouse strain with a truncated Apc (Delta474) by a novel COX-2 inhibitor, JTE–522. *Carcinogenesis*. **21**, 953–8.

Sasaki, T., Irie-Sasaki, J., Horie, Y., Bachmaier, K., Fata, J.E., Li, M., Suzuki, A., Bouchard, D., Ho, A., Redston, M., Gallinger, S., Khokha, R., Mak, T.W., Hawkins, P.T., Stephens, L., Scherer, S.W., Tsao, M., Penninger, J.M. (2000). Colorectal carcinomas in mice lacking the catalytic subunit of PI(3)K gamma. *Nature*. **406**, 897-902.

Sattler, M. Liang, H., Nettesheim, D., Meadows, R.P., Harlan, J.E., Eberstadt, M., Yoon, H.S., Shuker, S.B., Chang, B.S., Minn, A.J., Thompson, C.B., Fesik, S.W. (1997). Structure of Bcl-xL–Bak peptide complex: recognition between regulators of apoptosis. *Science*. **275**,

983–986.

Schmelz, E.M., Sullards, C., Dillehay, D.L., Merrill, A.H. Jr. (2000). Colonic cell proliferation and aberrant crypt foci formation are inhibited by dairy glycosphingolipids in 1, 2-dimethylhydrazine-treated CF1 mice. *J Nutr*. **130**, 522-527.

Searle, G.D., Skokie, Co. (1999). The economic burden associated with colon cancer in the United States. *Clin Ther*. **21**, 370-9.

Sengupta, A., Ghosh, S., Bhattacharjee, S. (2005). Dietary cardamom inhibits the formation of azoxymethane-induced aberrant crypt foci in mice and reduces COX-2 and iNOS expression in the colon. *Asian Pac J Cancer Prev*. **6**, 118-22.

Shelly, C., Herrera, R. (2002). Activation of SGK1 by HGF, Rac1 and integrin-mediated cell adhesion in MDCK cells: PI-3K-dependent and -independent pathways. *J Cell Sci*. **115**, 1985-1993.

Sheppard, K.E. Autelitano, D.J. (2002). 11Beta-hydroxysteroid dehydrogenase 1 transforms 11dehydrocorticosterone into transcriptionally active glucocorticoid in neonatal rat heart. Endocrinology. **143**, 198–204.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., Ben-Zeev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A*. **96**, 5522-7.

Simon, P., Schneck, M., Hochstetter, T., Koutsouki, E., Mittelbronn, M., Merseburger, A., Weigert, C., Niess, A., Lang, F. (2007). Differential regulation of serum- and glucocorticoid-inducible kinase 1 (SGK1) splice variants based on alternative initiation of transcription. *Cell Physiol Biochem*. **20**, 715-728.

Smalley, M.J., Dale, T. C. (1999). Wnt signaling in mammalian development and tumorigenesis. *Cancer Metastasis Rev*. **18**, 215–230.

Smith, E.J., Leone, G., DeGregori, J., Jakoi, L., Nevins, J.R. (1996). The accumulation of an E2F-p130 transcriptional repressor distinguishes a G0 cell state from a G1 cell state. *Molecular and Cellular Biology*. **16**, 6965–6976.

Smits, R., Kielman, M.F., Breukel, C., Zurcher, C., Neufeld, K., Jagmohan-Changur, S., Hofland, N., van Dijk, J., White, R., Edelmann, W., Kucherlapati, R., Khan, P.M., Fodde, R. (1999). Apc1638T: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. *Genes Dev*. **13**, 1309–21.

Stahl, M., Dijkers, P.F., Kops, G.J., Lens, S.M., Coffer, P.J., Burgering, B.M., Medema, R.H. (2002). The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J Immunol*. **168**, 5024-31.

Steelman, L.S., Abrams, S.L., Whelan, J., Bertrand, F.E., Ludwig, D.E., Basecke, J., Libra, M., Stivala, F., Milella, M., Tafuri, A., Lunghi, P., Bonati, A., Martelli, A.M., McCubrey, J.A. (2008). Contributions of the Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways to leukemia. *Leukemia*. **22**, 686-707.

Styles, N.A., Zhu, W., Li, X. (2005). Phosphorylation and down-regulation of Bim by muscarinic cholinergic receptor activation via protein kinase C. *Neurochem Int*. **47**, 519–527.

Su, L.K., Kinzler, K.W., Vogelstein, B., Preisinger, A.C., Moser, A.R., Luongo, C., Gould, K.A., Dove, W.F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science*. **256**, 668–70.

Sulis, M.L., Parsons, R. (2003). PTEN: from pathology to biology. *Trends Cell Biol*. **13**, 478-483.

Takahashi, M. Fukuda, K., Sugimura, T., Wakabayashi, K. (1998). Beta-catenin is frequently mutated and demonstrates altered cellular location in azoxymethane-induced rat colon tumors. *Cancer Res*. **58**, 42–46.

Takahashi, M., Nakatsugi, S., Sugimura, T., Wakabayashi, K. (2000). Frequent mutations of the beta-catenin gene in mouse colon tumors induced by azoxymethane. *Carcinogenesis*. **21**, 1117-20.

Taketo MM. (2006). Mouse models of gastrointestinal tumors. *Cancer Sci*. **97**, 355-61.

Tanaka, T., Kohno, H., Suzuki, R., Yamada, Y., Sugie, S., Mori, H. (2003). A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci*. **94**, 965-73.

Tangir, J., Bonafe, N., Gilmore-Hebert, M., Henegariu, O., Chambers, S.K. (2004). SGK1, a potential regulator of c-fms related breast cancer aggressiveness. *Clin Exp Metastasis*. **21**, 477-483.

Tran, H., Brunet, A., Grenier, J.M., Datta, S.R., Fornace Jr, A.J., DiStefano, P.S., Chiang, L.W., Greenberg, M.E. (2002). DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science*. **296**, 530–534.

Vallon, V., Wyatt, A.W., Klingel, K., Huang, D.Y., Hussain, A., Berchtold, S., Friedrich, B., Grahammer, F., Belaiba, R.S., Gorlach, A., Wulff, P., Daut, J., Dalton, N.D., Ross, J., Flogel, U., Schrader, J., Osswald, H., Kandolf, R., Kuhl, D., Lang, F. (2006). SGK1-dependent cardiac CTGF formation and fibrosis following DOCA treatment. *J Mol Med*. **84**, 396-404.

van der Voort van Zijp, J., Hoekstra, H.J., Basson, M.D. (2008). E volving management of colorectal cancer. *World J Gastroenterol*. **14**, 3956-67.

Vanhaesebroeck, B., Alessi, D.R. (2000). The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J*. **346**, 561-76.

Vaux, D.L., Cory, S., Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature*. **335**, 440-2.

Wagner, C.A., Ott, M., Klingel, K., Beck, S., Melzig, J., Friedrich, B., Wild, K.N., Broer, S., Moschen, I., Albers, A., Waldegger, S., Tummler, B., Egan, M.E., Geibel, J.P., Kandolf, R., Lang, F. (2001). Effects of the serine/threonine kinase SGK1 on the epithelial Na+ channel (ENaC) and CFTR: implications for cystic fibrosis. *Cell Physiol Biochem*. **11**, 209–218.

Wakeyama, H., Akiyama, T., Kadono, Y., Nakamura, M., Oshima, Y., Nakamura, K., Tanaka, S. (2007). Posttranslational regulation of Bim by caspase-3. *Ann N Y Acad Sci*. **1116**, 271-80.

Waldegger, S., Barth, P., Raber, G., Lang, F. (1997). Cloning and characterization of a putative human serine/threonine protein kinase transcriptionally modified during anisotonic and isotonic alterations of cell volume. *Proc Natl Acad Sci USA*. **94**, 4440–5.

Waldegger, S., Klingel, K., Barth, P., Sauter, M., Rfer, M.L., Kandolf, R., Lang, F. (1999). h-Sgk serine-threonine protein kinase gene as transcriptional target of transforming growth factor beta in human intestine. *Gastroenterology*. **116**, 1081–8.

Wang, H.G., Pathan, N., Ethell, I.M., Krajewski, S., Yamaguchi, Y., Shibasaki, F., McKeon, F., Bobo, T., Franke, T.F., Reed, J.C. (1999). $Ca^{2+}$-induced apoptosis through calcineurin dephosphorylation of BAD. *Science*. **284**, 339-43.

Wang, J.G., Wang, D.F., Lv, B.J., Si, J.M. (2004). A novel mouse model for colitis-associated colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sulfate sodium. *World J Gastroenterol*. **10**, 2958-62.

Wang, Y., Helland, A., Holm, R., Kristensen, G.B., Borresen-Dale, A.L. (2005). PIK3CA mutations in advanced ovarian carcinomas. *Hum Mutat*.

**25**, 322.

Webster, M.K., Goya, L., Ge, Y., Maiyar, A.C., Firestone, G.L. (1993).Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. *Mol Cell Biol*. **13**, 2031–2040.

Whiteman, E.L., Cho, H., Birnbaum, M.J. (2002). Role of Akt/protein kinase B in metabolism. *Trends Endocrinol Metab*. **13**, 444-451.

Williams, M.R., Arthur, J.S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P., Alessi, D.R. (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr Biol*. **10**, 439-48.

Willis, S.N., Adams, J.M. (2005). Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol*. **17**, 617–625.

Wolf, S.C., Schulze, M., Sauter, G., Risler, T., Lang, F. (2006). Endothelin-1 increases serum-glucose-regulated kinase-1 expression in smooth muscle cells. *Biochem Pharmacol*. **71**, 1175–1183.

World Health Organization. (2006). *Cancer*.

Wu, W., Chaudhuri, S., Brickley, D.R., Pang, D., Karrison, T., Conzen, SD. (2004). Microarray analysis reveals glucocorticoid-regulated survival genes that are associated with inhibition of apoptosis in breast epithelial cells. *Cancer Res*. **64**, 1757-1764.

Wulff, P., Vallon, V., Huang, D.Y., Volkl, H., Yu, F., Richter, K., Jansen, M., Schlunz, M., Klingel, K., Loffing, J., Kauselmann, G., Bosl, M.R., Lang, F., Kuhl, D. (2002). Impaired renal Na+ retention in the sgk1-knockout mouse. *J Clin Invest*. **110**, 1263-1268.

Xu, B.E., Stippec, S., Lazrak, A., Huang, C.L., Cobb, M.H. (2005). WNK1 activates SGK1 by a phosphatidylinositol 3-kinase-dependent and non-catalytic mechanism. *J Biol Chem*. **280**, 34218–34223.

Xu, J., Liu, D., Gill, G.., Songyang, Z. (2001). Regulation of cytokine-independent survival kinase (CISK) by the Phox homology domain and phosphoinositides. *J Cell Biol*. **154**, 699-705.

Yaffe, M.B., Rittinger, K., Volinia, S., Caron, P.R., Aitken, A., Leffers, H., Gamblin, S.J., Smerdon, S.J., Cantley, L.C. (1997). The structural basis for 14-3-3: phosphopeptide binding specificity. *Cell*. **91**, 961-971.

Yano, S., Tokumitsu, H., Soderling, TR. (1998). Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature*. **396**, 584-587.

You, H., Jang, Y., You, T., Okada, H., Liepa, J., Wakeham, A., Zaugg, K., Mak, T.W. (2004). p53-dependent inhibition of FKHRL1 in response to DNA damage through protein kinase SGK1. *Proc Natl Acad Sci U S A*. **101**, 14057-14062.

Zha, J., Harada, H., Yang, E., Jockel, J., Korsmeyer, S.J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell*. **87**, 619-28.

Zhang, Y.Z., Sheng, J.Q., Li, SR., Wu, ZT. (2005a). Hereditary predisposition of colorectal cancer and prevalence of hereditary nonpolyposis colorectal cancer in general population of colorectal cancer patients in China. *Zhonghua Yi Xue Za Zhi*. **85**, 2995-3000.

Zhang, L., Cui, R., Cheng, X., Du, J. (2005b). Antiapoptotic effect of serum and glucocorticoid-inducible protein kinase is mediated by novel mechanism activating IκB kinase. *Cancer Res*. **65**, 457-464.

Zhao, L., Vogt, P.K. (2008). Class I PI3K in oncogenic cellular transformation. *Oncogene*. **27**, 5486-96.

Zhu, Y., Richardson, J.A., Parada, L.F., Graff, J.M. (1998). Smad3 mutant mice develop metastatic colorectal cancer. *Cell*. **94**, 703–14.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A., Wang, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell*. **90**, 405–413.

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# 8. Curriculum Vitae

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# 9. List of publications

**Already published/accepted:**

Boini, K.M., Graf, D., Hennige, A.M., Koka, S., Kempe, D.S., **Wang, K.,** Ackermann, T.F., Föller, M., Vallon, V., Pfeifer, K., Schleicher, E., Ullrich, S., Häring, H.U., Häussinger, D., Lang, F. Enhanced insulin sensitivity of gene targeted mice lacking functional KCNQ1. Am J Physiol Regul Integr Comp Physiol.

Nasir, O., **Wang, K.** (co-first author), Föller, M., Gu, S., Bhandaru, M., Ackermann, T.F., Boini, K.M., Mack, A., Klingel, K., Amato, R., Perrotti, N., Kuhl, D., Behrens, J., Stournaras, C., Lang, F. Relative Resistance of SGK1 Knockout Mice Against Chemical Carcinogenesis. IUBMB Life.

**Wang, K.** (co-first author), Mahmud, H., Föller, M., Biswas, R., Lang, K.S., Bohn, E., Götz, F., Lang, F. Lipopeptides in the Triggering of Erythrocyte Cell Membrane Scrambling. Cell Physiol Biochem. 2008. 22, 381-6.

Föller, M., Sopjani, M., Koka, S., Gu, S., Mahmud, H., **Wang, K.**, Floride, E., Schleicher, E., Schulz, E., Münzel, T., Lang, F. Regulation of erythrocyte survival by AMP-activated protein kinase. FASEB J. 2009. 23, 1072-80.

Rexhepaj, R., Boini, K.M., Huang, D.Y., Amann, K., Artunc, F., **Wang, K.**, Brosens, J.J., Kuhl, D., Lang, F. Role of maternal glucocorticoid inducible kinase SGK1 in fetal programming of blood pressure in response to prenatal diet. Am J Physiol Regul Integr Comp Physiol. 2008. 294, R2008-13.

Hussain, A., Wyatt, A.W., **Wang, K.**, Bhandaru, M., Biswas, R., Avram, D., Föller, M., Rexhepaj, R., Friedrich, B., Ullrich, S., Müller, G., Kuhl, D., Risler, T., Lang, F. SGK1-dependent upregulation of connective tissue growth factor by angiotensin II. Kidney Blood Press Res. 2008. 31, 80-6.

**Submitted for review**

**Wang, K.**, Gu, S., Segditsas, S., Nasir, O., Föller, M., Ackermann, T.F., Klingel, K., Kuhl, D., Tomlinson, I., Stournaras, C., Lang, F. SGK1-dependent intestinal tumor growth in Apc-deficient mice.

Bhandaru, M., **Wang, K.** (co-first author), Föller, M., Nasir, O., Kempe, D.S., Stournaras, C., Lang, F. Relative resistance of PDK1 hypomorphic mice

against colonic tumors induced by chemical cancerogenesis.

Nasir, O., **Wang, K.**, Föller, M., Sandulache, D., Artunc, F., Ackermann, T.F., Ebrahim, A., Palmada, M., Klingel, K., Saeed, A.M., Lang, F. Downregulation of angiogenin transcript levels and inhibition of colonic carcinoma by Gum Arabic.

Nasir, O., Artunc, F., **Wang, K.**, Rexhepaj, R., Föller, M., Ebrahim, A., Kempe, D.S., Biswas, R., Bhandaru, M., Walter, M., Mohebbi, N., Wagner, C.A., Saeed, A., Osman, M., Lang, F. Downregulation of intestinal $Na^+$-coupled glucose transporter SGLT1 by Gum Arabic.

Föller, M., Mahmud, H., Gu, S., **Wang, K.**, Floride, E., Kucherenko, Y., Luik, S., Laufer, S., Lang, F. Participation of leukotriene C4 in the regulation of suicidal erythrocyte death.