

AD _____

AWARD NUMBER: W81XWH-08-1-0332

TITLE: Six1-Eya-Dach Network in Breast Cancer

PRINCIPAL INVESTIGATOR: Susan Farabaugh

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

REPORT DATE: May 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 1 May 2009		2. REPORT TYPE Annual Summary		3. DATES COVERED 15 Apr 2008 – 14 Apr 2009	
4. TITLE AND SUBTITLE Six1-Eya-Dach Network in Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0332	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Susan Farabaugh E-Mail: Susan.Farabaugh@ucdenver.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Colorado Health Sciences Center Aurora, CO 80045				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The Six1 homeodomain-containing transcription factor stimulates the proliferation and survival of progenitor cells and plays a role in the epithelial to mesenchymal transition (EMT) during development. Using cell culture and mouse models, we have previously shown that Six1 not only promotes proliferation and survival, contributing to tumorigenesis, but also upregulates the TGFβ pathway, brings about an EMT-like transformation, and promotes metastasis. There are no known intrinsic activation or repression domains in Six1. The Eya family (Eya1-4), coactivators of Six1, play important roles in Six1-mediated transcriptional activation in development. Here, we preliminarily show that Eya2 is required for Six1-induced upregulation of the TGFβ pathway as well as for most Six1-induced epithelial to mesenchymal transition (EMT) phenotypes. These results suggest that Eya2 is a critical coactivator that is necessary for Six1-induced tumorigenic properties. Thus, as Six1 and Eya are not normally expressed in most adult tissues, the Six1-Eya interaction may be a valuable future therapeutic target for the 50% of primary breast tumors and 90% of metastatic lesions that overexpress Six1.					
15. SUBJECT TERMS Six1, Eya2, Dach, epithelial to mesenchymal transitions (EMT), tumorigenesis, mammary gland, TGFβ, -catenin, E-cadherin, metastasis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4-8
Key Research Accomplishments.....	8-9
Reportable Outcomes.....	9
Conclusion.....	9
References.....	9-11
Appendices.....	11

INTRODUCTION:

The homeobox transcription factor Six1 is a critical mediator of embryonic development, where it stimulates proliferation and survival of progenitor populations as well as contributes to the epithelial to mesenchymal transition (EMT)^{1,2}. Using cell culture and mouse models, we and others have shown that, when expressed out of context in adult cells, Six1 not only promotes proliferation and survival^{3,4,5}, contributing to tumorigenesis⁶, but also brings about an EMT-like transformation which has been linked to metastatic and invasive cancers^{6,7}. Six1 has no known intrinsic regulatory domains¹, and as such, is part of the Six-Eya-Dach transcriptional complex. Dach1, co-repressor of Six1, acts in direct opposition to Six1, repressing breast tumor growth and transformation and having reduced expression in metastatic breast cancers^{8,9}. The Eya family (Eya1-4), coactivators of Six1, play important roles in Six1-mediated transcriptional activation both in normal development^{10,11,12,13,14} and in various diseases where the Six1-Eya1/4 interaction is disrupted^{15,16,17,18,19}. Additionally, Eya family members have been found to change Six1-Dach from a repressor to an activating complex^{12,20}. Together, these findings suggest that Six1 may require an Eya cofactor to mediate its tumorigenic effects. We are currently analyzing the role of Eya in breast cancer by knocking down Eya levels and asking whether loss of Eya is able to reverse Six1-induced tumorigenic properties. Due to the instability of long term overexpression of Six1 in the MCF12A mammary epithelial cell line, we have moved from this cell line into the MCF7 breast carcinoma cell line for easier initial analysis of the coactivator role of Eya. The MCF7 cells are tumorigenic but non-metastatic. Our lab has shown that overexpression of Six1 in the MCF7 cells induces the TGF β pathway, properties of EMT, as well as the development of metastasis when orthotopically injected into immunocompromised mice²¹. Aim2 has changed slightly to accommodate Six1-induced phenotypes in the MCF7 cells rather than MCF12A cells but follows the main principles and same timeline originally outlined.

BODY:

Aim 1: Determine whether Eya2 is present in MCF7 cells.

(A) Examine Eya2 protein levels in MCF7-Six1 and MCF7-Ctrl Clones (Months 1-2)

This subaim has been completed. We have examined Eya1 and Eya2 levels in the MCF7-Six1 and MCF7-Ctrl clones as these are the two Eya family members found to significantly correlate with Six1 in human breast cancer patients (as discussed below in Aim3). While Eya1 mRNA was not found to be expressed, Eya2 is expressed at similar mRNA levels in both MCF7-Six1 and MCF7-Ctrls (Fig1). Expression of Eya2, but not Eya1, suggests that Eya2 may be the relevant Eya co-activator for Six1-mediated tumorigenesis in breast cancers.

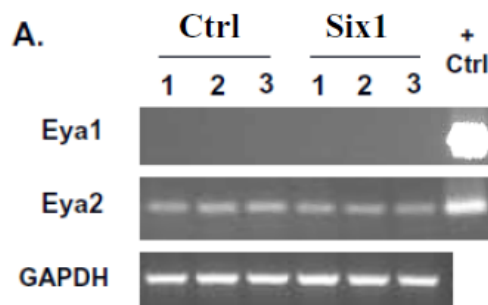


Fig 1. Eya2 is expressed in MCF7-Six1 and MCF7-Ctrl clones. Eya1 and Eya2 expression were analyzed by RT-PCR in MCF7 clones overexpressing Six1 and compared to MCF7 control clones.

(B) Determine if Eya2 interacts with Six1 in MCF7 clones (Months 3-7).

Since it has been well published that Six1 interacts with the Eya family²², this subaim is not being pursued. Immunoprecipitation of wildtype Eya2 with Six1 does not add any additional information at this time.

Aim 2: Determine the role of Eya2 in promoting Six1-induced tumor progression in MCF7 cells.

(A) Knockdown Eya2 in MCF7 cells overexpressing Six1 (Months 8-9).

This subaim has been completed. We have efficiently knocked-down Eya2 utilizing the SureSilencing system from SABiosciences. 5 Eya2 shRNA constructs and one scrambled negative control, all contained within the pGeneClip vector, were stably transfected into MCF7-Six1 or MCF7-Ctrl cells. Cells were cultured under puromycin selection and individual clones chosen. 2 individual clones from each of two working Eya2 shRNA constructs, as well as 2 scrambled negative control clones, were used to identify the role of Eya2 in Six1-mediated tumor progression (Fig 2). As shown in Figure 2, we have made Eya2 knockdowns in the MCF7-Ctrl line as well as the Six1 overexpressing line to control for any role Eya may play that is not linked with Six1. As we have yet to discover a Six1 independent phenotype in the Eya2 knockdowns, data in the following subaims will only reflect the Eya2 shRNA1 and shRNA2 clones with the MCF7-Six1 parental line as well as the scrambled control in both MCF7-Six1 and MCF7-Ctrl.

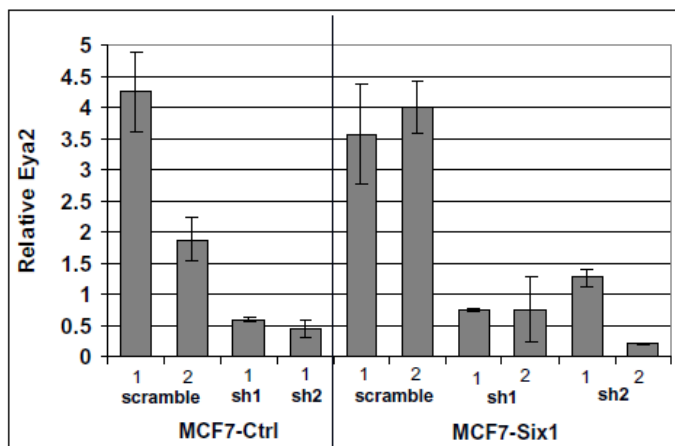


Fig 2. Eya2 is efficiently knocked down in MCF7-Six1 and Ctrl cells. Real Time PCR of Eya2 expression in knockdown and scrambled clones.

(B) Determine if Eya2 mediates Six1-induced TGF β signaling in MCF7-Six1 cells (Months 10-12).

Six1 overexpression in MCF7 cells activates the TGF β pathway²¹. We are in the process of analyzing the Eya2 knockdowns to determine if Eya2 is a necessary component for Six1-induced TGF β signaling. Thus far, we have found that lack of Eya2 reverses TGF β responsive transcription in the MCF7-Six1 cells back to, or slightly more than, levels detected in the MCF7-Ctrls as measured by 3TP reporter activity (Fig 3). These results suggest that Eya2 is indeed necessary for the ability of Six1 to activate the TGF β pathway. We are in the process of further analyzing this pathway to determine which stages the Eya2-Six1 complex is necessary. Expression of the TGF β receptors, as well as activation of the Smad proteins, under Eya2 knockdown conditions are currently being tested.

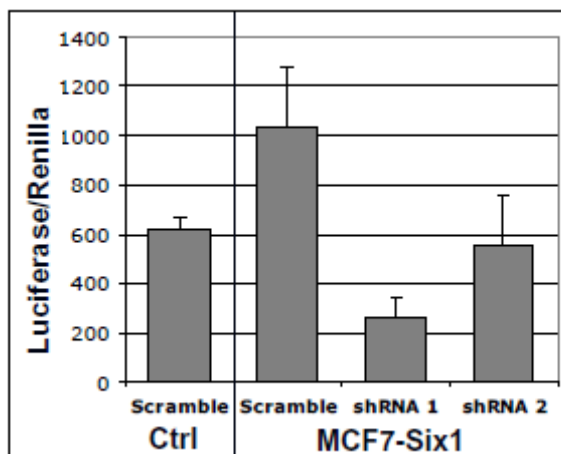


Fig 3. Loss of Eya2 in MCF7-Six1 cells decreases TGF- β responsive transcription as compared to Six1 and Ctrl scramble controls. Responsiveness was tested using luciferase activity of the 3TP reporter construct and normalized to renilla luciferase activity. Data points show the mean of two individual clones from two representative experiments and error bars represent the standard deviation.

(C) Determine if Eya2 mediates Six1-induced features of EMT in MCF7-Six1 cells (Months 13-15)

MCF7 cells overexpressing Six1 acquire mesenchymal properties while losing epithelial ones²¹. Lack of Eya2 in MCF7-Six1 cells reverses the Six1-induced increase in the mesenchymal marker fibronectin (Fig 4a). Eya2 is required for Six1-induced β -catenin responsive transcription as shown by reversal of TOP-FLASH reporter activity in the Eya2 knockdown clones to levels below that of the Ctrl scrambled clones (Fig 4b). Thus far, these data suggest that Eya2 is required for Six1-induced properties of EMT. Work is continuing on this subaim to determine if Eya2 is required for Six1-induced relocalization of β -catenin to the nucleus or if Eya2 is regulating β -catenin responsive transcription through another mechanism.

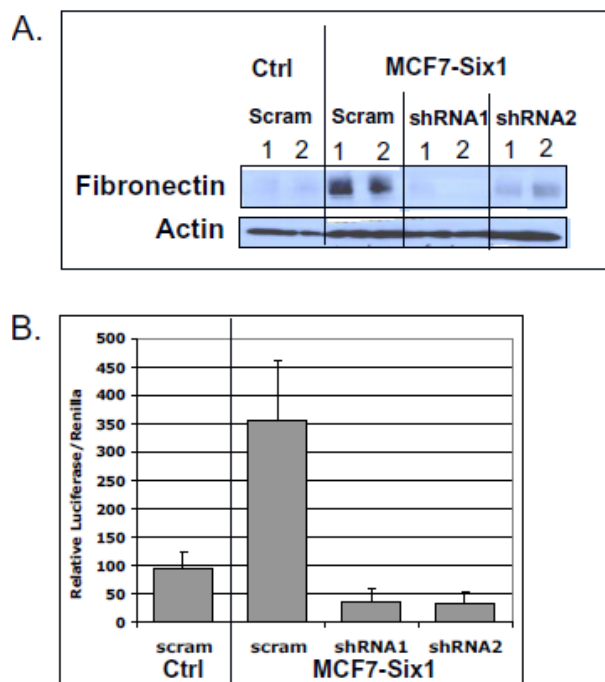


Fig 4. Loss of Eya2 reverses some Six1-induced EMT properties. (A) Western blot analysis was performed on whole cell lysates using Fibronectin and β -actin antibodies. (B) β -catenin induced transcriptional responsiveness was tested using luciferase activity of the TOP-FLASH reporter construct and normalized to renilla luciferase activity. Data points show the mean of two individual clones from two experiments and error bars represent the standard deviation.

In addition to the above mentioned EMT properties, we've analyzed the epithelial marker Cytokeratin 18 (CK18) but found that loss of Eya2 does not reverse the Six1-induced expression of this marker (data not shown). Additionally, Six1 overexpression in MCF7 cells results in a decreased adhesion to different matrixes, a property of a more mesenchymal cell, that is not reversed by loss of Eya2 (Fig 5). These data suggest that either Eya2 is not required for all Six1-induced EMT properties or that some properties, such as decreased adhesion, are more permanent and not reversible merely by interfering with Six1 function. We are in the process of knocking down Six1 in the MCF7-Six1 clones utilizing with pSuperRetro shRNA constructs already available in the to determine which Six1-induced phenotypes are reversible and which are more permanent.

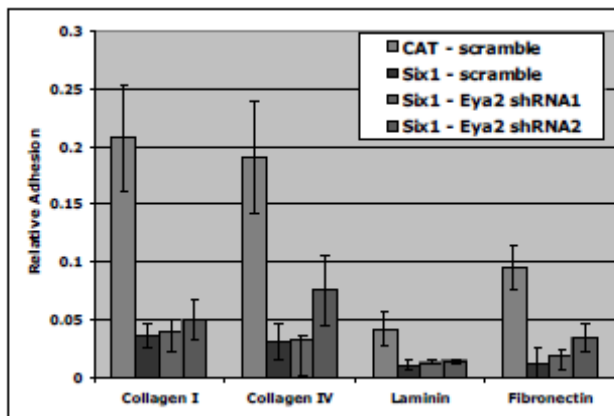


Fig 5. Eya2 is not required for loss of adhesion in MCF7-Six1 cells. Relative cell adhesion was measured by crystal violet staining. Data points show the mean of two individual clones from and are representative of two adhesion experiments. Error bars represent the standard deviation.

(D) Determine if Eya2 mediates Six1 induced metastasis (Months 16-27)

Our lab has shown that Six1 overexpressed in the tumorigenic but non-metastatic MCF7 cells induces metastasis when orthotopically injected into immunocompromised mice, both Nude and NOD/SCID²¹. This aim is ongoing. We have chosen one of the two clones each from shRNA1 and shRNA2 and both clones of the MCF7-Six1 and Ctrl scrambled clones. These clones already contain a ZSGreen fluorescent tag for easier visualization of metastases. The cells were orthotopically injected into the number 4 mammary gland of 10 NOD/SCID mice for each set of clones. When the primary tumors reach 2cm³ we will analyze the mice for metastatic burden as well as for location of metastasis. Both the primary tumor and the metastases will be analyzed by immunohistochemistry for Six1 and Eya2 expression as well as epithelial and mesenchymal markers and TGF- β pathway proteins such as Smad3.

Aim3. Determine whether Eya2 is relevant to human breast cancer.

(A) Examine expression of Six1 and Eya2 in human breast tumor samples (Months 28-31).

Not only are Eya2 and Six1 overexpressed in the same cancers^{23,24}, but additionally they both individually correlate with increased proliferation and shortened patient survival in ovarian cancer²⁴ suggesting that these proteins may initiate the same protumorigenic pathway. The goal of this aim is to determine if Eya2 is overexpressed in breast cancer and whether it is co-expressed with Six1 by analyzing Cloneteck Breast Cancer Profiling Arrays. We have developed our own Eya2 antibody and are in the process of testing this antibody on tissue sections. We will continue with this aim after antibody conditions are worked out.

(B) Correlate expression of Six1 and Eya2 with clinical parameters (Months 32-36).

This subaim will follow after the data for Subaim 3A is obtained.

In the meantime, while troubleshooting antibody conditions for Subaim 3A, we have been mining publicly available microarray data sets in collaboration with Alana Welm at the University of Utah. Together, we have shown that although high levels of Six1 significantly shortens time to metastasis in breast cancer patients, presence of the Eya1/Six1 or Eya2/Six1 transcriptional complex not only further reduces time to metastasis but also significantly decreases survival time in breast cancer patients (Fig 6). Presence of Eya3 and Eya4 do not correlate with Six1. This data in conjunction with the to-date Eya2 knockdown data suggests that Eya2 is a critical cofactor of Six1 in human breast cancer.

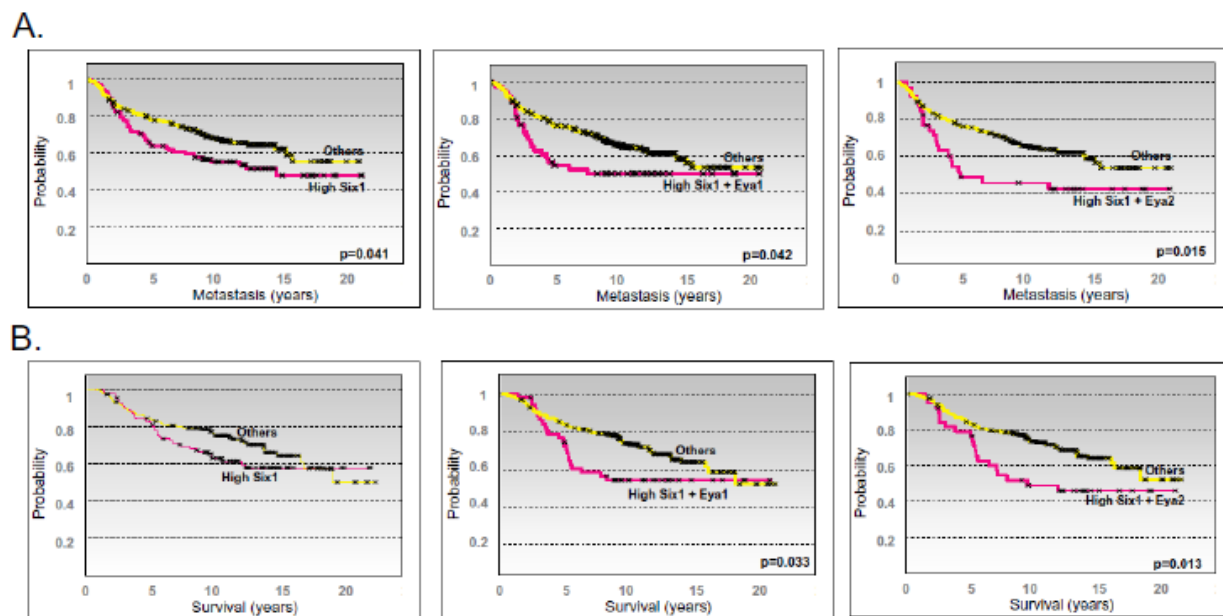


Fig 5. High Six1 with high Eya1 or Eya2 expression correlates with shortened time to metastasis and decreased survival in human breast cancers. In a microarray analysis of 295 women with early stage breast cancer, high Six1 expression correlates with (a) reduced time to metastasis while Eya1/Eya2 expression with high Six1 correlates with an even further reduction in time to metastasis. Additionally, (b) high Eya1/Eya2 with high Six1 expression significantly correlates with shortened survival time while high Six1 alone does not. The median value for Six1 and/or Eya1/Eya2 expression was used to divide the samples into high and low Six1 and/or Eya1/Eya2 expressors.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified that Eya2, but not Eya1, is expressed in MCF7 cells.
- Determined that Eya2 mediates Six1-induced TFG β responsive transcription.
- Determined that cells overexpressing Six1 require Eya2 for increased expression of the mesenchymal marker Fibronectin.
- Determined that Eya2 mediates Six1-induced β -catenin responsive transcription.

- Determined that loss of Eya2 was not sufficient to reverse the downregulation of the epithelial marker Cytokeratin 18 or the decreased adhesion which are both acquired by Six1 overexpression.
- Determined that high Six1 expression along with high Eya1 or Eya2 expression in early stage breast tumors correlates with an enhanced shortened time to metastasis and statistically significant decreased survival as compared to high Six1 without Eya correlation.

REPORTABLE OUTCOMES:

Research presented in the form of a poster at the Student Research Forum, University of Colorado Denver Anschutz Medical Campus, January 2009. See appendix for poster abstract.

Grants that resulted from this work includes a grant from the Breast Cancer Research Program, Synergistic DOD Idea Award entitled “*Structural and Functional Analyses of the Six1 Transcriptional Complex for Anti-Breast Cancer Drug Design*” (BC084105). PIs: Heide L. Ford and Rui Zhao.

CONCLUSIONS:

The results from studies completed thus far suggest that Eya2 is required for most Six1-induced tumorigenic properties in MCF7 cells. Loss of Eya2 results in a reversal of TGF- β responsive transcription, suggesting that Eya2 may be required for Six1-induced upregulation of the whole TGF- β pathway. In addition, our research suggests that Eya2 is necessary for most Six1-induced properties of the epithelial to mesenchymal transition. It is still unclear as to whether complete reversal of these EMT phenotypes is because Eya2 is not required or whether the phenotypes are just more permanent after initial Six1 overexpression. Overall, thus far, Eya2 appears to be a very important Six1 coactivator that is necessary for Six1-induced tumorigenic properties, suggesting that Eya2 may be a legitimate therapeutic target for the 50% of primary breast tumors and 90% of metastatic lesions that overexpress Six1³. As Six1 is overexpressed in a large number of breast tumors and metastases, and not expressed in most adult tissues^{3,25}, and as Eyas are developmentally restricted, we believe that targeting the Six1-Eya interaction in a clinical setting may successfully treat cancer while limiting side effects. Additionally, we are most interested in future studies with Eya as the Eya family contains phosphatase activity which may be an easier small molecule inhibitor target than the Six1-Eya interaction itself.

REFERENCES:

- ¹ Kawakami, K., Sato, S., Ozaki, H. & Ikeda, K. Six family genes--structure and function as transcription factors and their roles in development. *Bioessays* **22**, 616-626. (2000).
- ² Grifone, R. *et al.* Six1 and Six4 homeoproteins are required for Pax3 and Mrf expression during myogenesis in the mouse embryo. *Development* **132**, 2235-2249 (2005).

3 Ford, H. L., Kabingu, E. N., Bump, E. A., Mutter, G. L. & Pardee, A. B. Abrogation of
the G2 cell cycle checkpoint associated with overexpression of HSIX1: a possible
4 mechanism of breast carcinogenesis. *Proc Natl Acad Sci U S A* **95**, 12608-12613. (1998).
Coletta, R. D. *et al.* The Six1 homeoprotein stimulates tumorigenesis by reactivation of
5 cyclin A1. *Proc Natl Acad Sci U S A* **101**, 6478-6483 (2004).
K. Behbakht, L. Q., C. Aldridge, R. Coletta, S. Davidson, A. Thorburn, H. Ford. Six1
over-expression in ovarian cancers causes resistance to TRAIL-mediated apoptosis and is
6 associated with poor survival. *Manuscript Submitted* (2006).
Coletta, R. D. *et al.* Six1 overexpression in mammary cells induces genomic instability
and is sufficient for malignant transformation. *Cancer Res* **68**, 2204-2213 (2008).
7 Yu, Y. *et al.* Expression profiling identifies the cytoskeletal organizer ezrin and the
developmental homeoprotein Six-1 as key metastatic regulators. *Nat Med* **10**, 175-181
(2004).
8 Wu, K. *et al.* Cell fate determination factor DACH1 inhibits c-Jun-induced contact-
independent growth. *Mol Biol Cell* **18**, 755-767 (2007).
9 Wu, K. *et al.* DACH1 is a cell fate determination factor that inhibits cyclin D1 and breast
tumor growth. *Mol Cell Biol* **26**, 7116-7129 (2006).
10 Laclef, C., Souil, E., Demignon, J. & Maire, P. Thymus, kidney and craniofacial
abnormalities in Six1 deficient mice. *Mech Dev* **120**, 669-679 (2003).
11 Ando, Z., Sato, S., Ikeda, K. & Kawakami, K. Slc12a2 is a direct target of two closely
related homeobox proteins, Six1 and Six4. *Febs J* **272**, 3026-3041 (2005).
12 Li, X. *et al.* Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional
effects in mammalian organogenesis. *Nature* **426**, 247-254 (2003).
13 Xu, P. X. *et al.* Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis
of organ primordia. *Nat Genet* **23**, 113-117. (1999).
14 Xu, P. X. *et al.* Six1 is required for the early organogenesis of mammalian kidney.
Development **130**, 3085-3094 (2003).
15 Abdelhak, S. *et al.* Clustering of mutations responsible for branchio-oto-renal (BOR)
syndrome in the eyes absent homologous region (eyaHR) of EYA1. *Hum Mol Genet* **6**,
2247-2255. (1997).
16 Ruf, R. G. *et al.* SIX1 mutations cause branchio-oto-renal syndrome by disruption of
EYA1-SIX1-DNA complexes. *Proc Natl Acad Sci U S A* **101**, 8090-8095 (2004).
17 Zhang, Y., Knosp, B. M., Maconochie, M., Friedman, R. A. & Smith, R. J. A
comparative study of Eya1 and Eya4 protein function and its implication in branchio-oto-
renal syndrome and DFNA10. *J Assoc Res Otolaryngol* **5**, 295-304 (2004).
18 Schonberger, J. *et al.* Mutation in the transcriptional coactivator EYA4 causes dilated
cardiomyopathy and sensorineural hearing loss. *Nat Genet* **37**, 418-422 (2005).
19 Wayne, S. *et al.* Mutations in the transcriptional activator EYA4 cause late-onset
deafness at the DFNA10 locus. *Hum Mol Genet* **10**, 195-200 (2001).
20 Ikeda, K., Watanabe, Y., Ohto, H. & Kawakami, K. Molecular interaction and synergistic
activation of a promoter by Six, Eya, and Dach proteins mediated through CREB binding
protein. *Mol Cell Biol* **22**, 6759-6766 (2002).
21 Micalizzi, D. S., *et al.* *Under Revision for the J of Clinical Investigation.*
22 Ohto, H. *et al.* Cooperation of six and eya in activation of their target genes through
nuclear translocation of Eya. *Mol Cell Biol* **19**, 6815-6824. (1999).

- 23 Li, C. M. *et al.* Gene expression in Wilms' tumor mimics the earliest committed stage in
the metanephric mesenchymal-epithelial transition. *Am J Pathol* **160**, 2181-2190 (2002).
24 Zhang, L. *et al.* Transcriptional coactivator Drosophila eyes absent homologue 2 is up-
regulated in epithelial ovarian cancer and promotes tumor growth. *Cancer Res* **65**, 925-
932 (2005).
25 Zimmerman, J. E. *et al.* Cloning and characterization of two vertebrate homologs of the
Drosophila eyes absent gene. *Genome Res* **7**, 128-141. (1997).

APPENDICIES:

Poster abstract submitted for the Student Research Forum at the University of Colorado Denver
Anschutz Medical Campus, January 2009.

The Role of Eya as Cofactors of Six1 in Human Breast Cancer.

S.M. Farabaugh, Alana Welm, H.L. Ford, Department of Biochemistry and Molecular Genetics,
University of Colorado Denver Anschutz Medical Campus, Aurora, CO.

The Six1 homeodomain-containing transcription factor stimulates the proliferation and survival of progenitor cells and plays a role in the epithelial to mesenchymal transition (EMT) during development. Using cell culture and mouse models, we have shown that, when expressed out of context in adult cells, Six1 not only promotes proliferation and survival, contributing to tumorigenesis, but also upregulates the TGF β pathway, brings about an EMT-like transformation, and promotes metastasis. Most importantly, reducing Six1 levels decreases cancer cell proliferation and metastases in several models of cancer, strongly suggesting that Six1 will make an excellent cancer therapy target. There are no known intrinsic activation or repression domains in Six1. The Eya family (Eya1-4), coactivators of Six1, play important roles in Six1-mediated transcriptional activation in development. The fact that Eyas promote cellular proliferation through Six1, that the Six1-Eya interaction is lost in several diseases, that both Eya and Six1 are implicated in the same cancers, that Eya is needed to revert Six1 and its corepressor Dach to an activating complex, and that Eya phosphatase activity is required for Six1 activation, suggests that Eya may be a relevant co-activator for Six1-induced breast tumorigenesis. Here we show that while Six1 correlates with shortened time to metastasis in breast cancer patient tumors, presence of both Eya2 and Six1 together further reduces time to metastasis as well as significantly correlates with decreased survival. Additionally, decreasing Eya2 levels in MCF7 cells overexpressing Six1 reverses Six1-induced upregulation of the TGF β pathway and most Six1-induced EMT phenotypes, suggesting that Eya2 is required for the ability of Six1 to mediate tumor progression. As Eya2 appears to be required for Six1 activity in breast cancers and as Six1 and Eya are not normally expressed in most adult tissues, we believe that targeting Eya phosphatase activity or the Six1-Eya interaction with small molecule inhibitors could result in new chemotherapeutics for breast cancer patients.