Doctoral Thesis

Synthetic lethal screening in Drosophila melanogaster reveals novel kinases that can suppress Pten loss-of-function in vivo

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Synthetic lethal screening in *Drosophila melanogaster* reveals novel kinases that can suppress Pten loss-of-function *in vivo*

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Abstract

The Pi3K signaling pathways control key aspects of cellular growth, survival and proliferation. In these pathways, mutations of several components are known to be implicated in various types of cancer. One example is the tumor suppressor Pten whose loss-of-function leads to over-activation of Pi3K signaling, and hence to uncontrolled growth and proliferation. A better understanding of how different signaling inputs are integrated into the Pten-Pi3K networks will provide potential targets for novel therapeutic treatments of cancer.

With *Drosophila* as a model organism, we attempted to identify novel interactors of Pten, using a cell-based RNAi microarray approach coupled to the principle of synthetic lethality. Here we report the results of RNAi screening performed with the kinase/phosphatase protein families in *Drosophila* Kc167 cells. This led to the discovery of Heartless, Tssk1, and Sik2 as novel synthetic lethal kinases for Pten which can additionally suppress overgrowth *in vivo* induced by Pten loss-of-function. These kinases are potentially exciting candidates as anti-cancer targets, and will provide new insights into control and regulation of Pi3K signaling.
Zusammenfassung


1. Introduction

The term cancer is used to refer to a class of diseases in which a group of cells display uncontrolled growth, invasion, and metastasis. It can occur in nearly every tissue of the body, and is mostly characterized by the presence of malignant tumors, except in certain cases like leukaemia. Cancer is one of the leading causes of death worldwide; in 2007, it accounted for an estimated 7.9 million deaths worldwide and the projected number of deaths is predicted to increase to nearly 12 million by 2030. In 2005, tracheal, bronchial, and lung cancer was the leading cause of cancer-related deaths among both men and women in the United States (source: WHO).

Cancer occurs due to genetic abnormalities arising in transformed cells. These abnormalities may be inherited, or caused due to carcinogens like smoke, radiation, chemicals or infectious agents such as viruses. The heritability of cancers involves complex interactions between the genome and the environment, and it is now clear that several epigenetic mechanisms play a crucial role in several cancers. The mechanisms leading to uncontrolled growth and proliferation in tissues usually involve two different groups of genes. Oncogenes are typically hyperactivated in cancers leading to cell growth and division, protection against apoptosis, loss of maintenance of cell-cell boundaries, and ability to survive in diverse tissue environments. Tumor suppressors, on the other hand, are inactivated in cancers thereby disrupting normal functions of the cell such as accurate replication, cell cycle control, cell adhesion, and protective interactions with the immune system.
Oncogenes and tumor suppressors encompass an extremely diverse array of genes within the cell that are components of signaling pathways involved in essential functions such as DNA replication, cell cycle, cell growth, and cell death. Dysregulation of these pathways is the fundamental basis of tumor formation but the combination of causes leading up to the dysregulation and the mechanism by which it can be renormalized remain elusive. Mutations in one of the alleles of a tumor suppressor are not sufficient to lead to tumors, because of the presence of a second wild type copy of the allele. Usually a second hit is required in another gene (Knudson’s “two hit model”, 1971), or in some cases several genes, in order to for the cell to commit to a transformed state. Treatment for cancer thus remains a challenge because of the tremendous heterogeneity involved in the onset and progression of disease.

1.1. The Insulin-PI3K pathway in growth, survival, and metabolism

Insulin and insulin-like growth factors (IGFs) are well known as key regulators of energy metabolism and growth. There is now considerable evidence that these hormones and the signal transduction networks they regulate have important roles in neoplasia.

Epidermiological, clinical and laboratory research methods are being used to investigate novel cancer prevention and treatment strategies. Mutations of several pathway components are implicated in various cancers (reviewed in Barry & Krek, 2004). Despite the importance of these pathways for normal cell and organismal function we know very little about how hormonal signaling is interconnected with nutrient and oxygen sensing cues.
The Insulin/IGF system evolved millions of years ago, prior to vertebrate evolution. Thus conservation of nearly all essential components of the pathway can be observed from C.elegans to Homo sapiens. Binding of either insulin or IGFs to Receptor Tyrosine kinases (RTK), together with the Insulin Receptor Substrate (IRS) and IGF Binding proteins, transduces a signal which operates downstream via two main branches: the Akt branch and the Ras/MAPK branch. Binding and activation of the Insulin Receptor Substrate IRS causes activation of PI3K which catalyzes the conversion of Phosphatidylinositol-4,5-bisphosphate (PIP2) to Phosphatidylinositol-3,4,5-trisphosphate (PIP3). This in turn activates PDK1 kinase which phosphorylates and activates Akt/PKB on Threonine-308. Akt is also activated by phosphorylation at Serine473 by PDK2 (Vanhaesebroeck & Alessi, 2000; Alessi et al., 1997) Akt is a key player in mediating cell survival and growth and phosphorylates multiple targets including mitochondrial Target of Rapamycin Complex (mTORC), Tuberous Sclerosis Complex 1 and 2 (TSC1/2), Glycogen synthase kinase 3β (GSK3β), NF-κB, and Bcl2-Antagonist of cell Death (BAD) proteins. The signaling cascade induced leads to increased translation, inhibition of apoptosis, and recruitment of Forkhead transcription factors (FKHR) to the cytoplasm (reviewed in Stambolic and Woodgett, 2006). Akt is one of the most frequently mutated oncogenes in cancer. Three closely homologous Akt isoforms are found in humans and mice, all of which have been specifically implicated in tumors. In knockout mice, the different isoforms produce different developmental defects and alterations in insulin sensitivity (Cho et al., 2001; Peng et al., 2003; Easton et al., 2005; Tschopp et al., 2005). Genomic amplification of Akt2 is seen in pancreatic, breast, and ovarian cancers, while Akt3 is overexpressed in hormone-insensitive breast and ovarian cancers by an unknown
mechanism. Pten is an upstream inactivator of Akt and therefore a major inhibitor of the pathway, and is discussed in the next section. An overview of the Pi3K signaling network is shown in Figure 1, and shows the complexity of the network in controlling and coordinating multiple inputs into cellular survival, growth and metabolism.

Figure 1: Model of the complex interactions within the Pi3K network in regulation of cell growth, survival, and metabolism. (from Vivanco and Sawyers, 2002)

While activation of Akt occurs through the action of PDK1 and PDK2, inhibitory effects are mediated mainly by Pten. It is interesting to note that there are Akt independent, Pten dependent functions of Pi3K and PIP3 in controlling survival and cytoskeletal rearrangement.
mediated by Rac/CDC42, and the glucocorticoid-inducible kinase SGK (Liliental et al., 2000; Brunet et al., 2001).

1.2. Phosphatase and Tensin Homolog (Pten)

The phosphatase and tensin homolog protein Pten is a tumor suppressor and an important negative regulator of the Pi3K pathway. A tyrosine phosphatase, it dephosphorylates the substrate Phosphatidyl-inositol-3,4,5-trisphosphate (PIP3) resulting in the formation of the biphosphate product PIP2, thereby inhibiting the downstream phosphorylation and activation of Akt. The structure of PTEN solved in 1999 reveals a phosphatase domain harboring the catalytic site and a C2 domain, responsible for membrane binding and for allowing access to PIP3. The phosphatase domain shares similarity with dual-specificity phosphatases which can act on both serine/threonine and tyrosine/phosphotyrosine residues. The domain is embedded in an actin-binding motif homologous to that found in the focal-adhesion associated tensin (Lee, et al., 1999).

The molecular biology of the Pten tumor suppressor is as multifaceted as the range of human malignancies in which it is implicated. Multiple mechanisms of mutation or inactivation of Pten are seen in both heritable and sporadic cancers. Germline mutations of Pten cause autosomal dominant hamartoma tumors, while sporadic mutations occur frequently in central nervous system (20%), endometrial (39%), colorectal (9%), skin (17%), prostate (14%) and breast (6%) cancers. Monoallelic loss of PTEN can contribute to tumor growth in the context of other somatic mutations and protein levels correlate with disease severity, suggesting that Pten is functionally haploinsufficient (Yuan & Cantley, 2008; Salmena et al., 2008). The high frequency of Pten inactivation in tumors cannot be explained by structural
genomic changes alone, and epigenetic mechanisms have been predicted to play a role in gene silencing. Evidence for the latter has proven difficult to find due to the large size of the upstream regulatory region (>250 kb), the presence of a highly conserved processed pseudogene, and the technical challenges of linking epigenetic events to protein expression (Salvesen et al., 2001).

The tumor suppressor functions of Pten including G1 arrest and stimulation of apoptosis, are mediated by a cascade that maintains Akt in its dephosphorylated state. PTEN also targets Focal Adhesion kinase, thereby promoting cell migration and invasiveness, and regulates subcellular cytoskeletal organization in multiple cell types. Pten mutant mice exhibit early embryonic lethality (E7.5-E9.5) and the heterozygotes display a predisposition to tumor development (Di Cristofano et al., 2008). The C.elegans homolog of Pten is Daf-18, which is associated with the insulin signaling pathway. It has a conserved phosphatase domain and also a long non-conserved C-terminal domain. daf-18 mutants can suppress the mutant phenotypes of daf-2, the C. elegans insulin-like receptor, and age-1, the C. elegans PI3K homolog (Mihaylova et al., 1999; Ogg & Ruvkun, 1998).

The Drosophila Pten(DPten) homolog was identified independently by two different groups. Huang et al., in 1999 identified DPten by screening of a cDNA library with a low stringency human Pten probe and mapped the gene to the cytological location 31C-D on the second chromosome. The second method, (Goberdhan et al., 1999), involved a detailed genetic and mutational analysis of the chromosomal locus harboring Pten as well as several other signaling genes including chico, the IRS homolog and basket, the c-Jun homolog. The Drosophila Pten transcription unit is just over 4 kb in length, contains 11 introns and produces
a transcript that is about 2.2 kb in length that conceptually encodes a protein of 509 amino acids. There are two alternatively spliced mRNAs differing by a few amino acids at their C-terminal end. Comparison of the predicted DPten sequence with the human homolog reveals 65% homology in the N-terminal half which contains the phosphatase domain within the actin-binding domain, and an overall sequence homology of 44% (see Figure 2). There are also less conserved similarities in the C-terminal region which may be involved in regulation of the protein. Most importantly, all the key residues that are known to be mutated in cancers or in Pten-linked dominant genetic disorders in humans are conserved in Drosophila, thus making it a powerful in vivo model for the study of Pten function, and its role in the insulin signaling pathway.

Most of the known Pten mutations in human tumors cluster around the phosphatase domain, however there are other mutations distributed along the entire length of the gene, suggesting that most regions of the protein are important for its growth-suppressing function. A critical mutation G129E in the phosphatase binding domain, affects the lipid-phosphatase function but not the protein phosphatase activity in vitro. This mutation also inactivates Pten’s tumor suppressor function, indicating the crucial role played by lipid substrates. Although Pten has been shown to dephosphorylate Focal Adhesion kinase and Shc in cell culture (Gu et al., 1999), the in vivo evidence so far points to the lipid phosphatase function as the dominant one in mediating tumor suppressor activity.

Clonal analysis of Pten in the Drosophila compound eyes has shown that Pten mutant cells are upto three times bigger in size than their wild type counterparts (Goberdhan et al., 1999). While Pten mutant flies are not viable, heterozygous allelic combinations produce viable but
giant-sized flies which are about 50% larger than wild type and have bigger cells, implicating Pten in overall cell and tissue growth (Goberdhan, unpublished data). In addition to the increase in cell size, Pten mutant cells also show accelerated G1/S transitions and elevated proliferation rate. Genetic analysis has shown that the growth regulatory effects of Pten are mediated through the Pi3K pathway by antagonizing Dp110 and by inhibiting nutritionally regulated Insulin receptor (InR) signaling. Epistatic analysis has placed Dpten in a clear linear pathway upstream of Tor/S6K, and the Tsc1/2 complex (Stocker et al., 2003). While Pten and InR signaling play an essential role in growth control in flies, there are other global and cell-type specific functions of the pathway. In particular, inhibition of the InR pathway reduces fertility, slows down development and adult aging, resulting in flies that survive nearly twice as long as wild type flies. Metabolism is also affected in these flies as indicated by elevated levels of circulating sugars. InR signaling has also been shown to regulate these processes in nematodes and mammals pointing to the role of Pten as an evolutionarily conserved inhibitor of the pathway.

1.3. Synthetic lethality in cancer

Cancer cells accumulate growth-promoting mutations in several genes, and their ability to mutate rapidly makes treatment extremely difficult. In the past few years, a lot of research has focused on the concept of synthetic lethality as a possible therapeutic strategy using a range of cytotoxic agents to specifically target cancer cells.
Figure 2: The Pten protein is conserved across species. The predicted DPTen protein was compared with human Pten, *C. elegans* Pten (DAF-18; the carboxy-terminal portion of this 965-amino-acid protein is omitted; and the yeast Pten homolog TEP1 (yTEP1 or YNL128W). Identical amino acids are shaded in black and conservative changes in gray. The extent of the tensin domain is demarcated by arrows. Three regions within this domain that are required for phosphatase activity are underscored, including the highly conserved phosphatase signature motif. The positions of missense mutations identified in cancer or in dominant genetic disorders are marked by asterisks. All but one of the affected residues are identical in DPTen. Crosses above the sequence highlight Pten-specific amino acids that are not found in other known members of either the phosphatase or tensin families (from Goberdhan et al., 1999).
The term synthetic lethality comes from classical genetics wherein a combination of two separate mutations can lead to cell death, although the single mutation has no effect on the cell. Thus, using chemical agents or gene inactivation to cause lethality in cells already in a cancer setting would allow normal non-transformed cells to remain unscathed (reviewed by Kaelin, 2005).

In *S. cerevisiae*, where the phenomenon was first studied, approximately 20% of the genes are individually essential, but genetic screens have demonstrated that in the remaining 80%, synthetic lethal interactions can occur in the order of 10 interactions per gene. Synthetic lethal interactions were first described for loss-of-function alleles, but can also involve gain-of-function alleles. Such interactions were also studied later in human cells for potential therapeutic strategies. It is known that several proteins implicated in cancer have both a pro-apoptotic and antiapoptotic function. One such example is E2F which is negatively regulated by its own promoter; in such a setting blocking the E2F-Cyclin A interaction causes death of transformed cells but not their wild-type counterparts (Chen *et al*., PNAS, 1999; Chen *et al*., Cancer Res., 2003).

Similarly, cells lacking p53 (involved in G1 phase control) are more sensitive to caffeine than wildtype cells because the caffeine mediated inhibition of specific S phase proteins such as ATR leads to chromosomal condensation and apoptosis. (Sarkaria *et al*., 1999; Nghiem *et al*., 2001). Synthetic interactions may not only involve gene inactivation (as is the case with tumor suppressors) but gain-of-function as well. Many cancers demonstrate the phenomenon of “oncogene addiction” whereby the hyperactivation of a gene or a pathway is essential to sustain tumor cell survival (the same is true for inactivation of tumor suppressors). These
genes typically have proliferative as well as antiproliferative functions. In a tumor cell, the proliferative function predominates. Acute inhibition of this function causes the antiproliferative effects of the gene to become predominant and leads to impaired growth and/or cell death. These effects are apparent only when there is a temporal difference i.e when the survival signal is lost faster than the anti-survival signal in the cell. Alternatively, many synthetic lethal interactions may be time dependent. During tumor progression certain mutations are acquired only in the later stages of tumor growth in a sequential manner relying on preceding mutations. Therefore if these prior mutations were to be corrected by restoring the gene function, the effect of acquiring a second mutation may be lethal for the cell. For example, restoring p53 function in a p53 mutant cancer cell, which has since accumulated mutations in pRb, may cause these cells to senesce or die because while Rb loss promotes E2F dependent cell cycle progression, it also promotes p53 mediated apoptosis.

The concept has been extended in the last few years for a variety of small-molecule screens to identify specific drug targets that can kill cells carrying mutations in several known oncogenes and tumor suppressors. Many of these screens were “sensitized” i.e performed in defined cellular contexts such as treatment with known pharmacological agents or under specific cellular stresses. In 2004, Wang et al., showed that Myc overexpression, a common phenomenon in many cancers, sensitizes multiple human cell types to apoptosis caused by the TRAIL receptor DR5 agonists both in vitro and in cultured xenografts in mice. More recently, a large scale RNAi screen in cancer cell lines dependent on hyperactivated KRAS mutations revealed a novel serine/threonine kinase that was synthetic lethal with oncogenic KRAS (Scholl et al., 2009). The availability of a wide range of small molecule and RNAi libraries
means that several screens can now be carried out across different cell types to discover potential new drug targets and therapeutic strategies.

1.4. Synthetic lethal screening using Drosophila as a model system

Using the rationale described above, we decided to screen the Drosophila genome for synthetic lethal interactions with the tumor suppressor Pten in an attempt to discover novel genes that would be of interest not only as potential anti-cancer targets, but as new signaling branches of Pten signaling. A schematic of the principle is shown in Figure 3. We were interested in discovering RNAi which in the absence of Pten led to lethality but which had no effect on cell wild type for Pten. The kinase/phosphatase genome was selected for the screening since these constitute the most functionally relevant class of signaling molecules in the cell. Screening was performed using Kc167 cells. This is a spontaneously immortalized non-clonal embryonic cell line derived from the dorsal closure stage of development from the parental Kc line. It is important to remember that the cause of immortalization in these cells is unknown and all results must be viewed under this caveat. Drosophila cells are easily transfectable by large double-stranded RNAs (dsRNA) ranging anywhere between 300-700 bp in length. This eliminates the cost of siRNA synthesis and the problems associated with the design and selection of RNAi templates in mammalian cells. dsRNA once taken by cells is cleaved into several component siRNA fragments 21-23 bps in length by a specific RNAse Dicer (Bernstein et al., 2001). This ensures not only efficient knockdown but a much higher specificity of gene deregulation. The mechanism of dsRNA uptake in Drosophila remains
poorly understood but receptor-mediated endocytosis has been shown to be involved in mediating the process (Saleh et al., 2006).

The choice of *Drosophila* as the model screening system of choice was due to the following reasons:

- Genome-wide conservation of all major signaling pathway components
- Smaller subset of genes (about 300 odd kinases and phosphatases in the *Drosophila* genome as compared to about 900 odd kinases in the human genome)
- Less genomic redundancy, which allows the discovery of new genes of interest that could be missed in human cell lines
- *Drosophila* cells are easily amenable to RNAi, and subsequent validation *in vivo* is also convenient due to the RNAi transgenic fly library available from the VDRC since 2006.

The screening approach used for the primary screen was the cell-based array methodology which was reported in 2004 by the Sabatini group. The array system allows for high-throughput screening of upto 5000 RNAi spots per slides. The array can be conveniently customized for subsequent immunofluorescence protocols and imaging (Wheeler et al., 2004). The kinase and phosphatase genes in *Drosophila* were annotated using automated gene predictor methods, by surveying the 13,000-odd genes in fly for overall sequence homology to kinase/phosphatase proteins (Morrison et al., 2000). In all, 251 kinases and 86 phosphatases were identified, of which nearly 50% had been undetected in *Drosophila* research so far, thus providing a rich source of as-yet unknown proteins with potentially...
important physiological functions. The subsequent secondary screen was performed by live imaging of cells with the apoptosis-specific marker Annexin V (Miller, 2003) in a 96-well plate format to identify genes which showed increased apoptosis upon loss of Pten.

The kinases and phosphatases can be broadly sub-divided into distinct groups based on their structural and functional properties. A detailed description of their classification can be viewed in Morrison et al., 2001, representative examples are mentioned below.

![Pten wild type vs Pten depleted](image)

**Figure 3: Synthetic lethal screening in Drosophila.** The RNAi library is screened in Kc167 cells either wildtype or depleted for Pten, for genes causing lethality (red spots).

We used templates for dsRNA synthesis commercially available from Open Biosystems for all 337 genes representing the *Drosophila* set of kinase/phosphatase proteins. *In vitro* transcription of templates with the T7 RNA polymerase, was used to generate functional dsRNA that could be used for all future experiments.

**1.5. Summary of the project**

We performed synthetic lethal screening of the *Drosophila* kinase/phosphatase family of genes. 337 genes were screened for synthetic lethal interactions with Pten, and based on the
results we identified 3 genes that could suppress the Pten loss-of-function phenotype *in vivo*. In *Drosophila*. These novel candidates may have potential therapeutic functions in tumors where Pten has been inactivated. The effects of dysregulation of these genes and the mechanisms involved in their interaction with Pten will provide interesting avenues for further investigations in mammalian *in vitro* and *in vivo* models.
2. Results

2.1. Cell-based array screening

2.1.1. DIAP array in Kc167 cells-optimization of cell array

For performing a cell-based array screen, we tested different glass slides for their stability and adherence properties for Kc cells. We finally chose DS8 slides from Erie Scientific as the preferred system, as the cells incubated on these slides showed least variability, maximum adherence, minimum loss from washing steps, and were suitable for all subsequent staining and microscopy procedures. Based on the protocol developed by the Sabatini group in 2004 (Wheeler et al., 2004), we performed the first array optimization experiments using RNAi against the *Drosophila* Inhibitor of Apoptosis (DIAP1), which acts as a potent inducer of cell death (Igaki et al., 2002). DS8 slides were spotted using the Perkin Elmer piezoelectric array spotter with DIAP RNAi and GFP RNAi (in a final concentration of 100 ng/µL) in a 5X5 pattern. Kc167 cells were seeded on the slide and visualized after 24 hours with the nuclear dye Hoechst 33342. The DIAP1 spots are indicated by absence of cells, and the “holes” observed are indicative of cell death (Figure 4). GFP RNAi was used as a negative control, and it can be seen from the figure that the cells growing over the GFP RNAi spots grew in a smooth lawn indistinguishable from the rest of the slide. We decided to screen for such “holes” in the primary screening of 337 candidate genes. The array spotting technology provides an efficient and convenient method of RNAi delivery to a small quantity of cells.
Results

Between 5 and 10 nL of RNAi is printed per spot. At an average spot size of 250 um, this implies that about 200-300 Kc cells take up the desired RNAi.

![Image](image_url)

**Figure 4: DIAP1 RNAi in Kc167 leads to “hole” formation on DS8 slides.** Hoechst 33342 staining of Kc167 cells spotted on DS8 slide (overview) spotted with DIAP and GFP RNAi. Spot-to-Spot spacing 250 um. RNAi treatment was performed for 24 hrs and 25 spots were printed per RNAi; scale bar =1mm, montage view generated from 5X magnification

2.1.2. Pten downregulation in Kc167 cells

We next wanted to verify whether Pten could be efficiently knocked down in Kc167 cells using dsRNA. RNAi against Pten lead to efficient down-regulation after 24 hrs (78%
Results

efficiency; Figure 5A). The downregulation remains constant over 48 hours (74% Figure 5B) and 72 hours (not shown) thus showing that Pten mRNA remains knocked down for the entire period of the screening. We quantitated the down-regulation before each independent experiment with quantitative real time PCR.

![Graph showing Pten down-regulation](image)

Figure 5: Pten down-regulation Kc167 cells is constant over 48 hours quantitative real time PCR of Pten knockdown cells after A) 24 hours and B) 48 hours of RNAi treatment (** denotes P-value,0.01)

It should be noted that cells treated with Pten dsRNA did not show significant increase in cell number, or cell size, compared to GFP RNAi treated cells over 48 hours (data not shown). Thus loss of Pten in Kc167 cells did not lead to an obvious growth/proliferation phenotype in our screening.

2.1.3. Synthetic lethal screening with cell based array

337 dsRNAs comprising the Drosophila set of kinases and phosphatases were synthesized from templates commercially available from Open Biosystems.
Results

These comprised 251 kinases and 86 phosphatases. DS8 slides were spotted with the dsRNAs and synthetic lethal hits were identified by Hoechst 33342 staining as shown in the Figure 6, in cells downregulated for Pten. Three representative hits showing synthetic lethality are shown in presence of Pten RNAi in Kc167 cells. We performed 4 independent screens, and in each, spots were printed in duplicate for every gene. The presence of “holes” indicated spots where cells did not grow or were lost due to apoptosis, and/or necrosis. Pten downregulation was verified using quantitative real-time PCR.

Figure 6: Synthetic lethal screening in Kc167 cells reveals novel targets. Kc167 cells treated with control GFP RNAi (left panel) and Pten RNAi (right panel) for 24 hrs, seeded on spotted slide arrays for 24 hrs, and stained with Hoechst 33342. Representative pictures of 3 dsRNAs with their Drosophila gene id are shown are shown A) CG14305, B) CG7223, C) CG4290. Each dsRNA was represented as duplicate spots on the slide (magnification 10X).

The results of 4 independent screens are summarized below. We performed 4 rounds of screening in all and obtained in 55 genes out of 337 that could be shown to be synthetic lethal with Pten. The number of genes obtained in each round is shown in Figure 7A, and was
respectively 23, 12, 13, and 14 hits per screen. Figure 7B shows the overlap between the 4 experiments.

As can be seen from Figure 7B, the overlap between independent screens was quite low. This high variability between experiments may be attributed to the heterogeneity in the level of Pten knockdown between experiments, which cannot be completely eliminated. The Pten knockdown efficiency was estimated by qPCR for each experiment, and was about shown to be between 74-78% as shown in Figure 5B. However, there is no way to establish that the cells growing over a particular dsRNA spot show the same level of Pten downregulation in every case. Also, the semi-adherent nature of Kc167 cells leads to detachment and loss of cells from the slide surface during the fixation and staining. Although this problem was largely circumvented by the use of specially coated DS8 slides, there still remains the possibility that cells did not remain uniformly attached to the slide over repeated experiments.

The percentage of genes classified according to 8 sub-categories of GO annotation is summarized in Figure 7C. Since some genes appear in more than one category, the overall percentage adds up to more than 100%. The top three groups represented were: 28 serine-threonine kinases (60.8%), 8 phosphoprotein phosphatases (17.4%), and 7 tyrosine kinases (15.2%). For a detailed list of genes and their closest known human homologues see Appendix.
Figure 7: **Summary of cell-array based synthetic lethal screening.** A) Total number of synthetic lethal hits per experiment; B) overlap between 4 independent experiments; C) GO annotation of 55 genes obtained from screening, percentage of genes per category is indicated, 46 out of 55 genes were successfully annotated.
2.2. Synthetic lethal screening with Annexin V

The RNAi array-based strategy of screening is not specific for the exact mechanism of cell death. This could occur due either apoptosis, or necrosis, lack of proliferation, or loss of cell-cell adhesion on the array. Thus we decided to screen in the next round for apoptosis specific genes. After the primary screening, we took all the 55 genes obtained from the 4 rounds of screening for our secondary screening assay. The secondary assay was performed using the apoptotic marker Annexin V-FITC which binds to exposed phosphatidylserine residues on the membranes of apoptotic cells. Using a double staining of Annexin V with Hoechst we quantified the total ratio of apoptotic cells to nuclei, in Pten positive and Pten negative cells screened against the 55 candidate genes.

2.2.1. DIAP RNAi induces apoptosis in Kc167 cells visualized using the apoptotic marker Annexin V.

As a proof of principle for the apoptosis screen we used RNAi to downregulate *Drosophila* Inhibitor of Apoptosis (DIAP) in Kc167 cells. Cells treated with DIAP RNAi for 24 hrs were stained with Annexin V-FITC, Propidium Iodide (PI) and Hoechst, and visualized using the BD Pathway screening microscope. As can be seen from Figure 8, there was a large increase in the percentage of Annexin positive and PI positive cells upon DIAP knockdown. We also observed that Pten RNAi did not cause any increase in apoptosis when compared to control GFP RNAi treated Kc cells. Thus we could be certain that any increase in apoptosis observed in the screening of Pten deficient cells against the selected genes was solely due to the effect
Results

of the double knockdown.

Figure 8: DIAP RNAi causes apoptosis in Kc167 cells. PI and Annexin V staining Kc167 cells; A) DIAP RNAi treated, B) GFP RNAi treated and C) Pten RNAi treated for 24 hours prior to imaging (Magnification 40 X, scale bar =100 µm).

Figure 8 shows Hoechst (blue), PI (red) and Annexin V (green) staining in Kc167 cells. There is a clear increase in PI positive cells upon loss of DIAP, which is known to induce cell death. PI staining does not distinguish between apoptotic or necrotic cells but Annexin V is a marker for apoptotic cells specifically and is seen to increase as well. Thus we could use Annexin V as an effective marker of apoptosis-induced cell death for our secondary screening. DIAP RNAi was subsequently used as a positive control in all experiments.
2.2.2. Apoptosis screening of candidate genes

Kc167 cells were screened against 55 genes after 24 hours of GFP RNAi and Pten RNAi knockdown using the BD Pathway 855 screening microscope. Cells were treated with the second RNAi for 24 hours. Total number of nuclei was calculated using the Imaris Object Recognition Software, while apoptotic (Annexin positive) cells were counted manually. The apoptotic ratio of Pten knockdown to GFP control cells was calculated per gene. The graph in figure 9 shows results of three independent experiments. The genes subsequently used for further validation are indicated.

![Graph showing fold change in apoptotic ratios between Pten Kd and control cells.](image)

**Figure 9: Synthetic lethal screening in Kc167 cells against 55 target genes.** Apoptotic ratio in Kc167 cells upon Annexin staining plotted versus well number. The bars are average +/- standard deviation of three independent experiments, for some genes we lacked enough data for calculating mean and standard deviation and these were excluded from the analysis. Genes of interest are indicated next to the well number, DIAP RNAi was included as the positive control.

We chose to follow up genes that gave a >1.5 fold increase in apoptotic ratio when compared to wild type. It should be noted that this is not as high an increase when compared to the apoptotic ratio in DIAP knockdown cells, which showed a 3.5 fold change in apoptotic ratio. As can be seen from figure 9, there were 14 genes which showed a high (> 1.5 fold) apoptotic
ratio in the Annexin screening, including CG4290 (well b3), CG14305 (well b7), CG7223 (well a3) and CG10177 (well f6).

We performed three independent experiments and each dsRNA was represented in duplicate in a single experiment. The apoptotic ratio was averaged per experiment and mean and standard deviation was calculated from the averaged values of the three independent experiments. The genes for which we lacked sufficient data to generate mean and standard deviation, or which were not significant were subsequently excluded from further analysis. The complete list of genes corresponding to well number can be seen in Appendix, Table 3. The narrowing down of candidates for follow-up was based on a combination of reasons. Firstly, we preferentially selected those genes which had appeared 2 or more times in the primary screening. From these, we selected the ones which a) showed efficient mRNA knockdown; (see figure 10B) and c) demonstrated the same effect when tested in S2 cells. This led to number of candidates dropping out from the final list. The results shown are for genes which passed all these criteria.

To compare the actual percentage of Annexin positive apoptotic cells in each well, it is important to look at the Absolute apoptotic ratios. Total number of Annexin positive cells to Hoechst were calculated for each of the 4 dsRNAs indicated in figure 10A in the presence or absence of Pten RNAi in Kc167 cells. In this case the experiment was performed differently - both dsRNAs were administered simultaneously to Kc167 cells and Annexin staining was performed after 48 hours. In each case, knockdown together with Pten loss gave an increased ratio of Annexin positive cells to Hoechst when compared to control cells treated with GFP RNAi. Cells treated with Pten RNAi alone did not show increased apoptosis. Looking at the
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In figure 10B we can see that in most cases the ratio of Annexin/Hoechst between GFP treated and Pten treated cells was comparable to that observed when Pten was downregulated prior to the second knockdown, i.e. between 1.5 - 2 fold change in apoptotic ratios (see fold change in apoptotic ratios of the genes in Figure 9). There was one exception, CG7223, where the apoptotic ratio was much higher than that observed in the screening. This is most likely due to the lag period before knockdown in these cells, during which time cells may adapt to the loss of Pten in a way that renders them less sensitive to the subsequent loss of CG7223. It should be mentioned however Pten knockdown is almost constant over the entire 48 hours of the assay (figure 5).

Knockdown of mRNA was verified using quantitative real-time PCR analysis for the genes indicated in Figure 10B. The fold change in knockdown was 0.23 for Pten, 0.32 for CG7223, 0.31 for CG14305, 0.23 for CG1077, and 0.35 for CG4290. Thus, the knockdown in most cases ranges from about 80%-70% efficiency which is consistent given the levels of dsRNA knockdown previously reported in Drosophila cells. The qPCR was performed for each gene in both presence (fig 10B) and absence of Pten RNAi (data not shown), to verify that the knockdown remained the same in both cases, and was not affected by the loss of Pten mRNA. Data is shown as relative fold change compared to Control (GFP RNAi treated) cells for each gene. All values were normalized to rp49 as the housekeeping control.
Results

**Figure 10: Increase in Apoptotic ratio in Kc167 cells upon Pten knockdown.** A) Absolute Annexin/Hoechst ratios in Kc167 cells treated indicated RNAi in Control (GFP RNAi) or Pten RNAi (P-values were calculated with respect to GFP RNAi control in each case); B) qPCR of indicated genes in Kc167 cells, relative mRNA levels were normalized to the Control (GFP RNAi) cells in each case; experiments were performed in triplicate (* denotes P-value <0.05, ** denotes P-value <0.01).
Results

The Annexin V FITC assay was then performed in S2 cells to confirm the robustness of the screen in a different *Drosophila* cell type. The results are shown in Figure 11. S2 cells were down-regulated for Pten for 24 hours before treatment with 1µg of the second RNAi in 96-well plates. Annexin V staining was performed after 24 hours as described previously. Apoptotic ratios are shown for the indicated RNA is screened against Pten positive and Pten negative cells. We could observe increase in apoptosis upon Pten knockdown for 4 genes discovered from the Kc167 and this increase was similar to that seen in Kc cells (compare to Figure 10A) and ranged from 1.5-2 fold. These genes were then used for all subsequent experiments *in vivo*.

![Figure 11: Annexin/Hoechst ratio is increased upon loss of Pten in candidate genes in S2 cells](image)

Figure 11 shows Annexin/Hoechst staining in S2 cells treated with the indicated dsRNA; three independent experiments were performed in 96-well plates; significance calculated with respect to GFP controls in each case (* denotes P-value<0.05, * denotes P-value<0.01).

These four genes were found to be homologues of the following proteins...
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- **CG14305: Testes Specific Serine Kinase 1 (Tssk1)**
- **CG7223: Heartless (Htl), FGFR1 homologue in Drosophila**
- **CG10177: p90 Ribosomal S6 kinase**
- **CG4290: Salt-inducible kinase 2 (Sik2)**

With the exception of Heartless which is annotated in the Fly genome, all others were uncharacterized genes whose closest human homologue was ascertained with a BLAST search against the non-redundant genome database from NCBI. These genes will henceforth be referred to by their gene names in subsequent experiments.

We verified the efficient knockdown of mRNA of the indicated genes using qPCR in S2 cells (Figure 12), and found the level of downregulation to be the same as that seen in Kc167 cells.

The expression levels are shown as relative mRNA values normalized to the house-keeping control. This gives allows us to adjudge not only the knockdown efficiency of each gene gives us an overview of the range of expression levels of the individual genes in these cells. As can be seen from figure 12, Htl and Pten are expressed upto 10 times more than Sik2/Tssk1/p90.
Results

Figure 12: Down-regulation of CG7223, CG4290, CG10177, CG14305, and Pten upon dsRNA treatment in S2 cells. qPCR for indicated genes in S2 cells after dsRNA knockdown; Results are average of three experiments (** denotes P-value<0.01, *** denotes P-value<0.001)

2.3. Suppression the Pten loss-of-function phenotype in Drosophila using tissue-specific RNAi

Transgenic RNAi flies were ordered from the VDRC in Vienna to test for possible in vivo interactions with Pten RNAi among our hits. We used the tissue specific Gal4 driver engrailed to examine the effect of Pten loss in adult wings which, during larval development is expressed in the posterior half of imaginal discs. Pten RNAi alone was shown to induce overgrowth in the posterior half of adult wings (Figure12, bottom left panel). This is consistent with clonal analysis of Pten mutant clones in the Drosophila eye as reported by
Goberdhan et al. (1999). To check whether this effect was due to increase in cell size or cell number, we calculated the trichome ratio between the posterior and anterior halves of the wing. Compared to control flies (yw crossed to engrailed-Gal4) there was a small but significant decrease (0.74 vs 1.01) in posterior:anterior trichome ratio shown in Figure 14, indicating that loss of Pten leads to a modest increase in cell size in the wing. In engrailed-flies expressing RNAi against Heartless, Tssk1, and Sik2, there was no effect on wing size or trichome ratio when compared to control flies. However, in double RNAi flies the effect of Pten overgrowth was completely rescued. Trichome ratios of double RNAi flies were the same as yw, thereby showing that the effect of Pten loss could be rescued upon loss of the gene identified from screening. In this and subsequent experiments, data are shown for adult female flies unless otherwise indicated. Male flies when tested showed the same results (data not shown). Interestingly we observed that this rescue was not seen for CG10177 (p90-S6 Kinase) RNAi flies(data not shown). Thus, this gene, although obtained as a hit from our screen does not appear to interact with Pten in vivo.

To check whether the increase in cell size observed upon loss of Pten was also accompanied by an increase in cell number, and whether this was also rescued upon knockdown of Heartless, Tssk1, or Sik2, we performed BrDU incorporation assays as described below.
Results

Figure 13: Tissue overgrowth caused by Pten loss is synthetically suppressed by Heartless, Tssk1, and Sik2. Dotted line demarcates the anterior (A) and Posterior (P) boundary; Black arrow head indicates the posterior region of interest, pictures are taken from adult female flies.
Results

Figure 14: Suppression of Pten-induced overgrowth in wings by Htl, Tssk1 and Sik2.
Posterior/Anterior trichome ratios for adult female flies expressing engrailed driven RNAi for the genotypes indicated.

The BrdU incorporation assay was performed on third instar wandering larvae to examine the effect of cell proliferation in the developing wing discs. As can be seen from Figure 15(top panel), loss of Pten in the posterior half of wing discs led to an increase in BrdU staining which was rescued in all three of the double RNAi lines tested. RNAi expression was driven by engrailed-Gal4-UAS GFP to distinguish the posterior/anterior boundary. The anterior portion of the discs served as a control in all stainings. No such effect was seen for the single RNAi alone for any of the genes tested (data not shown). Thus Htl, Tssk1, and Sik2 can suppress the increase in proliferation induced upon loss of Pten.

To further confirm the suppression of the Pten loss-of-function phenotype we used the Drosophila adult eye as a model system. RNAi was expressed under the control of the GMR
Results
driver which is expressed in the proliferating part of the eye during development. Adult eyes were examined using scanning electron microscopy to look at the size, number, and arrangement of ommatidia. Loss-of-function of Pten leads to a rough eye phenotype as indicated by increased size and number of ommatidia, when compared to wild type eyes. However the rough eye phenotype is rescued in the double RNAi flies. Heartless, Tssk1, and

Figure 15: Increase in proliferation induced by loss of Pten is suppressed by Htl, Tssk1, and Sik2 BrdU staining in third instar wing imaginal discs, from en-GAL4-UAS GFP flies crossed to the indicated RNAi. RNAi expression is marked by presence of GFP. Scale bar= 100µm
Results

Sik2 RNAi alone did not show any eye phenotype and were the same as wildtype (Figure 17). Thus the effect of Pten loss in the adult eye is synthetically suppressed upon further loss of Htl, Sik2, and Tssk1. This strengthens the theory that all three genes interact genetically with Pten in *Drosophila* in regulating tissue size and development. We checked whether

![Figure 16: Rough-eye phenotype caused by loss of Pten is suppressed by Htl, Tssk1, and Sik2. Scanning Electron micrographs of female adult eyes; transgenes are indicated in the image; representative pictures are shown for adult female flies; Control flies were yw crossed to GMR-Gal4, scale bar=100nm).](image-url)
Results

apoptosis was increased double RNAi wing discs with TUNEL staining and found no increase in apoptosis in these discs when compared to wild type (data not shown) suggesting that the synthetic interaction led only to the suppression of proliferation but not increase in cell death at least in vivo.

In order to verify whether the transgenes could actually lead to mRNA knockdown, the ubiquitous driver Actin-5C-Gal4 was used to drive RNAi in flies with the indicated genotype (Figure 17). Third instar wandering larvae were isolated and quantitative real-time PCR was performed to confirm the knockdown of gene expression.

Figure 17: Pten, Tssk1, Sik2, and Htl are downregulated in transgenic RNAi flies. qPCR of third instar larvae from Actin5C-GAL4 flies crossed to the indicated genotype; values are calculated with respect to control (yw crossed to Actin-5C-Gal4) which is set to 1 in all cases; results are average of three independent crosses.

These experiments were performed in whole larvae carrying ubiquitous knockdown with Actin-Gal4, since we could not isolate enough mRNA from wing discs to check the expression under engrailed. Thus while we cannot conclude that the level of downregulation

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is the same for engrailed, we can show that the UAS RNAi transgenes are successful in knocking down gene expression ubiquitously.

2.4. Expression levels of Sik2, FGFR2, and Tssk1 are up-regulated in DU145 cells upon Pten knockdown

We identified three genes that could suppress the Pten loss-of-function phenotype in vivo in Drosophila. We decided to further study the role played by these genes in specific cancer cell lines containing either wild type or mutant Pten, and examine whether they are indeed synthetic lethal in such a setting.

Preliminary experiments were performed in the prostate cancer cell line DU145. Using lentivirus mediated (LMP) knockdown of Pten with shRNA we obtained stable Pten negative cell lines (from P.Wild). Pten was efficiently knocked down with the shRNA; see Figure 18B, middle panel. We found that mRNA levels of FGFR2 and Tssk1 were upregulated upon Pten loss (Figure18A). The same was true of Sik2 protein levels as shown by Western blotting (Figure18B). However mRNA levels of FGFR1, the closest Htl homologue in humans, remained unchanged (data not shown). This dysregulation of protein expression could point to their potential role in maintaining cellular proliferation and survival upon loss of Pten, similar to the results reported for FGFR2 by Byron et al. in endometrial cancer. Interestingly we did not observe up-regulation of these genes in Kc167 cells where Pten was knocked down (See Appendix, Figure 20). We cannot completely dismiss the idea that this could be cell type specific phenotype. Since the DU145 cells did not seem to be amenable to siRNA mediated transfection however, we could not efficiently down regulate either Tssk1 or Sik2 mRNA
levels in these cells to demonstrate the synthetic effect. Therefore the next stage is to examine whether the same effect is true for other cancer cell lines where Pten is mutated and functionally inactivated.

Since Sik2 expression can be visualized with a commercially available antibody by immunohistochemistry, it is possible to analyze the expression levels of the gene across a range of tumor samples using Tissue Micro Arrays, and perform statistical correlations to known oncogene/tumor suppressor status. Since prostate cancer is one cancer type in which Pten mutation/inactivation is commonly observed (14% of the cases, see Introduction, Section 1.2), we started initially with prostate cancer tissue arrays to analyse Sik2 staining. When we examined the expression of the Sik2 gene across 459 patient samples we found a correlation between expression of the protein and the stage of cancer. Sik2 expression was increased in prostate carcinoma when compared to BPH (benign prostate hyperplasia) in Figure 19A, showing representative immunohistochemical staining from patient biopsies. Tumor regions are indicated by a loss of epithelial structure when compared to the benign stage (black arrowheads). Evaluation of staining intensity on the arrays was performed by two independent researchers and expression levels were arbitrarily graded from 0-3, with 0 denoting negative/no staining while 3 represents strong staining (for details, see Section 4.5; Materials and Methods).
Figure 18: Tssk1, FGFR2 mRNA and Sik2 protein levels increase in DU145 cells upon Pten knockdown. A) qPCR in DU145 cells with lentiviral-mediated hairpins: LMP-Pten and Non-silencing control (NS); (i) Tssk1 and (ii) FGFR2 mRNA levels normalized to 18S; results are average of three independent experiments (** denotes P-value < 0.01) B) Sik2 upregulation in Pten-shRNA treated DU145 cells: lane 1-NS, lane 2 – LMP-Pten.
Results

Figure 19B shows a cumulative frequency distribution of Sik2 staining across different stages of prostate cancer in a total of 429 samples: Metastasis (MTS), Hormone refractory prostate cancer (HRPC), Radical prostatectomy performed for localized prostate cancer (RPE), and Benign Prostate Hyperplasia (BPH).

It can be seen from figure 19B that strong expression of Sik2 is only seen in MTS, RPE and HRPC samples but not BPH. Also the percentage of intermediate-to-strong Sik2 staining is increased in MTS, HRPC, and to a lesser but significant in RPE compared to BPH samples. Furthermore, Sik2 expression was strongly correlated to Phospho-S6 levels, a hallmark of constitutive Pi3K activity (figure 19C). This analysis was performed on the St. Gallen cancer array containing localized prostate cancer (or RPE) and BPH samples. Interestingly, in this array as well we found a strong correlation between Sik2 expression and Carcinoma using the Mann Whitney-U test; (N=60, P-value=0.001). We checked whether Sik2 expression correlated to other known clinicopathological factors in prostate cancer such as PSA and Gleason score. The Gleason Score is used to evaluate prognosis and guide therapy in prostate cancer based on the microscopic appearance of the tumor; the prostate-specific antigen PSA, is a biomarker whose expression levels in the serum is used as a diagnostic tool for prostate cancer. We found that Sik2 expression did not correlate to Survival, Gleason Score, or PSA levels. (data not shown). Correlation data for Pten and Phospho-Akt staining is ongoing and will soon be added to the analysis. However we have the first hints the expression levels of Sik2 are dysregulated upon the advent of prostate cancer.
**Figure 19: Sik2 expression is correlated with cancer.** A) Sik2 immunohistochemical staining in Benign prostate hyperplasia (BPH, staining 0) and prostate carcinoma (staining 3) from two representative biopsies; scale bar = 500 µm. B) Cumulative frequency of Sik2 expression vs tumor stage; 0+ denotes negative-weak staining; 1+ denotes weak-intermediate, 2+ denotes intermediate-strong, and 3+ denotes strong expression. P-value = 0.019. C) Pearson Correlation Coefficient between Sik2 and phospho-S6 expression in 59 patient samples.
Based on the data described above, 3 novel genes were discovered that interact with Pten \textit{in vivo} from our synthetic lethality screening. It seems clear that the \textit{in vivo} function of Pten in \textit{Drosophila}, i.e. its role in controlling cell size and proliferation, requires the activity of Htl, Tssk1, and Sik2. Loss-of-function of Pten is a common feature in several human malignancies, so it is tempting to speculate that targeting one or more of these genes in such a setting could rescue the effect of Pten loss. We also tried to check the level of apoptosis in these flies with TUNEL staining but found no change in apoptosis when compared to control flies for any of the genotypes. (data not shown). The possible implications of these interactions and their known physiological roles are discussed at length in the next chapter.
3. Discussion

We employed the concept of synthetic lethal screening to identify novel proteins that interact with the tumor suppressor Pten. The rationale behind the project was twofold: to discover new genes that could induce cell death under conditions of tumor suppressor inactivation, and to find new kinases and phosphatases that might be implicated in novel signaling pathways regulating cell growth and proliferation. The synthetic lethality principle has gained a lot of traction in the past few years because of the possibility of discovering novel anti-cancer targets through a combination of gene and drug-based screening. The *Drosophila* genome provides a rich source of potentially exciting candidates since many genes remain unidentified with regard to their physiological function, and are ripe for discovery.

Two different screening approaches were used to identify genes of interest. The first screen performed using spotted RNAi arrays, identified genes that led to an absence of growth in the presence of Pten downregulation. However, this method did not distinguish between apoptosis, necrosis, lack of cell adhesion or simply lack of cell proliferation. We obtained a list of putative candidates of which 33 were annotated in the fly genome but 12 were of as yet completely unknown function (see Appendix). There were 48 Kinases and 7 phosphatases in the list. The 12 unannotated kinases presented potentially exciting targets for further study, and we analyzed the closest possible human homologue of each using BLAST.

Before proceeding further, a brief note on the benefits and limitations of our approach. The RNAi approach has clear advantages in terms of high throughput and convenience in large
scale screening. With a robust assay, it is possible to discover novel candidates implicated not only in essential cellular functions such as growth and survival but also as regulatory components in established signaling pathways. The caveat of course is that knockdown levels vary and cannot completely replicate a complete gene knockout situation. Knockdown levels were seen to vary between 70-80% efficiency in all cases. It could be therefore be speculated that in the presence of a complete knockout the lethality may be even more pronounced.

*Drosophila* cell lines are not easily amenable to stable transfections so heterogeneity in down-regulation within a given batch of cells is another factor to be considered. Furthermore RNAi has been shown to have non-specific targets in several cases (Jackson et al, 2003) and these can lead to an incorrect interpretation of the results. Although this problem is largely circumvented in *Drosophila* because of dsRNA-mediated gene knockdown, there still remains the possibility of non-specific targets.

To further narrow down the mechanisms of cell death, we performed the second round of screening with the Apoptosis specific marker Annexin V, and after preliminary follow-up and validation assays we obtained a list of 4 genes that we used to test in *Drosophila* for genetic interaction with Pten. 3 out of the 4 genes were among the 12 unannotated candidates we found. We found that Gene Id CG10177 whose closest human homologue was the p90 ribosomal S6 kinase, did not show any genetic interaction with Pten in the fly (data not shown). This could be a tissue-specific effect or could be indicative of this gene having different functions in the fly as opposed to cultured *Drosophila* cells. However the other three (Heartless/ Tssk1/Sik2) could all rescue the Pten deficient phenotype when knocked down. When we performed experiments with the human prostate cancer cell line DU145, we
observed an up-regulation of FGFR2 and Tssk1 transcripts and Sik2 protein upon Pten knockdown. No such effect was seen in Kc167 cells upon Pten knockdown. This could thus be a context or cell-type specific effect. However, this could more likely be a difference between *Drosophila* and human cells, where different signaling pathways may be up- or down-regulated upon Pten loss. Loss of Pten has Pi3K-independent phenotypes in the fly (Goberdhan *et al.*, 1999) related to Pten-control of cytoskeletal architecture; moreover, the role of the weakly conserved C-terminal region of the protein is still unclear and may have species-specific functions. Therefore, it is tempting to speculate that the up-regulation of these genes points to a possible role for increased activation of Htl/Tssk1/Sik2 dependent pathways upon Pten loss, and their subsequent inactivation could lead to cell death. Here we will discuss the known functions and possible role that these genes could play in regulating cell survival.

Interestingly, of the 3 genes we found, Tssk1 and Sik2 belonged to the group of AMPK related kinases. AMPK is a key cellular sensor of reduced energy supply that is activated by an increase in the cellular ration of AMP/ATP. Stress or pathological conditions which cause a drop in the cellular levels of ATP, lead to allosteric activation of AMPK upon AMP binding, and the subsequent phosphorylation and activation of AMPK on Thr-172 by the tumor suppressor LKB1 (Hong *et al.*, 2003, Woods *et al.*, 2003). Activated AMPK can through multiple mechanisms promote catabolism and decrease anabolism to restore the cell’s depleted energy levels, and thus aid cellular recovery and survival. However, AMPK has also been shown to mediate apoptotic cell death under certain conditions. AMPK is a complex heterotrimeric enzyme containing a catalytic alpha subunit and the regulatory beta and gamma
Discussion

subunits, all of which are conserved across evolution. In humans there are two or three distinct genes encoding each subunit (excluding splice variants) while in *Drosophila* there is a single gene for each subunit. (Hardie *et al.*, 2003).

Recently, based on their sequence, several other proteins have been shown to belong to the AMPK subfamily in humans. These include BRSK1 &2, the Sik genes, and MARK 1,2 3&4. All of these proteins carry the conserved T-Loop Thr 172 residue which is the site for LKB1 – mediated phosphorylation. Although functionally very little is known about the other AMPK related kinases , it was shown that LKB1 can phosphorylate the Thr 172 residue in all the proteins *in vitro*, and thus may play heretofore unknown roles in mediating the physiological roles of LKB1 including its tumor suppressor function(Lizcano *et.al.*, , 2004).

The *Drosophila* kinase CG4290 was identified as a homologue of the human Sik2 gene. There are three isoforms of the gene in humans that are members of the SNF/AMPK subfamily. (Takemori & Okamoto, 2007). Expression data from rats showed that SIK1 expression is restricted to the adrenal glands, Sik2 is expressed in brown and white adipose tissue and to a lesser extent in testes, while Sik3 appears to be ubiquitously expressed. All three isoforms are known to be phosphorylated by LKB1 on the classical T-loop Thr182 residue. Sik has been shown to phosphorylate SREBP and negatively regulate hepatic lipogenesis (Yoon *et al.*, 2009). Sik can also phosphorylate the TORC complex thereby leading to repression of CREB mediated transcriptional activity, and was shown to phosphorylate IRS1 on Ser 794 in 3T3-L1 and COS7 cells (Takemori & Okamoto, 2007; Horike *et al.*, 2003). Sik mRNA levels were upregulated in diabetic animals. While Sik2 was shown to be upregulated in db/db mice in white adipose tissue, Sik1 mRNA, whose expression is normally restricted to adrenal glands
of wild type animals was seen to be markedly upregulated in brown adipose tissue, skeletal muscle and liver. Thus Sik may act as a crucial energy sensor in the maintenance of glucose homeostasis under physiological conditions. The phosphorylation of Sik and subsequent CREB repression is dependent on the priming LKB1 phosphorylation and was the first demonstration of feedback inhibition of the CREB transcriptional activity by a novel kinase cascade. Sik1 has been shown to play a role in survival of skeletal myocytes as a Class II HDAC kinase (Berdeaux et al., 2007), leading to derepression of the myocyte enhancer factor 2 (MEF2) family of transcription factors which are known to promote cellular differentiation and survival in skeletal muscle( Wu et al., 2001). The effect of Sik1 on MEF2 dependent transcription is potentiated by phosphorylation and inhibition of Sik1 by PKA upon cAMP activation (Takemori et al., 2002), and its subsequent activation by CREB upon decay of intracellular cAMP. SIK1 can also regulate its activity in a negative feedback loop via TORC2 phosphorylation. Since Sik1 and Sik2 have different patterns of tissue expression, it remains unclear whether Sik2 has an independent, tissue- specific role in survival. Sik2 was shown to be phosphorylated and activated by Akt2 in response to insulin in the liver. The phosphorylation of Sik2 leads to TORC inhibition and degradation, and subsequent inhibition of hepatic glucose production(Dentin et al., 2007). This could provide a mechanism to maintain blood glucose levels during inappropriate induction of the gluconeogenic program, and point to a role of Sik2 dysregulation in insulin resistance and diabetes. In flies, where TORC is mainly expressed in the brain, Sik2 has been implicated in survival, whereby Sik2-mediated TORC phosphorylation led to TORC inhibition upon re-feeding, and neuronal depletion of Sik2 led to enhanced survival and increased resistance to starvation by increasing
TORC activity in an Insulin dependent manner (Wang et al, 2008). Thus Sik2 clearly plays a role in maintaining the energy balance in cells in response to insulin stimulation. It is noteworthy that depletion of Sik2 at least in the Drosophila brain, led to increased survival which apparently contradicts a role of Sik2 in apoptosis. However, it should be noted that these studies were performed in flies under conditions of fasting –re-feeding wherein insulin signaling is modulated by several feedback mechanisms to maintain cellular energy levels. The same condition may not be true for immortalized cultured cells which are continuously proliferating and where nutrient supply is non-limiting. Therefore Sik2 depletion in such a setting may lead to different effects from that seen in vivo, where tissue-specific functions are clearly more important. It will be extremely interesting to look at the effects of Sik2 knockdown on Phospho-TORC levels and CREB-regulated gene expression using reporter assays to assess the role of Sik2 in suitable cancer cell lines in the presence and absence of Pten. Furthermore changes in expression levels of Sik2 can be analyzed across prostate cancer samples from patients in different stages of progression from benign to metastatic, using Tissue Microarray analysis. The first step in this direction has already been made possible in collaboration with the Kantonspital in St Gallen of Zurich, who kindly provided us with a prostate cancer arrays comprising a total of 83 biopsy samples. We found that Sik2 expression levels correlated strongly to the diagnostic outcome of cancer. There was an overall increase in protein abundance between benign prostate hyperplasia and carcinoma. This increase was seen to be even higher in metastasis and hormone-refractory cases than in localized prostate cancers. Thus, it appears that Sik2 levels increase as the tumor progresses to more advanced stages. Sik2 expression also showed strong correlation with Phospho S6 staining in these
samples. Loss of Phospho S6 is a hallmark of constitutive Pi3K activation, and this suggests that Sik2-dependent regulation becomes hyperactive in this setting and may upon inactivation lead to cell death. Since Pten is one of the most frequently inactivated tumor suppressors in prostate cancer, it is very tempting to speculate that Sik2 plays a role in tumor progression when Pten is absent.

Tssk1 is one of a family of 5 identified serine-threonine kinases with exclusive or dominant expression in human testes, and involvement in spermatogenesis and male fertility (Xu et al., 2008). Because of their pattern of expression and also the testis-specific expression of the Tssk1-Tssk2 substrate TSKS, these proteins are being studied for their potential role as male contraceptive targets. Targeted deletion of Tssk1 and Tssk2 generated mice with reduced male fertility arising due to haploinsufficiency of either allele, and characterized by elongated spermatids, and apoptosis of spermatocytes and spermatids (Xu et al., 2008). Cloning and expression analysis of the human homologues revealed Tssk1 and 2 mRNA to be present exclusively in testes, as also was the substrate TSKS (Hao et al., 2004). Apart from their role in male fertility, very little is known about the function of these proteins including upstream and downstream targets, regulation, or potential implication in tumor function. Tssk1 is also distantly related to the subfamily of AMPK and AMPK related enzymes and contains the conserved T-loop Thr 172 residue. However, it was shown not to be a substrate for LKB1 and instead is believed to be autophosphorylated at its T-loop residue. Detailed examination of the function of Tssk1 has been difficult because of the lack of a functional antibody against the human protein, but it remains a target of interest, precisely because so little is known about it.
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The gene is uncharacterized in Drosophila, and it is still remains to be tested whether it has testis-specific functions in the fly as well.

AMPK is a critical node regulating metabolism and survival through multiple downstream effectors. It is now clear that there are a number of AMPK related family members in humans whose function remains elusive. Many have been shown to be LKB1 substrates (SIK, BRSK), and there is still the possibility that these proteins are components of novel signaling pathways involved in cell survival and proliferation. Expression analysis from human cancer samples can reveal whether these proteins are dysregulated in tumors and whether their dysregulation correlates to Pten loss.

The FGF-receptors constitute a group of 4 differentially expressed receptor tyrosine kinases which are implicated in multiple signaling pathways downstream of FGF signaling. The activation of FGFRs requires receptor dimerization, autophosphorylation, and the assembly of signaling complexes. Pathways activated by FGF include the Pi3K, Ras/Mapk & phospholipase C-gamma. Mutations leading to constitutive FGFR activation, altered dimerization, or altered ligand-binding specificity and affinity are implicated in a variety of congenital disorders and cancer. While FGFR2 mutations are commonly found in endometrial and gastric tumors, FGFR3 is mutated in urothelial carcinomas and multiple myelomas, and FGFR1 mutations have been found in glioblastomas. Apart from mutations, gene amplification of FGFR especially FGFR2 have also been observed in multiple cancer types such as breast, bladder and endometrial cancer (see Grose & Dickson, 2005 for a review). FGFR1 is commonly overexpressed in advanced prostate cancer, although FGFR2 and FGFr3 have not been well studied in this regard (Kwabi-Addo et al., 2004). The cytoplasmic domain
of mammalian FGFR1 contains at least seven tyrosine autophosphorylation sites. Autophosphorylation of two tyrosine residues in the catalytic domain is critical for the kinase activity of FGFR1. Another C-terminal tail tyrosine functions as a binding site for phospholipase C-gamma, and is essential for FGF-induced stimulation of phosphatidylinositol hydrolysis. Analysis of the crystal structure of the tyrosine kinase domain reveals that residues in the activation loop appear to interfere with substrate peptide binding but not with ATP binding, revealing a general autoinhibitory mechanism for receptor tyrosine kinases (Mohammadi, 1996).

*Drosophila* contains two FGFR genes, Breathless and Heartless, mutations in either of which cause lethality due to developmental defects. Heartless (HtL) is the *Drosophila* homolog of FGFR1(DFGFR1). Heartless and Breathless proteins contain, respectively, two and five immunoglobulin-like domains, in the extracellular region, and a split tyrosine kinase domain in the intracellular region. In early embryos, *htl* mRNA expression is specific to mesodermal primordium and invaginated mesodermal cells. At later stages, putative muscle precursor cells and cells in the central nervous system (CNS) express *DFGFR1* (Gisselbrecht *et al*., 1996, Shishido *et al*., 1997). Two genes have been implicated as possible ligands in the fly. These are Pyramus and Thisbe, which are both required for mesoderm development, but their differential expression patterns suggest distinct functions during development (Klingseisen *et al*., 2009).

Heartless was discovered as an synthetic lethal hit with Pten from the primary and secondary screens we performed in Kc167 cells. Interestingly, in 2008, Pollock *et al*., showed that
inhibition of activated FGFR2 in endometrial cancer cells could induce cell death in the context of Pten loss, thereby providing the first synthetic lethal interaction between these two genes in a cancer-specific setting. A study of 116 endometrial cancer samples from patients showed that Pten mutations frequently accompany activating FGFR2 mutations in these samples (77% of the cases). Two different endometrial cancer cell lines carrying hyperactivating FGFR2 mutations were further used to test the effect of shRNA and pharmologically mediated inhibition of FGFR2. In AN3CA cells which exhibit Pten abrogation, FGFR2 inhibition led to increased cell death as measured by Annexin V. The same was true, albeit to a lesser extent, for MFE296 cells which are wildtype for Pten, and this effect occurred via a Pi3K-independent/ErK dependent mechanism. Interestingly pharmacological inhibition using a pan-FGFR inhibitor induced apoptosis selectively in the Pten null cell line but not in Pten wild type cells which instead went into cell cycle arrest, thus showing that in endometrial tumors, targeted inhibition of FGFR2 can be therapeutically beneficial even in the context of Pten loss.

Our putative candidates were subjected to in vivo functional assays. For this purpose we obtained transgenic Fly lines carrying RNAi transgenes against these hits. The Pten RNAi flies were a kind gift from E. Hafen. Loss of function of Pten in mutant clones expressed in Drosophila eye lead to an increase in the size and number of ommatidia (see Introduction). We found that Pten RNAi when expressed tissue-specifically in the posterior wing compartment can cause an overgrowth of the tissue. However the three genes appear to be non-essential during wing and eye development since RNAi against the genes singly does not have any phenotype. This points to the idea that the genes function redundantly in Pten-
independent pathways in regulating growth and proliferation. Loss of the genes concomitantly with Pten leads to a suppression of Pten mediated overgrowth, in an as yet unknown mechanism. It is tempting to speculate on a model wherein Pten inhibits survival and proliferation through multiple downstream effectors including Akt in vivo. In this model, loss of Pten leads not only to Pi3K pathway activation but also to increased dependence on other survival pathways through unidentified signaling mechanisms leading to an imbalance in proliferation. Subsequent inhibition of genes driving such a cascade could restore the balance in proliferation and bring the cells back to wild type conditions. As described previously, in an endometrial cancer model, shown by Byron et al., loss of FGFR2 signaling in the context of Pten loss and constitutive Pi3K activation, can lead to lethality independent of Pi3K signaling. Thus while Pten inactivation can lead to uncontrolled growth on the one hand, it can also sensitize cells to other signaling cascades which can become potential new targets for anticancer treatments. Importantly, we did not observe apoptosis or enhanced growth suppression in the double RNAi flies. This could be a reflection of the differences between an in vivo model and in vitro cell culture. In the organism, lethality may be suppressed because the cell relies on several different homeostatic mechanisms to sustain the balance between survival and apoptosis in vivo. Pten knockdown in Kc cells was not accompanied by a corresponding growth phenotype as seen in flies where Pten mutations are lethal, and effects can only be studied tissue-specifically or with homozygotic clones of cells. This is clearly not the case in cell culture studies, and therefore it will be of great interest to study what kind of effects such interactions may have in mammalian settings.
Negative feedback loops and cross talk of Pi3K signaling with other pathways such as Ras/MAPK may provide a clue as to how such synthetic interactions may be mediated. For example, negative feedback inhibition of the IRS receptor expression in Tsc1/2 deficient cells by phosphorylated S6K is a mechanism to regulate constitutive Akt activity and downstream mTOR mediated growth, thus preventing hyperproliferation in cells. Similarly, FGFR signaling is negatively regulated by a) ubiquitination and proteasomal degradation of the receptor by c-Cbl recruitment in cells where FGF signaling is constitutively activated, and b) Phosphorylation and inhibition of the FGFR docking protein FRS2alpha leading to attenuation of the FGFR signal (Lax et al., 2002; Wong et al. 2002). Interestingly, recruitment of Cbl to the plasma membrane was recently found to attenuate Pi3K activity by proteasomal degradation and lead to apoptosis in osteoblasts where FGFR2 signaling is hyperactivated. In this context, cross talk between FGF signaling and Pi3K signaling regulates the balance between cellular survival and apoptosis (Dufour et al., 2008). Similarly, the Sik genes have also been shown to be involved in energy regulation via CREB and regulate their own activity through feedback regulation, as well as phosphorylation by Akt and PKA (described previously). We found that FGFR2 mRNA levels were upregulated in prostate cancer cells upon shRNA-mediated Pten knockdown (see Results, Figure 5). However we did not observe any change in FGFR1 levels in this setting (data not shown). The multiple isoforms of FGF-receptors in humans (four genes in humans compared to two genes in Drosophila) probably generates a lot of functional redundancy and context specific physiological roles which could account for the effect seen. The benefits of Drosophila as a model system become clear in this regard. Many potential interactions with Pten can be
Theme missed in a mammalian setting due to multiple isoforms and redundancy. This is true for all three genes discovered and validated in our screening. However, this does not exclude dysregulation of one or more of the different isoforms in tumor growth and progression. The exact function of Tssk1, Sik2, and Heartless specifically in a cancer setting is a phenomenon that requires further investigation.

We tried to analyse these functions in DU145 cells as described previously. However, siRNA–mediated transfection was not efficient in these cells. This problem could be addressed using cell lines more amenable to siRNA or lentiviral transfections. The most common cancers carrying Pten inactivation include prostate, endometrium and breast cancer. The endometrial cell lines AN3CA and MFE96 are being used for further experiments with these genes. Future experiments in this direction include knockdown of the genes in presence and absence of Pten and analysis of downstream components in the Pten pathway including phospho-Akt, and Phospho-S6K. Since synthetic lethality in many cases involves cross-talk between different signaling pathways, the role of other signaling regulators such as ERK/Ras/MapK needs to be investigated as well. The mechanistic explanation of how these novel genes lead to synthetic lethality will provide new insights into the control and regulation of survival in the cell.
4. Materials and Methods

4.1. Preparation of the kinase/phosphatase library

The selection of *Drosophila* kinase and phosphatase genes was made in accordance with the list published by Morrison *et al* (J. Cell. Biol., 2000). DNA templates of the selected genes were ordered from the Open Biosystems *Drosophila* dsRNA library and based on the available templates 337 genes were finally selected to form the kinase/phosphatase RNAi library. The templates were then used to synthesize dsRNA using the Ambion High Yield *invitro* transcription kit as per the manufacturer’s protocol. The resultant dsRNAs were purified, quantitated on a nanodrop spectrophotometer, suspended in 500mM NaCl, 10mM Tris Buffer (pH 7.0) and sent for spotting. Concentration of dsRNAs ranged from 50-500 ng/ul. 4µm Rhodamine dye (Sigma) was suspended in a solution of 100 mg/mL poly-D,L-lactic acid in methyl salicylate (Sigma) in was used to mark the borders of the cell array for microscopy.

4.1.1. Preparation of slide arrays

DS8 slides from Erie Electroverre were spotted with the non-contact microdispensing piezoarray from Perkin Elmer from a 384-well plate containing 20 µL of dsRNA solution. All slides were prepared at the Functional Genomics Center in Zurich. Spot-to spot spacing was 750 µm and the average diameter of each spot was 250 µm.
4.1.2. *Drosophila* Cell Culture and maintenance

Embryonic *Drosophila* Kc167 cells were used for all experiments. Cells were cultured at 25°C in Schneider’s medium supplemented with 10% Foetal Calf Serum (Amimed) and 1% Penicillin/Streptomycin.

4.1.3. RNAi Treatment

Cells were serum starved for 4 hours, then incubated in either 20µg of PTEN dsRNA, or an equivalent amount of control GFP dsRNA for 1 hour, followed by replacement with fully supplemented medium. After 24 hours cells were transferred onto spotted DS8 slides placed in a Sterilin petridish, at a density of 1 X10^7 cells and incubated at 25°C for 48 hrs.

4.1.4. Staining and microscopy

The slides were removed from medium and washed in Phosphate Buffered Saline (PBS) containing CaCl₂ and MgCl₂ (henceforth referred to as PBS+). Cells were fixed in 4% PFA in sucrose for 20 minutes. Following fixation, slides were rinsed, and incubated in Hoechst 33342 from Sigma for 10 minutes in PBS+. Slides were then mounted in VectaShield mounting medium, and stored at 4°C until imaging. All images were acquired with the Zeiss Lifecell Microscope at 10 and 20X magnification. Images were acquired at each spot using Slidebook, and scanned manually to look for absence of Hoechst staining. Spots were printed in duplicate on each slide.
4.2. Annexin V Screening

4.2.1. RNAi treatment

Kc167 cells were pretreated with PTEN dsRNA or control GFP dsRNA for 24 hrs (as described previously), and seeded in 96-well plates at a cell density of $0.5 \times 10^5$ per well. 1µg of candidate RNAi was added to each well. After 24 hrs incubation at $25^0$C the cells were stained with Annexin V and imaged live without fixation.

4.2.2. Annexin Staining

For the measurement of apoptosis and cell number in, medium was removed from the wells which were rinsed twice in 1X PBS buffer. Annexin-FITC and Hoechst 33342 were added to each well, at dilutions of 1:500 and 1:1000 respectively in Binding Buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM CaCl$_2$). After incubation for 15 minutes at $25^0$C the staining solution was removed, rinsed once in Binding Buffer and imaged immediately without fixation.

4.2.3. Image Analysis and Quantification

Images were acquired on the BD Pathway 855 screening microscope at 20X magnification. 4 images were acquired per well in a fixed area around the well center. For image analysis, we used the object recognition feature of the Imaris software, to identify and count the number of nuclei in each image. However, this tool did not work as well for identifying FITC positive cells, which were then counted manually for each image. The ratio of Annexin positive cells to the total number of nuclei was calculated, and this ratio was then compared between cells treated with PTEN dsRNA and GFP dsRNA, to obtain the fold change in apoptosis.
4.2.4. Quantitative PCR

qPCR in Kc167 cells was performed as follows: cells were seeded in 6-well plates at 1 X 10^6 cells/ml, incubated with the desired RNAi (singly or in combination) for 48 hrs, washed once with sterile PBS buffer before pelleting the cells and proceeding to lysis and mRNA isolation as per the manufacturer’s protocol (Nucleospin II). 2 µg of mRNA were used for cDNA generation in each case. Primers were self designed and ordered from Microsynth from mRNA sequences annotated in Flybase (see Appendix Table1))

4.3 *Drosophila* maintenance

4.3.1. *Drosophila* stocks

Fly RNAi lines were ordered from the VDRC. Stocks: yw;enGAl4-UASGFP;+/+ (from C.Frei), yw;+/+;PtenRNAi/TM6B (from E. Hafen), yw;GMR-Gal4/Cyo; MKRS/TM6B, yw; actin-5C-Gal4/Cyo-GFP;+/+ (C.Frei). Transgenic flies carrying homozygous UAS-RNAi transgenes were purchased from VDRC. CG7223, CG10177 (Chromosome II insertion), CG14305, CG4290 (Chromosome III insertion). Flies were balanced using double balancer yw; sp/cyo; MKRS/TM6B for subsequent crosses. Genotypes of flies were yw; en-Gal4-UAS-GFP/CG7223; PtenR/+;yw;en-Gal4-UASGFP/+; Pten/CG14305, yw; en-Gal4-UAS GFP/+; PtenR/CG4290, yw, en-Gal4-UAS GFP/CG10177; PtenR/+;yw; GMR-Gal4/CY7223; PtenR/+;yw; GMR-Gal4/CY10177; PtenR/+;yw; GMR-Gal4/+; PtenR/CY14305, yw; GMR-Gal4/+; PtenR/CG4290.
4.3.2. Fly Husbandry
Flies were maintained at 25°C, 60% humidity and a 12 hour day-night light cycle. For larval experiments, 6-day old wandering third instar larvae were used unless otherwise indicated.

4.3.3. Trichome analysis
Adult wings were dissected and placed in an eppendorf tube containing 200 ul of 95% ethanol for 1 hr, after which 800 ul of glycerol was added to the tube. Wings were mounted on glass slides with coverslips sealed with nailpolish.

For trichome ratios, 5 wings were selected per genotype for male and female flies; trichomes were counted over a given area randomly selected from the anterior and posterior part of the wing using Adobe Photoshop, to obtain the average ratio.

4.3.4. BrDU staining
3rd instar larvae were dissected in PBS and incubated in 100 µg BrdU for 60 minutes at 25°C. Larvae were then fixed in Carnoy’s fixative(chloroform:ethanol:acetic acid, 6:3:1) for 30 minutes and washed sequentially in 0.1% Tween 20 in PBS containing 70%, 50% and 30% ethanol respectively. Following this, larvae were treated with 2N HCl for 1 hour, then rinsed in PBST( PBS containing 0.1% Tween 20) for further staining.

4.3.5. Immunofluorescence
Larvae were incubated with primary antibody solution in 10% NGS for 1 hour at 25°C, washed three times in PBST, and incubated with secondary antibodies conjugated to fluorescent Alexa Fluors probes from Molecular Probes at specified concentrations for 40 minutes, followed by incubation in Hoechst 33342 (1µg/mL) for 10 minutes. Larval wing
discs were dissected and mounted in 70% glycerol in PBS on glass slides, and sealed with nailpolish. Slides were visualized on a Zeiss Microscope at 20X magnification, with automatic exposure settings in all cases.

4.3.6. Quantitative PCR

Third instar larvae were used for RNA isolation using the Nucleospin RNA kit from Mackerei Nagel. 5 larvae were taken for each genotype and q-PCR was performed on 2 µg of mRNA per genotype. Normalization was performed using the *Drosophila* rp49 gene encoding ribosomal subunit 5S. For Primer sequences used in PCR see Appendix II.

4.3.7. Electron Microscopy

Adult flies were frozen in dry ice, and visualized on the Jeol Scanning electron microscope. 5 flies were analyzed per genotype, and representative pictures were taken.

4.4. Mammalian Cell Culture

4.4.1. Cell Culture and maintenance

DU145 prostate cancer cells carrying stably transfected lentiviral hairpins were a kind gift from Peter Wild. Cells were maintained in Dulbecco’s Modified Eagle’s Medium from Invitogen supplemented with 10% Foetal Calf Serum (Amimed), 1% Penicillin/Streptomycin, 1% L-Glutamate and 0.5 µg/mL Puromycin as the selection marker, and incubated at 37ºC and 5% CO₂.

4.4.2 Western Blotting and Antibodies

The following antibody concentrations were used:
Materials and Methods

Rat monoclonal anti-BrDU (1:1000) from Oxford Biotechnology
Mouse monoclonal anti-GFP (1:1000) from Roche Applied Sciences
Rabbit monoclonal anti- Pten (1:1000) from Cell Signaling
Rabbit polyclonal anti cleaved-Caspase 3 (1:500) from Cell Signaling,
Rabbit Polyclonal Phospho-Ser505-AKT (1:500) from Cell Signaling.
Goat Cdk2 from Santa Cruz (1:1000)
Rabbit Polyclonal Sik2 (1:200) from Abcam.
FITC-conjugated mouse secondary from Molecular Probes (1:500),
Cy3-conjugated Rat secondary from Molecular Probes (1:1000).
Western Blotting was performed according to standard procedures. 50 ug of total lysate was
loaded in Laemml Buffer on 10% SDS gels in all cases. Primary antibody incubation was
performed overnight at 4°C and secondary antibodies were incubated for 1 hour at room
temperature in all cases. All washes were performed in Tris Buffered Saline buffer containing
1% Tween-20.

4.5. Tissue MicroArray Analysis

4.5.1. Prostate cancer tissue microarray

Tissue microarrays contained a consecutive series (non-selected) of formalin-fixed, paraffin-
embedded, 348 radical prostatectomy specimens, 29 hormone-refractory prostate cancer
samples, 18 lymph node metastases, 28 distant metastases (bone, lung, urinary bladder) and
50 benign prostatic hyperplasia samples. Prostate tissues were from the Institute of Surgical
Pathology, University of Zurich, Zurich, Switzerland. The Phospho-S6 staining was done on a
2nd cancer array made at the University Hospital Zurich, from samples provided by the
Kantospital in St.Gallen and comprised 48 radical prostatectomy specimens and 35 Benign
Prostate Hyperplasia. Before construction of the Zurich tissue microarray, H&E-stained slides of all specimens were re-evaluated by experienced pathologists to identify representative areas. Tumor stage and Gleason score of the Zurich cohort were assigned according to the International Union Against Cancer (UICC) and WHO/ISUP criteria. In total, clinical follow-up data were available for 637 of 678 prostatectomy patients (94%) with a median follow-up period of 72 months (range, 0-167 months). The local scientific ethics committee approved the study.

4.5.2. Immunohistochemistry

The expression of Sik2 was analyzed immunohistochemically by using tissue microarrays (TMAs). The following primary antibody was used: anti-Sik2 (rabbit polyclonal, Abcam, dilution 1:100), Phospho-S6 (1:50, Cell Signaling, dilution 1:50). For staining the ChemMate detection kit (DAKO, Glostrup, Denmark) was used. After antigen retrieval (microwave oven for 10 min at 250 W) immunohistochemistry was carried out in a NEXES immunostainer (Ventana, Tucson, AZ) following manufacturer’s instructions. For negative controls, the primary antibody was omitted. Blind evaluation of the slides for Sik2 without knowledge of clinical data was carried out by two independent researchers. Causes of non-interpretable results included lack of target tissue, presence of necrosis or crush artifact.

4.5.3. Statistical analysis of Array

SPSS version 17.0 (SPSS, Chicago, IL, USA) was used for statistical analyses. P-values <0.05 were considered significant. Contingency table analysis, Chi-square and two-sided Fisher’s exact tests were used to study statistical associations between clinicopathological and immunohistochemical data. For the comparison of two independent samples the non-
parametric Mann-Whitney test was calculated. Time to PSA recurrence (cutoff ≥ 0.1 ng/ml) was selected as end point. Only patients with primary prostate cancer undergoing radical prostatectomy (RPE) were used for survival analysis. Recurrence-free survival (RFS) curves were calculated by the Kaplan-Meier method with significance evaluated by two-sided log-rank statistics. Patients were censored at the time of their last tumor-free clinical follow-up visit. Patients not reaching PSA nadir (<0.1 ng/ml) postoperatively were excluded.
5. Appendix

5.1. Primer sequences used for q-RT-PCR

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer(5’-3’)</th>
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<tr>
<td>CG14305</td>
<td>CATGATGAATGCCAAGATGC</td>
<td>TAGATTCCAGCGAGCATGTG</td>
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<td>CG7223</td>
<td>CTGACGATGGAGGATCTGGT</td>
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<td>CG10177</td>
<td>AGTCCATGAAAATCCACGAC</td>
<td>TGTCGCAGCACCTCTGCCC</td>
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<td>DPten</td>
<td>GACGAGGCATAGCGTGATGTA</td>
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<td>DRp-49</td>
<td>CAAGAAGTTCCTGGTCGACAA</td>
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Table 1: All primers were self-designed from mRNA coding sequences available from Flybase. Primers for hFGF1, hFGFR2, and hTssk1, and h18S were ordered from the predesigned sequences available in Primerbank.

5.2. Primers used for dsRNA synthesis in candidate genes:

<table>
<thead>
<tr>
<th>Gene ID</th>
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Table 2: All primers sequences are from The Open Biosystems Drosophila RNAi database used for screening. The complete list of sequences used for dsRNA synthesis and screening is available from [http://www.dkfz.de/signaling2/rnai/index.php](http://www.dkfz.de/signaling2/rnai/index.php). The T7 RNA polymerase extension tag was appended to all sequences for dsRNA synthesis.
5.3 List of candidate genes selected from the primary screening

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<tr>
<td>CG7838</td>
<td>H6</td>
<td>Bab1</td>
</tr>
</tbody>
</table>
Table 3: List of 55 candidate genes with their *Drosophila* gene Id and position of 96-well master plate for Annexin screening. *Drosophila* gene names are indicated where applicable. Asterisks indicate unannotated genes which were analysed for the closest human homologue using BLAST.

5.4. Transcript levels of Htl, Tssk1 and Sik2 remain unchanged upon Pten knockdown in Kc167 cells

![Graph showing fold change in mRNA levels for Pten, Htl, Tssk1, and Sik2](image)

Figure 21. Pten downregulation in Kc167 cells does not alter expression of Htl, Tssk1, and Sik2. qPCR of indicated genes in the presence of Pten RNAi. Fold change was calculated with respect to GFP RNAi in all cases; experiments were performed in triplicate.
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