PhD student recruitment

at the Ludwig Cancer Institute at the Karolinska Institute in Stockholm, Sweden starting in fall 2008 or January 2009. We are seeking for highly motivated and enthusiastic students to work on the molecular and cellular basis in tumor biology. The primary goal of the work is to understand the molecular basis of neuronal cell death that is deregulated in cancer that originated from neuronal precursors.

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PhD projects: Oxygen sensing and Cancer

Lay term abstract:

Neuroblastomas arise from the same primitive cells that give rise to parts of the nervous system (hence 'Neuro'). These primitive cells are referred to as 'neural crest'. Some families are at high risk for developing neural crest derived tumors because they have mutations (alterations) in specific genes. We recently discovered that the genes that, when mutated in some of these nervous system tumors all play essential roles in determining whether neural crest cells live or die. In particular, when these genes are mutated neural crest cells *that should have died as part of the normal development of the fetus* escape their death sentence and go on to cause tumor development later in life. We also showed that a gene called 'EglN3' plays a critical role in this setting. EglN3 promotes the death of neural crest cells whereas inhibiting EglN3 function has the opposite effect. Interestingly, EglN3 belongs to the family of prolyl hydroxylases that require molecular oxygen to perform its catalytic activity and therefore is termed as an oxygen sensor.

Future work will continue to elucidate the underlying basis that is common in the development of tumors arising from the neural crest. More specifically, we are

interested in how, mechanistically, EglN3 regulates KIF1Bβ, and how this translates into cell death. The identification of oxygen-dependent enzymes such as EglN3, which are involved in developmental apoptosis and linked to tumor development when disrupted, remains a fertile ground primed for future explorations. This line of investigation not only awakens questions as to why neurogenesis contains an oxygen-sensing mechanism in EglN3, but also inspires possible strategies to target cancer cells that are highly dependent on their pseudo-hypoxic environment to escape from apoptosis.

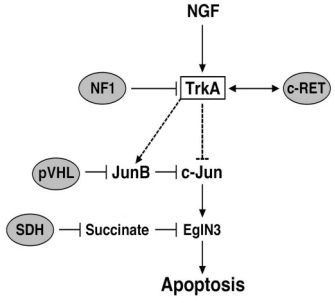
Introduction

Aerobic organisms require systems to ensure adequate cellular oxygenation. Decreased oxygen tension in tissue (hypoxia) is detected rapidly at the cellular level and affects a range of response pathways. Recent research has demonstrated that oxygen-sensing can be mediated via prolyl hydroxylation by a family of enzymes called EglN1, 2, and 3.

Prolyl hydroxylation by EglNs is a critical posttranslational modification that executes an oxygen-sensing pathway, the activity of which strictly depends on the molecular availability of oxygen (referred to as dioxygenase). Prolyl hydroxylation is not only required for adaptation to low oxygen environments, but also critical for normal development and apoptosis. For instance, the prolyl hydroxylase EglN1 is a critical regulator of the transcription factor HIF (hypoxia-inducible factor) that transactivates a variety of genes implicated in angiogenesis and energy metabolism, whereas EglN3 plays an important role in triggering apoptosis in neural precursors during development. Developmental apoptosis of neuronal precursors is important for determining the final number of terminally differentiated cells, and deregulation of this process is implicated in disease. During neural development, cells undergo apoptosis as growth factors such as nerve growth factor (NGF) become limiting. Abnormal NGF signaling has been linked to pediatric nervous system tumors such as neuroblastoma and medulloblastoma (Katsetos et al 2003, Nakagawara, 2001). Neuroblastomas arise from the neural crest, which gives rise to the peripheral nervous system and specialized cells found in many other organs such as the skin, heart, adrenal gland and gastrointestinal tract. Disease-associated mutations have been shown to enhance signaling by NGF receptors and promote neuronal survival.

Past Research Accomplishments

My past research has been driven by the hypothesis that failure of neuronal apoptosis during early development causes predisposition to certain benign and malignant tumors including neuroblastoma, neurofibrosarcomas, pheochromocytoma, islet cell tumors, medullary thyroid carcinomas, and familial melanoma, all of which arise from the neural crest. We recently discovered that known familial pheochromocytoma/ paraganglioma genes (*c-Ret, NF1, VHL,* and *SDH)* may act in a common pathway that is important during neuronal development. NF1 has been reported to act as a RAS-GAP for the NGF receptor TrkA, and we showed that activating c-RET mutations also stimulates NGF signaling, probably through crosstalk with TrkA. As a result, loss-of-function NF1 mutations or gain-of-function c-Ret mutations promote neuronal survival after NGF withdrawal. EglN3, a paralog of the HIF prolyl hydroxylase EglN1, is induced in sympathetic neurons after NGF withdrawal and



provokes apoptosis when over-expressed. Earlier studies showed that c-Jun is a critical mediator of apoptosis when cells are starved of NGF. We found that EglN3 acts downstream of c-Jun and is necessary for apoptosis when NGF is limiting. 2-oxoglutarate-dependent dioxygenases such as EglN3 produce succinate as an end-product and we demonstrated that EglN3 can be product-inhibited by succinate. We confirmed that inactivation of SDH (succinate dehydrogenase) inhibits neuronal apoptosis by blocking EglN3 activity through the abnormal accumulation of succinate. In summary, all the germline genetic lesions associated with the neural crest-derived tumor pheochromocytoma/paraganglioma are proposed to act on a common pathway that impinges upon EglN3-mediated apoptosis.

We have shown that EglN3 can induce apoptosis in a variety of neural crest-derived tumors, including neuroblastomas. To understand the mechanism by which EglN3 causes neuronal cell death, We performed an unbiased genome-wide shRNA screen to identify genes that suppress EglN3–induced neuronal apoptosis. This led to the identification of the kinesin KIF1Bβ as a downstream effector of EglN3. KIF1Bβ is both necessary and sufficient for neuronal apoptosis when NGF becomes limiting. KIF1Bβ maps to 1p36.2, a region of the genome that is frequently deleted in neural crest-derived tumors including neuroblastomas. Sequencing a large set of neuroblastomas and pheochromocytomas led to the identification of loss-of-function KIF1Bβ missense mutations, arguing that KIF1Bβ is a pathogenic target of these deletions. This work assigns a biological significance to EglN3-mediated neuronal apoptosis during development that is defective in certain neural crest-derived tumors. This may lead to the identification of the long-sought-after 1p neuroblastoma genes. This information could, in time, help in the design of therapies which would reactivate the apoptotic program in neuroblastomas.

Future PhD projects:

The recent work on EglN3- and KIF1Bβ-mediated neuronal apoptosis points to interesting questions regarding the development of tumors arising from the same cellular origin and the implication of oxygen-sensing pathways during development or in the adult in various diseases. The heart of future PhD projects will be:

1. *To understand how, mechanistically, EglN3 regulates KIF1Bβ, and how this translates into cell death.*
2. *To investigate whether KIF1Bβ is a tumor suppressor* in vivo.
3. *To ask if EglN3 can be pharmacologically reactivated in tumors that developed due to failure of EglN3 induced apoptosis.*

1. ***To understand how, mechanistically, EglN3 regulates KIF1Bβ and how this translates into cell death.***

We demonstrated that EglN3 and KIF1Bβ belong to the same neuronal apoptotic pathway, and that KIF1Bβ acts downstream of EglN3. Moreover, the chromosomal location of KIF1Bβ and my preliminary mutational data make KIF1Bβ a strong candidate to be a or the neuroblastoma gene on 1p36. Therefore, the aim is to understand how EglN3 regulates KIF1Bβ and how, mechanistically, KIF1Bβ induces apoptosis. This might identify other proteins linked to developmental apoptosis and cancer and also point to novel targets for therapeutic intervention.

Our recent findings show that KIF1Bβ is required for EglN3-induced apoptosis and is necessary and sufficient for apoptosis in developing neurons that are deprived of NGF. KIF1Bβ, which contains 1770 amino residues, is a member of the

kinesin 3 family. Kinesins are microtubule-dependent motors involved in the transport of organelles, vesicles, and protein or RNA. The KIF1Bβ motor domain is located within its N-terminus (residues 1-600) and its C-terminal region contains the cargo-binding domain. Preliminary data show that the KIF1Bβ cargo-binding region is sufficient to induce apoptosis. We plan to identify KIF1Bβ cargos by screening for associated proteins specific to the cargo domain in cell culture or yeast 2-hybrid systems.

Since EglN3 hydroxylase activity is required for KIF1Bβ induction and apoptosis, we hypothesize that EglN3 directly hydroxylates KIF1Bβ or perhaps a protein that, in turn, regulates KIF1Bβ accumulation. In the former scenario I would envision that hydroxylation of KIF1Bβ enhances its stability. Both genetic and biochemical approaches will be used to investigate if KIF1Bβ or its cargos are direct hydroxylation substrates of EglN3.

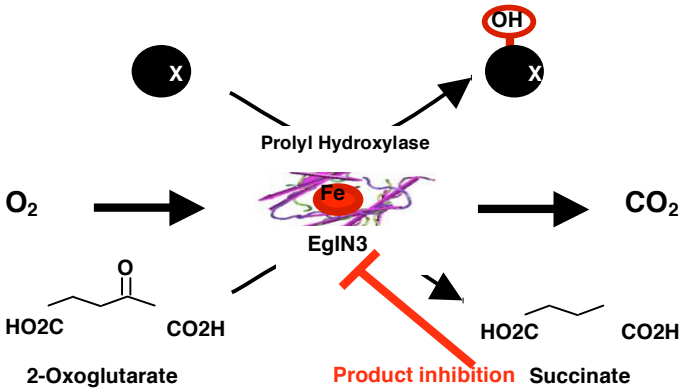
Further, a genetic approach will be used to identify shRNAs that suppress KIF1Bβ-induced neuronal apoptosis. RNA interference (RNAi) is a powerful tool to perform large scale loss-of-function screens. Our collaborators Rene Benards and Daniel Peeper have recently developed expression vectors to direct the synthesis of short hairpin RNAs (shRNAs) that are processed into short interfering RNA (siRNA) *in vivo* to stably suppress gene expression. A loss-of-function screen similar to that used with EglN3 (past research accomplishments) will be performed using the KIF1Bβ-Adenovirus (obtained from Akira Nakagawara, Japan).

2. *To investigate whether KIF1Bβ is a tumor suppressor* in vivo.

Neuroblastomas, like pheochromocytomas, are neural crest-derived tumors and frequently harbor deletions of chromosome 1p36 encompassing the KIF1B locus. The existence of multiple human tumor-suppressor genes on chromosome 1p has been suspected for decades. Our data strongly suggest that one such tumor suppressor gene is KIF1Bβ, and that this gene is especially relevant to certain tumors of neuronal origin. Nonetheless, others and we have observed that the remaining KIF1Bβ allele in 1p deleted tumors and cell lines is often wild-type, contrary to the Knudson 2-Hit scenario *(*Akira Nakagawara-personal communication). Moreover, the somatic medulloblastoma mutation we identified was not associated with loss of the remaining wild-type allele. Perhaps KIF1Bβ is haploinsufficient for tumor suppression in some contexts, especially when combined with loss of other contiguous 1p genes such as CHD5. Supporting this, we noted substantial protection against apoptosis with an shRNA that decreased KIF1Bβ levels by ~50%.

To investigate the nature of KIF1Bβ tumor suppressor capacity *in vivo*, we plan to inactivate this gene in model organisms to see if this promotes neuroblast survival and neuroblastoma development, either alone or in cooperation with other genes such as Myc-N. We already obtained the KIF1B transgenic mouse strain specifically targeting the KIF1B-beta form. The use of the KIF1Bβ knockout will help to further characterize the tumor suppressor role of KIF1Bβ in nervous system tissues. The KIF1Bβ mouse in cooperation with other transgenes such as N-Myc, might help to mirror neuroblastoma development.

3. ***To ask if EglN3 can be pharmacologically reactivated in tumors that developed due to failure of EglN3-induced apoptosis.***



Our recent findings provide mechanistic links between SDH mutations, EglN3 activity, and escape from neuronal apoptosis. We demonstrated that EglN3 activity can be inhibited by increased intracellular succinate levels in SDH deficient cells. Lack of SDH activity in the Krebs cycle causes accumulation of its substrate succinate.

Succinate, as the end-product of the 2-oxogluterate-dependent enzyme EglN3, can product-inhibit the EglN3 reaction. We demonstrated that succinate can product-inhibit EglN3 activity *in vitro* and *in vivo* and, importantly, its activity can be restored by addition of its substrate 2-oxogluterate by neutralizing the product-inhibition. SDHB and D have been reported to be familial pheochromocytoma tumor suppressors, and it is likely that they will also be |implicated in other familial neural crest-derived tumors like neuroblastoma.

Interestingly, cancer cells generate the majority of ATP from aerobic glycolysis and have reduced rates of oxidative phosphorylation. The high lactate production by cancer cells in the presence of oxygen (aerobic glycolysis) was first noted by Otto Warburg in 1924. As where healthy cells generate the majority of ATP through oxidative phosphorylation somewhere between 30–36 ATPs, glycolysis supplies only 2 ATP. The conundrum why cancer cells preferentially use aerobic glycolysis as the less efficient ATP source is still largely unknown. We propose a mechanism in which cancer cells benefit from aerobic glycolysis by decreasing the Krebs cycle metabolite 2-oxogluterate to enhance resistance to mitochondrial apoptosis. 2-oxogluterate is an important co-factor required for a variety of enzymatic reaction of demethylases and hydroxylases, including the EglN-prolyl hydroxylases (PHD's). Limitation in 2-oxogluterate subsequently inhibits their enzymatic activity. We hypothesized that cancer cells could benefit from decreased EglN hydroxylase activity by suppression of the EglN3 mediated apoptotic program through mechanisms that directly lead to the Warburg phenotype.

Addition of cell permeable 2-oxogluterate to cancer cells that are limited in 2-oxogluterate might restore EglN3 activity and trigger them to die. This information might help us to design therapies that would reactivate the apoptotic program in neuroblastomas.

Long term perspective:

How inactivation of mitochondrial enzymes like SDH can have an impact in tumor development has exciting implications. It was first indicated by the German scientist Otto Warburg in 1924 that cancer cells are highly glycolytic and predominantly generate ATP from glycolysis (referred to as Warburg effect). For a long time it was believed that this was due to the hypoxic environment of cancer cells; however, a more attractive hypothesis is that the cancer cells favor pseudo-hypoxia in order to

escape apoptosis or oncogene-induced senescence. This would explain why diffuse cancer cells that do not grow in solid tumors still appear to be pseudo-hypoxic regardless of the availability of oxygen. Our recent work showed how inhibition of mitochondrial enzymes can protect from neuronal apoptosis. Moreover, in preliminary data we have also observed how oncogene-dependent premature senescence can be reversed when cells are forced into glycolysis or a hypoxic environment. The identification of oxygen-dependent enzymes that are involved in developmental apoptosis and linked to tumor development when disrupted remains a fertile ground primed for future explorations. This line of investigation not only awakens questions as to why neurogenesis contains an oxygen-sensing mechanism such as EglN3, but also inspires possible strategies to target cancer cells that are highly dependent on their pseudo-hypoxic environment to escape from apoptosis or senescence.

Relevant Publications:

Schlisio S, Kenchappa RS, Vredeveld LCW, George RE, Stewart R, Shahriari K , Greulich H, Nguyen VN, Dahia PL, Pomeroy S, Maris JM, Look TA, Meyerson M, Peeper DS, Carter BD and Kaelin WG. The Kinesin Gene KIF1Bβ Acts Downstream of EglN3 to Induce Apoptosis and is a Potential 1p36 Tumor Suppressor. Genes Dev. 2008 Apr 1;22(7):884-93

Lee S, Nakamura E, Wei W, Linggi MS, Sajan MP, Farese RV, Freeman RS, Carter BD, Kaelin WG and Schlisio S. (2005) Neuronal Apoptosis linked to EglN3 Prolyl Hydroxylase and Familial Pheochromocytoma Genes. Developmental Culling and Cancer. Cancer Cell, August 2005, Vol. 8.

Young AP, Schlisio S, Minamishima YA, Zhang Q, Li L, Grisanzio C, Signoretti S and Kaelin WG. VHL loss actuates a HIF-independent senescence programme mediated by Rb and p400. Nat Cell Biol. 2008 Feb 24;

Yang H, Minamishima YA, Yan Q, Schlisio S, Ebert BL, Zhang X, Zhang L, Kim WY, Olumi AF, Kaelin WG Jr. (2007) pVHL Acts as an Adaptor to Promote the Inhibitory Phosphorylation of the NF-kappaB Agonist Card9 by CK2. Mol Cell. 2007 Oct 12;28(1):15-27.

Wei W, Jin J, Schlisio S. Harper JW, and Kaelin WG. (2005) The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. Cancer Cell, 8(1): 25-33.

Patents: http://www.wipo.int/pctdb/en/wo.jsp?wo=2007009044
U.S. Patent Application Number: 60/698,879
Entitled: “Treatment of Neuronal Disorders with Inhibitors of EglN3”
Inventors: William G. Kaelin, Susanne Schlisio, Archana Bommi-Reddy
Country: USA
Filing Date: July 13, 2005
DFCI Reference Number: 101601