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PRINCIPAL INVESTIGATOR: Erica McCoy

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Aurora, CO 80045

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14. ABSTRACT Homeobox transcription factor Six1 has been associated with tumorigenesis and metastasis in a number of organ systems and has been implicated in proliferation, survival, and migration during normal development. Our research is aimed at utilizing mouse models to understand the role of Six1 in the onset and progression of breast cancer. Most significantly, we have determined that Six1 is sufficient to induce tumor formation in the mammary glands of mice genetically engineered to inducibly overexpress the gene. The latency for tumor formation is between 12-21 months and the tumors that arise in these animals are very aggressive and have morphological features of an epithelial to mesenchymal transition (EMT), a phenomenon that has recently been suggested to contribute to metastasis. Further molecular analysis of these tumors has revealed a possible role for Six1 in initiating Wnt signaling, a pathway implicated in maintaining EMT and a stem cell fate that may contribute to tumorigenesis. Additionally, we have discovered that our inducible mouse model allows for leaky transcription of Six1 in the uninduced state. Interestingly, animals from this group acquire tumors at an increased frequency compared to those animals that are induced to express Six1, suggesting that even low levels of Six1 are capable, and may even be more efficient at initiating tumorigenesis compared to higher Six1 levels. These compelling results in combination with similar findings in our experiments involving Six1 in combination with PyMT have led to more studies to dissect the role of Six1 levels and its cofactors in breast cancer initiation and progression.					
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INTRODUCTION:

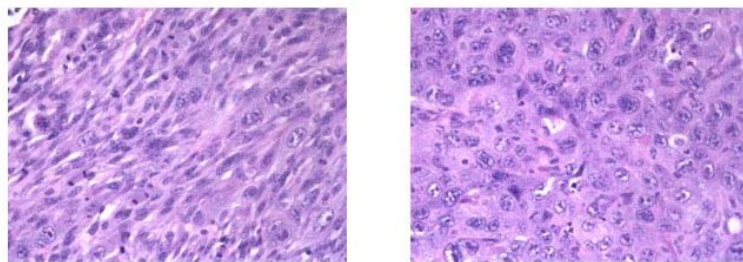
Homeobox transcription factor Six1 is a critical mediator of embryonic development, where it stimulates proliferation and survival of progenitor populations as well as migration of cells (1,2). Interestingly, Six1 expression is often found to be reinitiated in tumors where evidence suggests it stimulates proliferation and survival, and where we have most recently shown that it induces an epithelial to mesenchymal transition (EMT) in tumor cells. Together, these properties of Six1 may result in tumor progression and metastatic disease in tumors that overexpress the gene. Six1 overexpression is documented in a number of tumor types, including ovarian cancer, hepatocellular carcinoma, Wilms' tumor, rhabdomyosarcomas and breast cancer (2-7). Our research is aimed at utilizing mouse models to understand its role in the onset and progression of breast cancer. We are currently using an inducible mouse model to overexpress Six1 in differentiated cells of the mammary gland to determine if Six1 is sufficient for inducing tumor formation. Additionally, we are using a retroviral transplant model to overexpress Six1 in mammary progenitor cells to see if the gene is able to drive tumor formation differently if present in a less differentiated cell type. Future experiments will involve determining the downstream mediators that are responsible for Six1's role in the initiation and progression of human breast cancer.

BODY:

Aim 1: Determine the role of Six1 overexpression in mammary gland tumorigenesis. (Months 1-24)

(A) Evaluate the consequence of chronic Six1 overexpression in mammary gland epithelial cells using a tetracycline inducible mouse model (months 1-18).

Our progress for this aim has been substantial. Long term induction of Six1 expression (TOSIX bitransgenic animals overexpress the gene upon treatment with doxycycline) results in mammary tumor formation in a subset of animals after approximately 12-21 months. At this point, we have completed these studies and have adequate animal numbers to reach statistical significance. We have discovered that there is leak in our inducible system, such that we are getting low levels of Six1 expression in mammary glands taken from uninduced animals. Interestingly, tumors actually arise more frequently in these animals compared to those induced to express Six1, suggesting that there may be some dose-dependency to Six1's ability to initiate tumorigenesis. These tumors have been characterized by our collaborating pathologist, who determined that



Sarcomatoid- spindle cells

epithelioid

Fig. 1. Six1 induces an EMT in tumors in vivo. Two histological sections (40x) from a single tumor arising in a TOSIX1 mouse induced with Dox. The tumor has regions that are both sarcomatoid (containing many spindle cells) and epithelioid, demonstrating that regions of the tumor are undergoing EMT in vivo. Animal sacrificed 18 months after Dox induction.

they are high grade adenocarcinomas with features of EMT, including sarcomatoid regions and loss of E-cadherin expression (a marker of epithelial cells). (figures 1 and 2). Additionally, two animals presented with overt lung metastasis and studies are currently underway to identify if more of these animals have micrometastasis to the lung. These results further suggest that Six1 is not only capable of initiating tumorigenesis, but is also capable of directing its progression. Further analysis of molecular markers reveals the expression of nuclear β -catenin in these tumors, suggesting that Six1 may initiate the Wnt signaling pathway. In addition to nuclear β -catenin, we see nuclear slug, a transcription factor that is known to induce EMT. Finally, we see

cytokeratin 6 positive cells (a putative marker of mammary progenitor cells) and mixed luminal epithelial (cytokeratin 18 positive) and myoepithelial (cytokeratin 5 positive) populations, characteristics of tumors arising from mammary progenitor cells (10). Further analysis is underway to confirm this hypothesis and further characterize Six1's influence on Wnt, an important developmental pathway that is proven an important mediator of tumorigenesis, perhaps through its role in the maintenance of mammary stem cells.

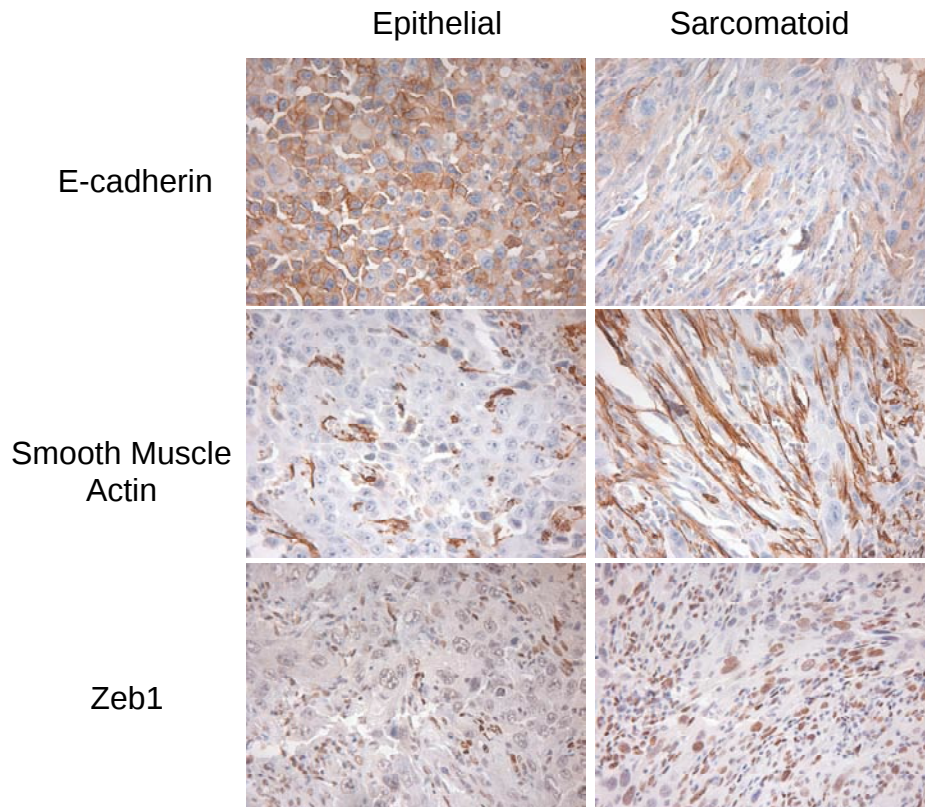


Fig. 2. Analysis of TOSIX tumor provides evidence of EMT. Epithelial regions express E-cadherin (epithelial marker), while the sarcomatoid regions express smooth muscle actin and nuclear Zeb1 (mesenchymal markers).

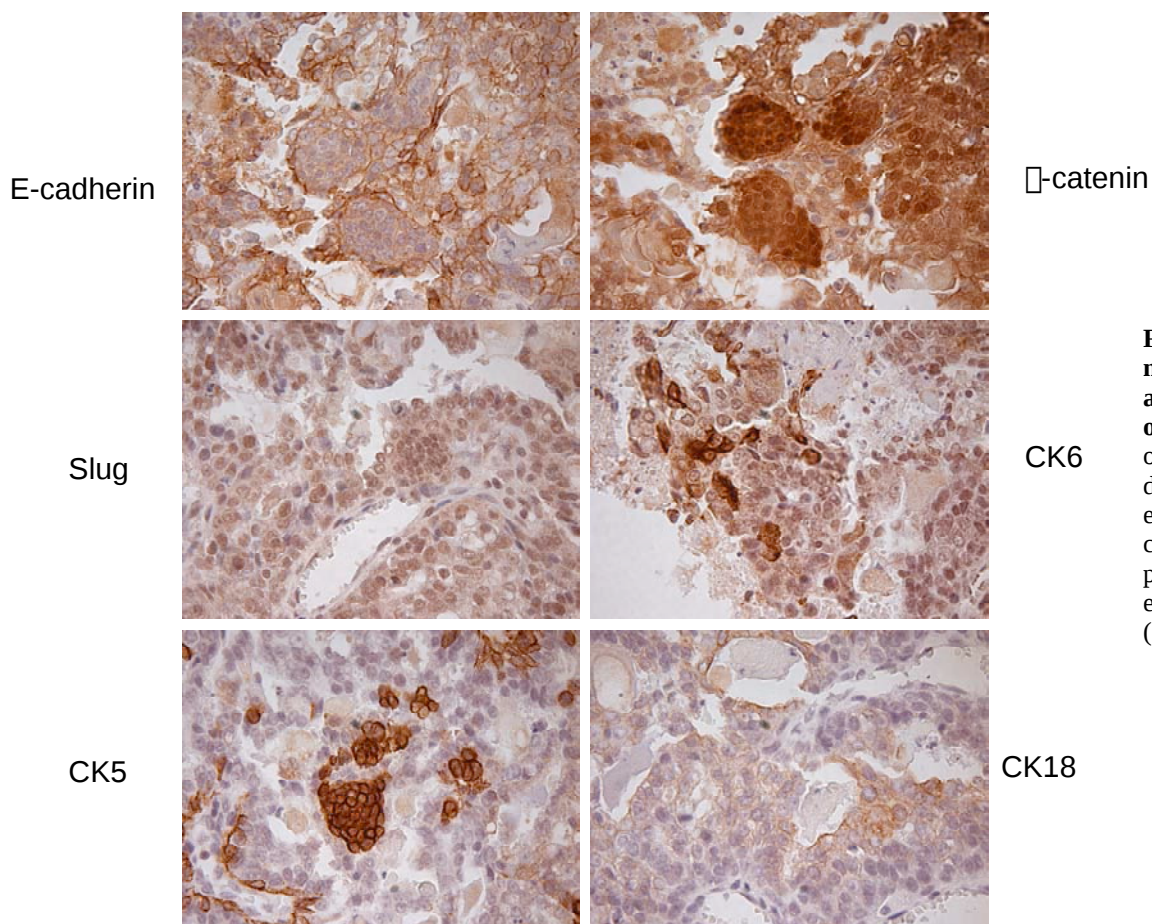


Fig. 3. TOSix tumors express molecular markers of Wnt activation and progenitor cell origin. Immunohistochemistry of serial sections of tumor demonstrating concurrent loss of e-cadherin, gain of nuclear β -catenin, gain of nuclear Slug, presence of CK6 and mixed expression of CK5 and CK18. (60X).

In addition to the tumor phenotype, we observe a hyperplastic phenotype in the mammary glands of animals induced to overexpress Six1 (previously described in 2007 report).

(B) Determine the effect of retroviral-mediated Six1 overexpression in mammary gland progenitor cells (months 1-18).

In the last year, we have been using a retroviral transduction method to overexpress Six1 in the mammary gland in combination with the polyoma middle T antigen (PyMT), a potent oncogene, to determine if Six1's expression is able to shorten tumor latency or increase PyMT-mediated lung metastasis. In order to do this, we have harvested primary mammary epithelial cells from wildtype animals and then retrovirally transduced them with Six1 and PyMT tagged with GFP (controls including Six1 alone or PyMT alone are included). These cells are then transplanted into the cleared fat pad of three week old recipient wildtype mice. Progenitor cells within the population will give rise to a mammary ductal network that will express Six1 along with PyMT.

PyMT is known to give rise to tumors with a very short latency in this model (approximately 3 months following transplant), and consistently leads to lung metastasis when tumors grow to a large size, or when the primary tumor is removed (9). In our original study, we obtained an infection efficiency of approximately 15%. In this study Six1 did not affect primary tumor latency or growth, but led to a significant increase in the frequency of lung metastasis, as well as an increase in metastatic burden. In a second experiment to confirm these results, we obtained a much higher infection efficiency of approximately 40%. Interestingly, in this experiment, the expression of Six1 prevented tumor formation and appears to have initiated mammary differentiation (table 1 and figure 3).

Table 1

Approximate Infection Efficiency	Phenotype	Cells Transplanted	Number
15%	Metastasis	PyMT	1/5
		PyMT + Six1	6/7
40%	Tumor	PyMT	9/10
		PyMT+Six1	2/10

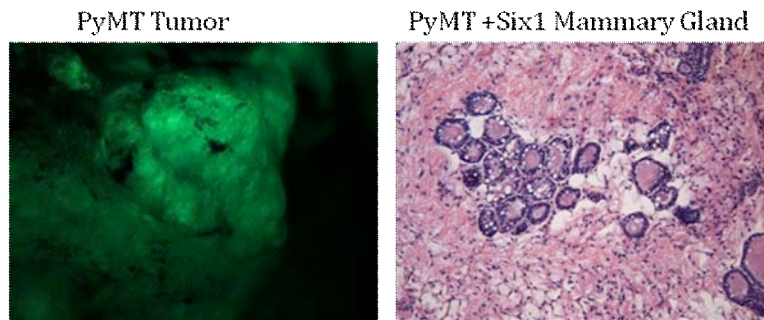


Fig. 4. PyMT with Six1 at high infection efficiency leads to mammary differentiation. Fluorescent image of tumor from animal injected with mammary cells transduced with PyMT alone (3 months after injection) (left). H&E of mammary gland from animal injected with mammary cells transduced with Six1 and PyMT (4 months after injection) showing differentiation (right).

These results suggest that perhaps, as already mentioned above, *SIX1* levels may be critical in mediating its effects on tumorigenesis. At this time, we are planning experiments to titrate the Six1 retrovirus and determine whether there is an optimal level of Six1 expression for its pro-tumorigenic role, and that, perhaps at higher levels, it is actually anti-tumorigenic. Other cases for oncogene dose-dependency have been cited in the literature involving Ras, as well as Oct3/4 (11, 12). We believe that carefully dissecting the importance of Six1 expression levels is critical for understanding its role in human breast cancer and for developing therapies for appropriately targeting its function.

Our studies involving combining Six1 with a dominant-negative form of p53 have not resulted in any tumors, suggesting that Six1 is not collaborating with p53 to lead to mammary tumorigenesis, at least in this model.

(C) Determine if mammary gland tumorigenesis (if observed) or additional phenotypes are dependent upon Six1 for maintenance using the inducible and retroviral overexpression models outlined in aims 1a and 1b (months 18-24).

At this time, due to the discovery that this inducible system allows for leaky expression of Six1 that is sufficient for inducing a phenotype, we do not believe that the completion of this aim will give us interpretable data.

Aim 2: Examine the dependency of Six1 on cyclin A1 for mammary gland proliferation and tumorigenesis. (Months 18-36)

As the tumor latency with Six1 overexpression is long, we have not begun the experiments associated with this aim where we will cross TOSIX1 mice with Cyclin A1 knockout mice to determine whether cyclin A1 is required for Six1-mediated tumorigenesis. We are generating MMTV-Six1 transgenic mice so that this experiment can be done more easily, without the necessity of bitransgenic lines and doxycycline treatment.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified that long term induction of Six1 expression in an inducible mouse model leads to hyperplasia as well as aggressive tumor formation that progresses to lung metastasis.
- Characterized the tumors to identify a Six1-induced epithelial-to-mesenchymal transition, as well as the presence of active Wnt signaling using molecular markers.
- Identified that the tumors also show markers of having arisen from more progenitor like cells, including keratin 6 expression, and mixed markers of myoepithelial and luminal epithelial lineages (keratin 5 and 18, respectively).
- Identified leaky expression in the inducible model that results in low levels of Six1 expression in the uninduced mammary glands, leading to increased tumor frequency, suggesting a Six1 dose-dependency.
- Noted that Six1 does not affect tumor latency or growth when combined with the PyMT oncogene.
- Discovered that at low infection efficiency, Six1 is able to enhance PyMT-mediated lung metastasis and that at high efficiency, Six1 is able to prevent PyMT-mediated tumorigenesis and may induce differentiation.
- Determined that Six1 does not collaborate with p53DN to initiate tumorigenesis in this model.

REPORTABLE OUTCOMES:

Research presented in the form of a short talk (invited based on abstract submission) and poster at the Gordon Conference for Mammary Gland Biology in Newport, RI, June 2007. See appendix for poster/talk abstracts. Additionally, research will be presented at the Keystone Meeting for “Signaling Pathways in Development and Cancer” in Steamboat Springs, CO, March 24-29, 2007 in the form of a poster, where I was selected for a Keystone Symposia travel award to attend the meeting, and will also be presented at the Era of Hope meeting in Baltimore, MD this June (also in the form of a poster).

Grants that resulted from this work include a grant from the American Cancer Society entitled “The Role of Six1 in EMT and Tumor Progression”. (\$150,000 per year direct costs/ for 4 years. The grant runs from 5/1/07-4/30/11 and 2R01-CA095277 -06 (Ford), “*The role of Six1 in EMT and Tumor Progression*” 9/29/2007 – 9/28/2012 from NIH/NCI (\$168,429 in directs, all overlap removed and budget reduced to avoid overlap with ACS grant).

CONCLUSIONS:

The results from studies completed thus far suggest that Six1 is capable of initiating mammary tumorigenesis. The tumors that arise in animals induced to express Six1 are very aggressive adenocarcinomas displaying high-grade regions with features of EMT and lead to lung metastasis in a subset of cases. Our research suggests that Wnt may be activated in these tumors and these molecular mechanisms are currently being studied. As EMT, as well as Wnt signaling, have been implicated in tumor progression, these results suggest that Six1 is an oncogene not only capable of initiating mammary tumor onset, but is capable of initiating the first stages of aggressive tumor progression. These results are quite compelling, given the clinical data that Six1 is overexpressed in 50% of primary breast cancers and 90% of metastatic lesions (1,2). Our ultimate goal is to identify Six1 as a legitimate therapeutic target. As Six1 expression is primarily expressed only in embryogenesis, lost in the adult, and re-expressed in cancers, targeting Six1 in a clinical setting may successfully treat cancer while avoiding damage to normal adult tissues, thus limiting side-effects. The importance of understanding a possible Six1 dose-dependency is critical for our future attempts to control Six1 as a cancer therapy.

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APPENDICIES:

Poster/talk abstract presented at the Gordon Conference on Mammary Gland Biology for June, 2007 (selected for short talk based on the submission of this abstract):

***In Vivo* Role of the Six1 Homeoprotein in Mammary Gland Tumorigenesis**

E.M. McCoy¹, N. Abbey², P. Jedlicka³, L. Chodosh⁴, H.L. Ford^{1,2}, ¹Program in Molecular Biology, ²Department of Obstetrics and Gynecology, ³Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado, USA ⁴Department of Cancer Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

Human Six1 is a homeodomain-containing transcription factor that is critical for cell proliferation, survival, and epithelial to mesenchymal transition (EMT) during normal development. In addition to its developmental role, overexpression of Six1 has been detected in a number of human cancers, including breast cancer, where it is linked to both proliferation and metastasis. As many as 50% of primary breast cancers and 90% of metastatic lesions overexpress the gene, in part due to gene amplification. Six1 can transform a mammary epithelial cell line, but no work has been done to show the effects of Six1 overexpression *in vivo*. We have established an inducible, mammary-specific Six1 overexpression model by crossing MMTV-rtTA mice to TetO-Six1 mice, and are using this model to test whether Six1 overexpression leads to mammary tumors, as well as to dissect the molecular mechanism by which Six1 influences tumorigenesis *in vivo*. In mice induced to constitutively overexpress Six1 in the mammary gland, marked hyperproliferation and abnormal alveologenesis is observed. In addition, tumor formation is observed after long latency (>1 year). Tumors formed are complex, but are best characterized as invasive ductal adenocarcinomas with complex features. The tumors contain regions with papillary and secretory differentiation, as well as high grade solid areas. Most importantly, sarcomatoid differentiation (spindle cell morphology) is observed, and E-cadherin expression is lost in high grade areas of the tumor. Thus, this transgenic model demonstrates that inappropriate expression of Six1 promotes high grade tumor formation and oncogenic EMT, suggesting that Six1 is important not only for tumor initiation, but also for tumor progression. This inducible model provides us with a system to examine whether removal of Six1 expression can reverse the phenotypes, thereby addressing whether Six1 is a viable drug target. Importantly, Six1 is not necessary for most normal adult tissues, and thus therapies directed against Six1 may not lead to the severe side effects seen with more conventional treatments.

Poster abstract submitted for Keystone Symposia on Signaling Pathways in Development and Cancer for March 24-29 and for Era of Hope meeting for June, 2007:

In Vivo Role of the Six1 Homeoprotein in Mammary Gland Tumorigenesis

E.M. McCoy, Alana Welm, Karen Heichman, P. Jedlicka, L. Chodosh, H.L. Ford. Program in Molecular Biology, University of Colorado Health Sciences Center, Aurora, Colorado, USA, 80045

Human Six1 is a homeodomain-containing transcription factor that is critical for cell proliferation, survival, and epithelial-to-mesenchymal transition (EMT) during normal development. In addition to its developmental role, overexpression of Six1 has been detected in a number of human cancers, including breast cancer, where it is linked to both proliferation and metastasis. As many as 50% of primary breast cancers and 90% of metastatic lesions overexpress the gene, in part due to gene amplification. Six1 can transform a mammary epithelial cell line, but no work has been done to show the effects of Six1 overexpression *in vivo*. We have established an inducible, mammary-specific Six1 overexpression model by crossing MMTV-rtTA mice to TetO-Six1 mice, and are using this model to test whether Six1 overexpression leads to mammary tumors, as well as to dissect the molecular mechanism by which Six1 influences tumorigenesis *in vivo*. Low levels of Six1 expression is observed in uninduced bitransgenic animals over long periods of time, suggesting leakiness in the inducible model. Interestingly, animals treated with doxycycline, as well as uninduced animals, develop marked mammary hyperproliferation and abnormal alveologenesis. In addition, tumor formation is observed after long latency (>1 year) in both induced and uninduced animals, suggesting that low levels of Six1 are sufficient to cause transformation in this model. Tumors formed are complex, but are best characterized as invasive ductal adenocarcinomas with complex features. Importantly, sarcomatoid differentiation (spindle cell morphology) is observed, and E-cadherin expression is lost, while the mesenchymal markers Zeb1 and b-catenin are detected in the nuclei of spindle-cell areas of the tumors. Nuclear localization of b-catenin in tumors overexpressing Six1 suggests that the Wnt pathway, a potent mediator of tumorigenesis, may be activated in Six1-driven tumors. Finally, lung metastasis has occurred in a subset of animals. Thus, this transgenic model demonstrates that inappropriate expression of Six1 promotes high-grade tumor formation, oncogenic EMT, and metastasis, suggesting that Six1 is a powerful oncogene that is important not only for tumor initiation, but also for tumor progression. Mining of clinical data sets reveals that expression of the Six1 transcriptional complex is an indicator of poor prognosis in a number of different cancers, suggesting that Six1 may play important roles in many different cancer types. As Six1 is not necessary for most normal adult tissues, therapies directed against Six1 may not lead to the severe side effects seen with more conventional treatments, making Six1 an attractive chemotherapeutic target.